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# **Investigating the ability of mammalian Membrane Associated RING-CH proteins to modulate respiratory virus infections**

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## Summary

Respiratory viruses generally infect epithelial cells lining the upper and lower airways, however subsets of airway immune cells are also susceptible to infection. Of interest, airway epithelial cells (AEC) and airway macrophages (AM $\phi$ ) are both susceptible to influenza virus infection, but only AEC support productive virus replication. Constitutive expression and/or induction of intracellular host proteins to limit or block virus replication is a well-known antiviral mechanism, however few such “restriction factors” have been well characterized and the antiviral activity of many putative restriction factors against respiratory viruses has not been reported. A recent RNA-seq study performed in our laboratory compared gene expression between AEC and AM $\phi$  in the presence or absence of influenza infection, allowing us to identify protein families with differential expression between these two cell types in steady-state and/or following virus infection. We hypothesized that amongst genes differentially expressed between AEC and AM $\phi$  would be those that represent novel restriction factors and these might contribute to the abortive versus productive phenotype observed in AM $\phi$  and AEC, respectively, in regard to influenza replication. One of the protein families identified were the membrane-associated RING-CH (MARCH) family proteins.

MARCH proteins are E3 ubiquitin ligases involved in the final step of the ubiquitination process. Amongst their functions, MARCH proteins modulate host immune responses by virtue of their ability to control the turnover of multiple immune molecules, including major histocompatibility complex (MHC) proteins. More recent evidence indicates that they can also modulate virus infection, acting as restriction factors against viruses such

as human immunodeficiency virus (HIV)-I, or as proviral factors for viruses such as hepatitis C virus (HCV). Based on these findings, this thesis aimed to investigate the ability of MARCH family proteins to modulate infections caused by influenza and other respiratory viruses.

First, we generated stable cell lines with doxycycline (DOX)-inducible expression of several human MARCH proteins (MARCH1/2/3/5/6/7/8/9) and evaluated their ability to modulate either the early or late stages of infections caused by influenza A viruses (IAV), respiratory syncytial virus (RSV) or human metapneumovirus (hMPV). Although none of the MARCH proteins tested affected the early stages of IAV infection (as measured by flow cytometry for newly-synthesized viral proteins), inducible expression of MARCH8 was associated with a significant reduction in titres of infectious IAV released from infected cells. Moreover, MARCH8 expression was also associated with a reduced percentage of RSV-infected cells, consistent with its ability to restrict at an early stage of the RSV replication cycle, although this was not explored further in this thesis. We did not observe any significant differences in the ability of any MARCH protein tested to modulate early or late stages of hMPV infection. Subsequent studies have focussed on understanding MARCH8-mediated restriction of influenza virus infections.

Next, we characterized the antiviral activity of MARCH8 against influenza viruses. MARCH8 was shown to mediate antiviral activity against a range of influenza viruses relevant to human health, including A/H1N1, A/H1N1pdm09 and A/H3N2 viruses, as well as against an influenza B virus. We demonstrated that expression of MARCH8, but not the closely related MARCH1, was associated with a reduction in the specific infectivity of virus particles released from IAV-infected cells. Moreover, virus particles



released from IAV-infected cells in the presence of MARCH8 exhibited altered protein composition and virion morphology, and MARCH8 was incorporated into the nascent virions. Of interest, MARCH8 expression did not alter cell-surface expression of the viral proteins HA, NA and M2, indicating no major defect in their synthesis and transport during viral replication. Overall, these findings are consistent with MARCH8 acting to block a step late in the virus replication cycle, possibly during virus assembly and/or budding from the cell surface.

Studies described in this thesis also attempted to determine features of MARCH8 and IAV relevant to MARCH8-mediated restriction of IAV. We used site-directed mutagenesis and reverse genetics to modify lysine residues in the cytoplasmic tails of the viral hemagglutinin (HA), neuraminidase (NA) and matrix protein (M)2 proteins, either alone or in combination. However, none of these substitutions were sufficient to overcome MARCH8-mediated restriction of IAV. We also modified several lysine residues in the viral M1 protein but again did not identify specific residues to abrogate MARCH8-mediated restriction. Currently, the identity of the particular viral and/or host proteins targeted by MARCH8 which result in inhibition of late stage IAV replication are not known.

To study MARCH8 itself, we generated mutants lacking E3 ligase activity and confirmed that this was essential for its anti-IAV activity. Furthermore, given that MARCH8, but not the closely related MARCH1, mediated potent anti-IAV activity against some strains of IAV, we generated cell lines with DOX-inducible expression of MARCH1-MARCH8 chimeric proteins in an effort to determine particular domains of MARCH8 critical to its anti-IAV activity. Chimeric proteins contained one or more domains/regions of MARCH1

substituted into the MARCH8 backbone. While all chimeric proteins retained activity against a common target protein (CD86), all of them also retained ability to restrict IAV replication. Based on these studies, we were unable to identify critical domains of MARCH8 that were essential for its antiviral activity against IAV.

Finally, we used a MARCH8 knockout (KO) mouse to evaluate the role of endogenous MARCH8 in modulating IAV infection *in vivo*. Following intranasal infection, MARCH8 KO mice exhibited enhanced weight loss and viral replication in the lungs at day 5 post-infection, although no major differences in virus titres or virus clearance were observed at later time-points. Moreover, we did not observe major differences in soluble inflammatory mediators or inflammatory cells at day 7 or 10 post-infection. These findings are consistent with a role for endogenous MARCH8 in controlling the early stages of IAV infection *in vivo* but suggest that it does not have a major impact on virus clearance or the development of adaptive immunity in this model. Of interest, siRNA knockdown of endogenous MARCH8 in a human epithelial cell line also resulted in a modest, but significant, reduction in titres of IAV released from infected cells *in vitro*.

Together, the studies presented in this thesis describe and characterize the antiviral activity of MARCH8 against influenza viruses. In addition, they provide preliminary data to indicate that MARCH8 can also mediate antiviral activity against the pneumovirus RSV, albeit by a distinct mechanism. Overall, these findings contribute to a growing body of evidence that MARCH8 plays an important role in modulating infections caused by a range of different viruses with relevance to human health.

## **Declaration**

This is to certify that,

- (i) the thesis comprises only my original work towards the PhD except where indicated in the preface;
- (ii) due acknowledgement has been made in the text to all other material used;
- (iii) the thesis is less than 100,000 word limit in length, exclusive of tables, maps, bibliographies and appendices as approved by the Research Higher Degrees Committee.

Fernando Javier Villalón Letelier

## Preface

The work presented in this thesis was conducted at The University of Melbourne, in the laboratory of Professor Andrew Brooks, under the supervision of Professor Patrick Reading, Dr. Sarah Londrigan and Professor Andrew Brooks. The project was funded by a grant from the National Health and Medical Research Council of Australia (Project Grant APP1143154). Fernando Javier Villalón Letelier was supported by the Research Training Program Scholarship (Stipend and Fee offset).

My contribution to experiments in each of the Chapters was as follows:

- **Chapter 3:** 100%

- **Chapter 4:** 95%

*Samples for transmission electron microscopy analysis were stained and visualized by Vicki Bennett-Wood (The University of Melbourne, Melbourne, Australia)*

- **Chapter 5:** 100%

- **Chapter 6:** 90%

*Infection of mice and tissue/sample collection from infected mice were performed by Professor Patrick Reading.*

I acknowledge these important contributions to the experiments presented in this thesis.

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## Table of contents

|                                                                                                             |           |
|-------------------------------------------------------------------------------------------------------------|-----------|
| Summary.....                                                                                                | I         |
| Declaration .....                                                                                           | V         |
| Preface .....                                                                                               | VI        |
| Acknowledgments.....                                                                                        | VIII      |
| Table of contents .....                                                                                     | XI        |
| List of Figures .....                                                                                       | XVIII     |
| List of Tables .....                                                                                        | XXII      |
| List of Abbreviations .....                                                                                 | XXIII     |
| <b>Chapter 1. Literature Review .....</b>                                                                   | <b>1</b>  |
| <b>1. <i>Orthomyxoviridae</i> family .....</b>                                                              | <b>2</b>  |
| 1.1. <i>General features.</i> .....                                                                         | 2         |
| 1.2. <i>Infection of host cells</i> .....                                                                   | 6         |
| 1.2.1. Early stages of influenza virus infection.....                                                       | 6         |
| 1.2.2. Late stages of influenza virus infection .....                                                       | 9         |
| <b>2. <i>Pneumoviridae</i> family.....</b>                                                                  | <b>10</b> |
| 2.1. <i>General features</i> .....                                                                          | 10        |
| 2.2. <i>Infection of host cells</i> .....                                                                   | 11        |
| 2.2.1. Early stages of pneumovirus replication .....                                                        | 13        |
| 2.2.2. Late stages of pneumovirus replication .....                                                         | 14        |
| <b>3. Paramyxoviruses. ....</b>                                                                             | <b>16</b> |
| <b>4. Type I interferons and antiviral immunity .....</b>                                                   | <b>18</b> |
| 4.1. <i>Induction of type I interferons.</i> .....                                                          | 18        |
| 4.2 <i>Viral strategies to counteract antiviral immune responses mediated by interferons.</i><br>.....      | 21        |
| <b>5. Cellular restriction factors that mediate antiviral activity against respiratory viruses</b><br>..... | <b>23</b> |

|                                                                                                                                         |           |
|-----------------------------------------------------------------------------------------------------------------------------------------|-----------|
| 5.1. Cellular restriction factors that act against influenza virus. ....                                                                | 24        |
| 5.1.1. Restriction factors that target virus attachment and entry .....                                                                 | 24        |
| 5.1.2. Restriction factors that target genomic transcription and replication. ....                                                      | 26        |
| 5.1.3. Restriction factors that target late stages of IAV infection .....                                                               | 28        |
| 5.2. Cellular restriction factors that act against other respiratory viruses. ....                                                      | 29        |
| <b>6. Approaches to study specific cellular restriction factors.....</b>                                                                | <b>30</b> |
| <b>7. Identifying ISGs and other antiviral factors with antiviral activity against influenza and/or other respiratory viruses. ....</b> | <b>32</b> |
| <b>8. Ubiquitination. ....</b>                                                                                                          | <b>34</b> |
| 8.1. Chain topology and ubiquitin signaling.....                                                                                        | 36        |
| 8.2. Ubiquitination specificity and E3 ubiquitin ligases.....                                                                           | 37        |
| <b>9. Membrane-Associated RING-CH (MARCH) proteins. ....</b>                                                                            | <b>38</b> |
| 9.1. Viral MARCH proteins. ....                                                                                                         | 38        |
| 9.2. Mammalian MARCH proteins: General features.....                                                                                    | 39        |
| 9.3. Mammalian MARCH proteins: Regulation of immune responses. ....                                                                     | 43        |
| 9.4. Mammalian MARCH proteins: Role as modulators of viral infections. ....                                                             | 45        |
| <b>10. Aims of this thesis.....</b>                                                                                                     | <b>47</b> |
| <b>Chapter 2. Materials and Methods .....</b>                                                                                           | <b>51</b> |
| 2.1. Cell lines. ....                                                                                                                   | 52        |
| 2.2. Viruses. ....                                                                                                                      | 52        |
| 2.3. Primers.....                                                                                                                       | 54        |
| 2.4 Antibodies .....                                                                                                                    | 56        |
| 2.5 Determination of titres of infectious virus .....                                                                                   | 57        |
| 2.5.1. Plaque assay for influenza viruses. ....                                                                                         | 57        |
| 2.5.2. ViroSpot assay for influenza viruses.....                                                                                        | 58        |
| 2.5.3. Plaque assay for RSV and PIV-3. ....                                                                                             | 58        |
| 2.5.4. ViroSpot assay for hMPV.....                                                                                                     | 59        |
| 2.6. Molecular cloning. ....                                                                                                            | 60        |

|                                                                                                                                         |           |
|-----------------------------------------------------------------------------------------------------------------------------------------|-----------|
| 2.6.1. Cloning of MARCH1/8 in pcDNA3.....                                                                                               | 60        |
| 2.6.2. Cloning of MARCH1/2/3/5/6/7/8/9 into pTRE-tight and FUV1mCherry plasmids.<br>.....                                               | 60        |
| 2.6.3. Transformation and plasmid purification. ....                                                                                    | 63        |
| <b>2.7. Transient transfection of 293T cells with pcDNA3 plasmids.....</b>                                                              | <b>63</b> |
| <b>2.8. Analysis of FLAG expression by flow cytometry. ....</b>                                                                         | <b>64</b> |
| <b>2.9. Analysis of FLAG expression by western blot. ....</b>                                                                           | <b>64</b> |
| 2.9.1. Preparation of cell lysates.....                                                                                                 | 64        |
| 2.9.2. Western blot. ....                                                                                                               | 65        |
| <b>2.10. Stable cell lines with DOX-inducible expression of MARCH proteins.....</b>                                                     | <b>66</b> |
| 2.10.1. Generation of lentiviral particles.....                                                                                         | 66        |
| 2.10.2. Transduction and cell sorting.....                                                                                              | 66        |
| 2.10.3. DOX-induction of stably transduced cell lines. ....                                                                             | 68        |
| <b>2.11. Detection of cell-surface CD86 by flow cytometry.....</b>                                                                      | <b>68</b> |
| <b>2.12. Detection of virus-infected cells by flow cytometry.....</b>                                                                   | <b>69</b> |
| <b>2.13. Assessment of productive replication of respiratory viruses in mammalian cell<br/>lines. ....</b>                              | <b>71</b> |
| <b>2.14 Generation of DOX-inducible cell lines expressing MARCH8 RING-CH mutants. 72</b>                                                |           |
| 2.14.1. Molecular cloning. ....                                                                                                         | 72        |
| 2.14.1a. Site-directed mutagenesis. ....                                                                                                | 72        |
| 2.14.1b. Cloning of MARCH8-W114 into pTRE-tight and FUV1-mCherry plasmids.<br>.....                                                     | 73        |
| 2.14.2. DOX-inducible 293T cells.....                                                                                                   | 73        |
| <b>2.15. Quantification of vRNA in supernatant from infected cells and calculation of<br/>infectivity per particle. ....</b>            | <b>73</b> |
| <b>2.16. Cell-surface expression of IAV glycoproteins by flow cytometry.....</b>                                                        | <b>75</b> |
| <b>2.17. Analysis of viral glycoproteins in concentrated supernatants and cell lysates from<br/>infected cells by western blot.....</b> | <b>76</b> |
| 2.17.1 Preparation of samples. ....                                                                                                     | 76        |

|                                                                                                                                             |           |
|---------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| 2.17.2 Protein quantification and western blot. ....                                                                                        | 77        |
| <b>2.18 Assessment of hemagglutination and neuraminidase activity in supernatants from virus-infected cells. ....</b>                       | <b>77</b> |
| 2.18.1. Preparation of samples. ....                                                                                                        | 77        |
| 2.18.2. Hemagglutination assay. ....                                                                                                        | 78        |
| 2.18.3. Enzymatic activity of the viral neuraminidase. ....                                                                                 | 78        |
| <b>2.19 Analysis of IAV particles by transmission electron microscopy. ....</b>                                                             | <b>79</b> |
| 2.19.1 Preparation of the samples. ....                                                                                                     | 79        |
| 2.19.2 Transmission electron microscopy. ....                                                                                               | 80        |
| <b>2.20. Generation of recombinant IAV by reverse genetics. ....</b>                                                                        | <b>80</b> |
| <b>2.21. Knockdown of endogenous MARCH8 using siRNA. ....</b>                                                                               | <b>81</b> |
| <b>2.22. Generation of MARCH8/MARCH1 chimeric proteins. ....</b>                                                                            | <b>82</b> |
| <b>2.23. IAV infection in mice. ....</b>                                                                                                    | <b>83</b> |
| 2.23.1. Total and upper respiratory tract infections. ....                                                                                  | 83        |
| 2.23.2. Viral titres in tissue samples. ....                                                                                                | 84        |
| 2.23.3. Detection of inflammatory mediators and cells in bronchoalveolar lavage fluids. ....                                                | 84        |
| 2.23.3a. Detection of inflammatory mediators in cell-free BAL fluid. ....                                                                   | 85        |
| 2.23.3b. Analysis of immune cell populations in BAL fluid ....                                                                              | 85        |
| 2.23.4. Isolation and infection of murine peritoneal macrophages. ....                                                                      | 86        |
| 2.23.4a. Immunofluorescence to detect IAV infection of PEC M $\phi$ . ....                                                                  | 86        |
| 2.23.4b. Growth of IAV in PEC M $\phi$ . ....                                                                                               | 87        |
| <b>2.24. Data analysis. ....</b>                                                                                                            | <b>87</b> |
| <b>Chapter 3. Investigating MARCH-family proteins as putative restriction factors against influenza and other respiratory viruses. ....</b> | <b>89</b> |
| <b>3.1. Introduction. ....</b>                                                                                                              | <b>90</b> |
| <b>3.2. Results. ....</b>                                                                                                                   | <b>92</b> |
| 3.2.1. Establishing parameters to study putative cellular restriction factors for respiratory virus infections. ....                        | 92        |

|                                                                                                                                                                                    |            |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| 3.2.2. <i>Generation of cell lines with ectopic expression of different MARCH-family proteins.</i> .....                                                                           | 97         |
| 3.2.3. <i>Functionality of DOX-inducible MARCH proteins.</i> .....                                                                                                                 | 103        |
| 3.2.4. <i>Use of cell lines with DOX-inducible expression of different MARCH proteins to investigate their potential to modulate IAV infection.</i> .....                          | 105        |
| 3.2.4a. <i>Effect of MARCH proteins on the early stages of IAV infection.</i> .....                                                                                                | 105        |
| 3.2.4b. <i>Effect of MARCH proteins on the late stages of IAV infection.</i> .....                                                                                                 | 107        |
| 3.2.5. <i>Use of cell lines with DOX-inducible expression of different MARCH proteins to investigate their potential to modulate infection by other respiratory viruses.</i> ..... | 109        |
| 3.2.5a. <i>Effect of MARCH proteins on the early stages of pneumovirus infection.</i> .....                                                                                        | 109        |
| 3.2.5b. <i>Effect of MARCH proteins on the early stages of paramyxovirus infection.</i> .....                                                                                      | 112        |
| 3.2.5c. <i>Effect of MARCH proteins on the late stages of pneumovirus infection.</i> ..                                                                                            | 112        |
| 3.2.5d. <i>Effect of MARCH proteins on the late stages of paramyxovirus infection.</i> .....                                                                                       | 114        |
| <b>3.3. Discussion</b> .....                                                                                                                                                       | <b>114</b> |
| <b>Chapter 4. Investigating the antiviral activity of MARCH8 against influenza viruses</b> .....                                                                                   | <b>125</b> |
| <b>4.1. Introduction.</b> .....                                                                                                                                                    | <b>126</b> |
| <b>4.2. Results</b> .....                                                                                                                                                          | <b>128</b> |
| 4.2.1. <i>Further characterization of the antiviral activity of MARCH8 against influenza viruses.</i> .....                                                                        | 128        |
| 4.2.1.1. <i>MARCH8, but not the closely related MARCH1, mediates antiviral activity during multicycle IAV infection.</i> .....                                                     | 128        |
| 4.2.1.2. <i>Induction of MARCH8 expression in different cell lines mediates antiviral activity against IAV</i> .....                                                               | 130        |
| 4.2.1.3. <i>MARCH8 mediates antiviral activity against different seasonal influenza viruses.</i> .....                                                                             | 132        |
| 4.2.2. <i>E3 ligase activity of MARCH8 is essential for its antiviral activity against IAV.</i> .....                                                                              | 134        |
| 4.2.3. <i>Insights into the mechanism/s by which MARCH8 mediates antiviral activity against IAV.</i> .....                                                                         | 136        |
| 4.2.3.1. <i>Virus particles released from IAV-infected cells in the presence of DOX-inducible MARCH8 show reduced infectivity per particle.</i> .....                              | 136        |

|                                                                                                                                                                          |            |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| 4.2.3.2. DOX-inducible MARCH8 expression does not result in downregulation of viral glycoproteins from the surface of IAV-infected cells. ....                           | 137        |
| 4.2.3.3. Investigating the protein composition of virus particles released from IAV-infected cells with induced MARCH8 expression. ....                                  | 140        |
| 4.2.3.4. Alterations in virion morphology following release of virions from IAV-infected cells with induced MARCH8 expression. ....                                      | 143        |
| 4.2.4. <i>Investigating if specific lysine residues in the viral HA, NA, M1 and M2 serve as targets for MARCH8-mediated ubiquitination.</i> ....                         | 145        |
| 4.2.5. <i>Investigating the role of endogenous MARCH8 against influenza viruses in vitro.</i> ....                                                                       | 151        |
| <b>4.3. Discussion.....</b>                                                                                                                                              | <b>153</b> |
| <br><b>Chapter 5. Generation and characterization of MARCH1/MARCH8 chimeric proteins to identify domains of MARCH8 required for antiviral activity against IAV .....</b> | <b>166</b> |
| <b>5.1. Introduction .....</b>                                                                                                                                           | <b>167</b> |
| <b>5.2. Results.....</b>                                                                                                                                                 | <b>169</b> |
| 5.2.1. <i>Design of MARCH1/8 chimeric mutants.....</i>                                                                                                                   | 169        |
| 5.2.2. <i>Chimeric mutants generated by swapping single domains of MARCH1 into MARCH8.....</i>                                                                           | 169        |
| 5.2.2a. Investigating the role of the C-terminal CT in determining the differential ability of MARCH1/8 to mediate anti-IAV activity. ....                               | 169        |
| 5.2.2b. Investigating the role of the DIRT, TMs and luminal connecting loop in determining the differential ability of MARCH1/8 to mediate anti-IAV activity..           | 174        |
| 5.2.2c. Investigating the role of the N-terminal CT and RING-CH domain in determining the differential ability of MARCH1/8 to mediate anti-IAV activity..                | 178        |
| 5.2.3. <i>Chimeric mutants generated by swapping multiple domains of MARCH1 into MARCH8.....</i>                                                                         | 182        |
| <b>5.3. Discussion.....</b>                                                                                                                                              | <b>187</b> |
| <br><b>Chapter 6. Evaluating the role of endogenous MARCH8 during IAV infection <i>in vivo</i> .....</b>                                                                 | <b>197</b> |
| <b>6.1. Introduction. ....</b>                                                                                                                                           | <b>198</b> |
| <b>6.2. Results.....</b>                                                                                                                                                 | <b>200</b> |

|                                                                                                                                                 |            |
|-------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| 6.2.1. Optimizing infectious dose of IAV for inoculation of wild-type and MARCH8 KO mice.....                                                   | 200        |
| 6.2.2. Characterizing the course of infection following TRT infection of wild-type and MARCH8 KO mice with $10^3$ PFU of IAV strain HKx31. .... | 204        |
| 6.2.3. Differences in NK cell makers expressed by wild-type and MARCH8 KO mice. ....                                                            | 210        |
| 6.2.4. In vitro infection of primary macrophages from wild-type and MARCH8 KO mice with IAV.....                                                | 212        |
| <b>6.3. Discussion.....</b>                                                                                                                     | <b>215</b> |
| <b>Chapter 7. General Discussion .....</b>                                                                                                      | <b>224</b> |
| Ubiquitination during respiratory viral infections.....                                                                                         | 226        |
| The anti-IAV activity of MARCH8: What we know and what we don't. ....                                                                           | 228        |
| Challenges of working with MARCH-family proteins. ....                                                                                          | 233        |
| Concluding remarks. ....                                                                                                                        | 236        |
| <b>References .....</b>                                                                                                                         | <b>239</b> |
| <b>Appendix.....</b>                                                                                                                            | <b>266</b> |
| Publications generated during this thesis.....                                                                                                  | 266        |

## List of Figures

|                                                                                                                                                                                                                      |            |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>Figure 1.1.</b> Schematic diagram of influenza A virion..                                                                                                                                                         | <b>3</b>   |
| <b>Figure 1.2.</b> Influenza A virus replicative cycle..                                                                                                                                                             | <b>7</b>   |
| <b>Figure 1.3.</b> Schematic diagram of pneumovirus virion..                                                                                                                                                         | <b>12</b>  |
| <b>Figure 1.4.</b> Induction of type I interferons (IFNs) by respiratory viruses..                                                                                                                                   | <b>20</b>  |
| <b>Figure 1.5.</b> Host restriction factors that modulate influenza virus infection.....                                                                                                                             | <b>25</b>  |
| <b>Figure 1.6.</b> Ubiquitination process.....                                                                                                                                                                       | <b>35</b>  |
| <b>Figure 1.7.</b> Phylogenetic analysis of human MARCH proteins..                                                                                                                                                   | <b>40</b>  |
| <b>Figure 1.8.</b> Expression levels of MARCH proteins in AEC and AMΦ from mice after IAV infection. ....                                                                                                            | <b>48</b>  |
|                                                                                                                                                                                                                      |            |
| <b>Figure 2.1.</b> Cloning strategy to generate pFUV1mCherry plasmids for the generation of lentiviral particles.....                                                                                                | <b>61</b>  |
| <b>Figure 2.2.</b> Generation of stable cells lines with DOX-inducible expression of MARCH proteins..                                                                                                                | <b>67</b>  |
|                                                                                                                                                                                                                      |            |
| <b>Figure 3.1.</b> Susceptibility of MDCK, A549 and 293T cells to infection by IAV, RSV, HMPV and PIV-3. ....                                                                                                        | <b>95</b>  |
| <b>Figure 3.2.</b> Growth of IAV, RSV, hMPV and PIV-3 in MDCK, A549 and 293T cells.....                                                                                                                              | <b>96</b>  |
| <b>Figure 3.3.</b> Expression of FLAG-tagged MARCH1 and MARCH8 following transfection of 293T cells..                                                                                                                | <b>98</b>  |
| <b>Figure 3.4.</b> Schematic representation of the human MARCH proteins studied in this thesis. ....                                                                                                                 | <b>101</b> |
| <b>Figure 3.5.</b> DOX-inducible expression of MARCH proteins in lentivirus-transduced 293T cells. ....                                                                                                              | <b>102</b> |
| <b>Figure 3.6.</b> Cell lines with DOX-inducible expression of MARCH1/2/3/8 proteins downregulate cell-surface expression of CD86..                                                                                  | <b>104</b> |
| <b>Figure 3.7.</b> DOX-inducible expression of different MARCH proteins in 293T cells does not result in major differences in the early stages of IAV infection, as assessed by intracellular expression of IAV-NP.. | <b>106</b> |



|                                                                                                                                                                                                                         |            |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>Figure 3.8.</b> The impact of DOX-inducible expression of different MARCH proteins on the release of infectious IAV from 293T cells. ....                                                                            | <b>108</b> |
| <b>Figure 3.9.</b> DOX-inducible expression of MARCH8 in 293T cells restricts early events in RSV infection, as assessed by intracellular expression of RSV-NP.....                                                     | <b>110</b> |
| <b>Figure 3.10.</b> DOX-inducible expression of different MARCH proteins in 293T cells does not result in major differences in early events in hMPV infection, as assessed by intracellular expression of hMPV-NP. .... | <b>111</b> |
| <b>Figure 3.11.</b> Impact of DOX-inducible expression of different MARCH proteins on cell-associated and cell-free pneumovirus particles released from 293T cells. ....                                                | <b>113</b> |
|                                                                                                                                                                                                                         |            |
| <b>Figure 4.1.</b> The impact of DOX-inducible expression of MARCH1 or MARCH8 on multicycle growth of IAV in 293T cells. ....                                                                                           | <b>129</b> |
| <b>Figure 4.2.</b> DOX-inducible expression of MARCH8 mediates antiviral activity against IAV in A549 and HeLa cells. ....                                                                                              | <b>131</b> |
| <b>Figure 4.3.</b> DOX-inducible expression of MARCH8 mediates antiviral activity against a broad range of influenza virus strains.....                                                                                 | <b>133</b> |
| <b>Figure 4.4.</b> DOX-inducible MARCH8 RING-CH mutants do not mediate antiviral activity against IAV in 293T cells .....                                                                                               | <b>135</b> |
| <b>Figure 4.5.</b> Effect of DOX-inducible expression of MARCH1 or MARCH8 in the copy number of vRNA matrix (M) gene segment and in the infectivity per particle of IAV released from infected cells.....               | <b>138</b> |
| <b>Figure 4.6.</b> Effect of DOX-inducible MARCH8 on expression of the influenza virus HA, NA and M2 proteins on the surface of IAV-infected cells .....                                                                | <b>139</b> |
| <b>Figure 4.7.</b> DOX-inducible expression of MARCH8 reduces incorporation of viral structural proteins into newly-synthesized IAV particles released from IAV-infected cells .....                                    | <b>142</b> |
| <b>Figure 4.8.</b> Effect of DOX-inducible expression of MARCH8 on HA and NA activity in supernatants from IAV-infected cells .....                                                                                     | <b>144</b> |
| <b>Figure 4.9.</b> DOX-inducible expression of MARCH8 alters the morphology of newly-synthesized virions released from IAV-infected cells.....                                                                          | <b>146</b> |
| <b>Figure 4.10.</b> Recombinant IAV with K→R substitutions in the cytoplasmic tails HA, NA and M2 proteins remain susceptible to MARCH8-mediated antiviral activity....                                                 | <b>148</b> |

|                                                                                                                                                                        |            |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>Figure 4.11.</b> Recombinant IAV with K→R substitutions in the M1 protein remain susceptible to MARCH8-mediated antiviral activity.....                             | <b>150</b> |
| <b>Figure 4.12.</b> Effect of MARCH8 siRNA knockdown on the viral titres of infectious IAV released from 293T cells.....                                               | <b>152</b> |
| <b>Figure 5.1.</b> Sequence analysis of MARCH1 and MARCH8 proteins.....                                                                                                | <b>170</b> |
| <b>Figure 5.2.</b> Generation of a chimeric MARCH8 protein expressing the C-terminal CT of MARCH1. ....                                                                | <b>172</b> |
| <b>Figure 5.3.</b> Impact of the C-terminal CT of MARCH1 on MARCH8-mediated restriction of IAV infection.....                                                          | <b>173</b> |
| <b>Figure 5.4.</b> Generation of chimeric MARCH8 proteins expressing either the DIRT, TM1, loop or TM2 from MARCH1.....                                                | <b>176</b> |
| <b>Figure 5.5.</b> Impact of the DIRT, TM1, loop and TM2 domains of MARCH1 on MARCH8-mediated restriction of IAV infection .....                                       | <b>177</b> |
| <b>Figure 5.6.</b> Generation of chimeric MARCH8 proteins expressing either the N-terminal CT or RING-CH domain of MARCH1 .....                                        | <b>179</b> |
| <b>Figure 5.7.</b> Impact of the N-terminal CT and RING-CH domains of MARCH1 on MARCH8-mediated restriction of IAV infection .....                                     | <b>181</b> |
| <b>Figure 5.8.</b> Generation of chimeric MARCH8 proteins expressing either the TM1-TM2 or the TM1-loop-TM2 domains of MARCH1.....                                     | <b>183</b> |
| <b>Figure 5.9.</b> Impact of the TM1-TM2 and the TM1-loop-TM2 domains of MARCH1 on MARCH8-mediated restriction of IAV infection.....                                   | <b>185</b> |
| <b>Figure 5.10.</b> Generation of chimeric MARCH8 proteins expressing All Cytoplasmic Domains of MARCH1.....                                                           | <b>186</b> |
| <b>Figure 5.11.</b> Impact of all cytoplasmic domains of MARCH1 on MARCH8-mediated restriction of IAV infection .....                                                  | <b>188</b> |
| <b>Figure 6.1.</b> Weight loss and virus replication in the airways following a total respiratory tract (TRT) infection of wild-type and MARCH8 KO mice with IAV ..... | <b>201</b> |
| <b>Figure 6.2.</b> Weight loss and virus replication in the airways following an upper respiratory tract (URT) infection of wild-type and MARCH8 KO mice with IAV ..   | <b>203</b> |

|                                                                                                                                                    |                |
|----------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| <b>Figure 6.3.</b> Airway inflammatory responses following a TRT infection of wild-type and MARCH8 KO mice with IAV at day 5 post-infection .....  | <b>205</b>     |
| <b>Figure 6.4.</b> Airway inflammatory responses following a TRT infection of wild-type and MARCH8 KO mice with IAV at day 7 post-infection .....  | <b>207</b>     |
| <b>Figure 6.5.</b> Airway inflammatory responses following a TRT infection of wild-type and MARCH8 KO mice with IAV at day 10 post-infection ..... | <b>209</b>     |
| <b>Figure 6.6.</b> Differential expression of cell-surface receptors in NK cells from WT and MARCH8 KO mice .....                                  | <b>211</b>     |
| <b>Figure 6.7.</b> <i>In vitro</i> infection of peritoneal macrophages from wild-type and MARCH8 KO mice with different IAV.....                   | <b>214</b>     |
| <br><b>Figure 7.1.</b> Overview of studies investigating the anti-IAV activity of MARCH8 described in this thesis. ....                            | <br><b>234</b> |

## List of Tables

|                                                                                                  |           |
|--------------------------------------------------------------------------------------------------|-----------|
| <b>Table 1.1.</b> Tissue distribution and cellular location of the mammalian MARCH proteins..... | <b>41</b> |
| <b>Table 2.3.1.</b> Primers used for cloning into pTRE-tight plasmid .....                       | <b>54</b> |
| <b>Table 2.3.2.</b> Primers and probe used for vRNA quantification.....                          | <b>54</b> |
| <b>Table 2.3.3.</b> Degenerate primers used to generate recombinant IAV .....                    | <b>54</b> |
| <b>Table 2.3.4.</b> Primers used to determine relative expression of MARCH8 .....                | <b>55</b> |
| <b>Table 2.3.5.</b> Primers used to generate MARCH8-MARCH1 chimeric mutants. ....                | <b>55</b> |
| <b>Table 2.4.1.</b> Antibodies used to detect virus-infected cells by flow cytometry.....        | <b>56</b> |
| <b>Table 2.4.2.</b> Antibodies used to detect IAV viral proteins by western blot .....           | <b>56</b> |
| <b>Table 2.4.3.</b> Antibodies used to detect immune cell populations by flow cytometry .....    | <b>56</b> |

## List of Abbreviations

### A

|             |                                           |
|-------------|-------------------------------------------|
| A:          | alanine                                   |
| AEC:        | airway epithelial cells                   |
| AMPK:       | adenosine monophosphate- dependent kinase |
| AM $\phi$ : | airway macrophages                        |
| ANOVA:      | analysis of variance                      |
| APC:        | antigen presenting cell                   |
| ATCC:       | American Type Culture Collection          |
| ATP:        | adenosine triphosphate                    |

### B

|      |                         |
|------|-------------------------|
| BAL: | bronchioalveolar lavage |
|------|-------------------------|

### C

|             |                                                           |
|-------------|-----------------------------------------------------------|
| C-terminal: | carboxyl-terminal                                         |
| C:          | cysteine                                                  |
| Cas9:       | CRISPR-associated protein 9                               |
| CBA:        | cytometric bead array                                     |
| CCL2:       | C-C motif chemokine ligand 2                              |
| CD:         | cluster of differentiation                                |
| cDNA:       | complementary deoxyribonucleic acid                       |
| cOVA:       | cytoplasmic ovalbumin                                     |
| CRISPR:     | clustered regularly interspaced short palindromic repeats |
| cRNA:       | complementary ribonucleic acid                            |
| CT:         | cytoplasmic tail                                          |
| CX3CR1:     | CX3C-chemokine receptor 1                                 |
| CXCL10:     | C-X-C motif chemokine 10                                  |
| Cyp:        | cyclophilin                                               |

## **D**

|        |                                                       |
|--------|-------------------------------------------------------|
| D:     | aspartic acid                                         |
| DC:    | dendritic cell                                        |
| DENV:  | dengue virus                                          |
| DIRT:  | domain in between the RING-CH and the first TM domain |
| DNA:   | deoxyribonucleic acid                                 |
| DOX:   | doxycycline                                           |
| dsRNA: | double-stranded ribonucleic acid                      |

## **E**

|        |                                                    |
|--------|----------------------------------------------------|
| Env:   | envelope protein                                   |
| ER:    | endoplasmic reticulum                              |
| ESCRT: | endosomal sorting complexes required for transport |

## **F**

|       |                                     |
|-------|-------------------------------------|
| F:    | fusion protein                      |
| FACS: | fluorescence-activated cell sorting |
| FBS:  | fetal bovine serum                  |
| FDA:  | Food and Drug Administration        |
| FW:   | forward                             |

## **G**

|       |                                       |
|-------|---------------------------------------|
| G:    | glycoprotein                          |
| GAGs: | glycosaminoglycans                    |
| GBP:  | guanylate-binding protein             |
| GE:   | gene end                              |
| gMFI: | geometric mean fluorescence intensity |
| GS:   | gene start                            |

## **H**

|    |           |
|----|-----------|
| H: | histidine |
|----|-----------|

|         |                                                              |
|---------|--------------------------------------------------------------|
| HA/H:   | hemagglutinin                                                |
| HCV:    | hepatitis C virus                                            |
| HECT:   | Homologous to E6-AP Carboxyl Terminus                        |
| HEp-2:  | human epithelial type 2                                      |
| HeV:    | Hendra virus                                                 |
| HIV-1:  | human immunodeficiency virus 1                               |
| hMPV:   | human metapneumovirus                                        |
| HN:     | hemagglutinin-neuraminidase                                  |
| HPAI:   | highly pathogenic avian influenza                            |
| hr:     | hour                                                         |
| HRS:    | hepatocyte growth factor-regulated tyrosine kinase substrate |
| HTLV-1: | human T-lymphotropic virus type 1                            |

## I

|         |                                          |
|---------|------------------------------------------|
| I:      | isoleucine                               |
| IAV:    | influenza A virus                        |
| IB:     | inclusion bodies                         |
| IBV:    | influenza B virus                        |
| IFITM:  | interferon-induced transmembrane protein |
| IFN:    | interferon                               |
| IFNAR:  | interferon $\alpha/\beta$ receptor       |
| Ig:     | immunoglobulin                           |
| IL1RAP: | interleukin-1 receptor accessory protein |
| IRF:    | interferon regulatory factor             |
| ISG:    | interferon-stimulated gene               |

## J

|      |              |
|------|--------------|
| JAK: | janus kinase |
|------|--------------|

## K

|    |        |
|----|--------|
| K: | lysine |
|----|--------|

KO: knockout  
KSHV: Kaposi's Sarcoma-associated herpesvirus

## **L**

L: leucine  
LB: Luria-Bertani

## **M**

M: matrix protein  
mAb: monoclonal antibody  
MARCH: membrane associated RING-CH  
MAVS: mitochondrial antiviral signaling protein  
MDA-5: melanoma differentiation associated gene 5  
MDCK: Madin-Darby canine kidney  
MDM: monocyte-derived macrophages  
MeV: measles virus  
MHC: major histocompatibility complex  
min: minute  
mL: milliliter  
MLMS: mouse linkage marker set  
MOI: multiplicity of infection  
MOV10: Moloney leukemia virus 10  
mRNA: messenger ribonucleic acid  
MuV: mumps virus  
Mx: myxovirus resistance

## **N**

N: asparagine  
N-terminal: amino-terminal  
NA: neuraminidase  
NAI: neuraminidase inhibitors



|                 |                                                                |
|-----------------|----------------------------------------------------------------|
| NEP:            | nuclear export protein                                         |
| NF- $\kappa$ B: | Nuclear Factor kappa-light-chain-enhancer of activated B cells |
| NiV:            | Nipah virus                                                    |
| NK:             | natural killer                                                 |
| NLR:            | NOD-like receptor                                              |
| NOD:            | nucleotide-binding oligomerization domain                      |
| NP/N:           | nucleoprotein                                                  |
| NS:             | non-structural protein                                         |

## O

|      |                                  |
|------|----------------------------------|
| OAS: | 2'- 5' oligoadenylate synthetase |
| ORF: | open reading frame               |

## P

|        |                                                            |
|--------|------------------------------------------------------------|
| P:     | phosphoprotein                                             |
| p.i.:  | post-infection                                             |
| PA:    | polymerase acidic protein                                  |
| pAb:   | polyclonal antibody                                        |
| PACT:  | protein activator of the interferon-induced protein kinase |
| PAI:   | plasminogen activator inhibitor                            |
| PAMPs: | pathogen-associated molecular patterns                     |
| PB:    | polymerase basic protein                                   |
| PBS:   | phosphate buffered saline                                  |
| PCR:   | polymerase chain reaction                                  |
| pdm:   | pandemic                                                   |
| PEC:   | peritoneal exudate cells                                   |
| PFU:   | plaque-forming units                                       |
| PIV:   | parainfluenza virus                                        |
| PKP:   | plakophilin                                                |
| PKR:   | protein kinase R                                           |
| PLA:   | proximity ligation assay                                   |

PRR: PAMP recognition receptors  
PVDF: polyvinylidene difluoride

## **Q**

Q: glutamine  
qPCR: quantitative polymerase chain reaction

## **R**

R: arginine  
RFU: relative fluorescence units  
RIG-I: retinoic acid-inducible gene I  
RING: really interesting new gene  
RLR: RIG-I-like receptors  
RNA: ribonucleic acid  
rpm: revolutions per minute  
RSV: respiratory syncytial virus  
RT: room temperature  
RV: reverse

## **S**

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2  
SD: standard deviation  
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis  
SH: small hydrophobic protein  
shRNA: short hairpin ribonucleic acid  
SIA: sialic acid  
siRNA: small interfering ribonucleic acid  
ssRNA: single-stranded ribonucleic acid  
STAT: signal transducer and activator of transcription

## **T**

|           |                                                                    |
|-----------|--------------------------------------------------------------------|
| T:        | threonine                                                          |
| TANK:     | TRAF family member Associated NF-kB Activator                      |
| TDRD7:    | tudor domain-containing 7                                          |
| TfR:      | transferrin receptor                                               |
| TGN:      | trans Golgi network                                                |
| TLR:      | Toll-like receptor                                                 |
| TM:       | transmembrane                                                      |
| TRAF:     | TNF receptor associated factors                                    |
| TRAIL-R1: | tumor necrosis factor-related apoptosis inducing ligand receptor 1 |
| TRE:      | tetracycline responsive element                                    |
| TRIM:     | tripartite motif containing                                        |
| TRT:      | total respiratory tract                                            |

## **U**

|      |                         |
|------|-------------------------|
| Ub:  | ubiquitin               |
| URT: | upper respiratory tract |

## **V**

|       |                            |
|-------|----------------------------|
| v:    | volume                     |
| V:    | valine                     |
| vRNA: | viral ribonucleic acid     |
| vRNP: | viral ribonucleoprotein    |
| VS:   | virospot                   |
| VSV:  | vesicular stomatitis virus |

## **W**

|      |                           |
|------|---------------------------|
| w:   | weight                    |
| WHO: | World Health Organization |
| WT:  | wild-type                 |

## **Z**

ZAP: zinc finger antiviral protein

ZIKV: Zika virus

μg: microgram

μL: microliter

μm: micrometer

°C: degree Celsius



## Chapter 1

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# Literature Review

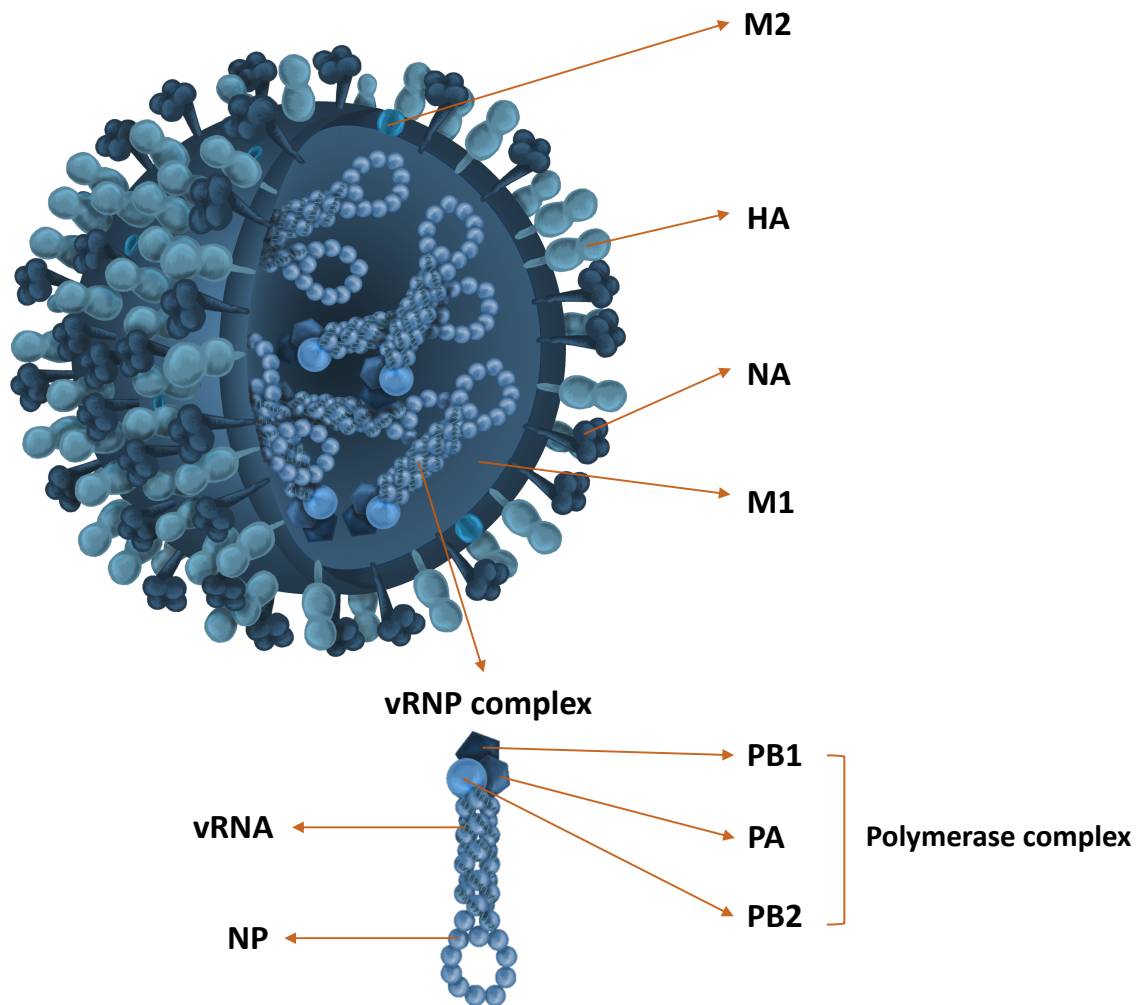
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In humans, acute respiratory infections of the upper and lower respiratory tract are generally caused by bacteria and viruses. Viruses commonly associated with respiratory disease in humans include influenza viruses, respiratory syncytial virus, parainfluenza viruses and rhinovirus, while adenoviruses, enteroviruses, metapneumoviruses, coronaviruses and others are also detected, but less frequently. For the purposes of this thesis, we will focus on members of the *Orthomyxoviridae*, *Pneumoviridae* and *Paramyxoviridae* families that are associated with respiratory infections in humans.

## **1. *Orthomyxoviridae* family**

### **1.1. General features.**

Within the *Orthomyxoviridae* family, influenza viruses are commonly associated with respiratory disease in humans. Influenza viruses are enveloped viruses with a genome comprising 8 segments of single-stranded negative sense RNA that encode up to 15 proteins, of which only 9 are incorporated into the virion (**Figure 1.1**). Among the structural proteins, three transmembrane proteins are expressed within the viral envelope: the hemagglutinin (HA), which mediates recognition of sialylated receptors, as well as fusion of the viral and endosomal membranes; the neuraminidase (NA), which is responsible for the release of newly-synthesized virions from the surface of virus-infected cells and the matrix protein (M)2, a small protein that acts as a pH-dependent ion channel which is important during early and late stages of virus replication. Multiple copies of M1 form a shell beneath the viral envelope which encapsulates the genetic material of the virus. Each segment of viral genomic RNA is encapsidated by multiple



**Figure 1.1. Schematic diagram of influenza A virion.** The transmembrane proteins hemagglutinin (HA), neuraminidase (NA) and matrix protein 2 (M2) are embedded within the host-derived viral envelope. Matrix protein 1 (M1) forms a layer beneath the viral envelope encapsulating the 8 segments of negative sense single-stranded viral RNA. Each viral (v)RNA is encapsidated by multiple copies of the nucleoprotein (NP) and tightly associated with the polymerase subunits PB1, PB2 and PA, forming viral ribonucleoprotein (vRNP) complexes. Adapted with permission from image prepared by Dr. Philippa Saunders, University of Melbourne.



copies of the nucleoprotein (NP) and tightly associated with the polymerase complex (comprised of viral proteins PB1, PB2 and PA) to form viral ribonucleoprotein (vRNPs) complexes. Among the non-structural (NS) proteins encoded by the virus, only the nuclear export protein (NEP) is incorporated into the virion. At least 6 more non-structural proteins are encoded by influenza viruses, including non-structural proteins NS1, PB1-F2, PB1-N40, PA-X, PA-N155 and PA-N182. These proteins play important roles when expressed within virus-infected cells, acting to modulate genomic replication, antagonize host immune responses and enhance viral pathogenesis [1-3].

Influenza viruses can be divided into types A, B, C and D based on differences in the viral NP and M genes. Type A, B and C viruses readily infect humans, whereas the main natural reservoir for influenza D viruses appears to be cattle [4]. Type A and B influenza viruses tend to be associated with severe disease in humans whereas type C viruses are rarely reported as a cause of human illness. Influenza A virus (IAV) can be further divided in subtypes depending on the genetic and antigenic properties of the HA and the NA glycoproteins and the genes that encode them. To date, 18 distinct HA subtypes (H1-H18) and 11 NA subtypes (N1-N11) have been identified, with most isolated from aquatic birds, although some have been identified in bats and other mammals [5]. Influenza B virus (IBV) only infect humans and are not classified into subtypes. Instead, IBV are classified into two distinct lineages based on differences in the HA glycoprotein: B/Victoria, named after the B/Victoria/2/1987 strain, and B/Yamagata, named after the B/Yamagata/16/1988 strain (reviewed in [6]).

Both type A and type B influenza viruses are associated with seasonal epidemics. These generally arise following accumulation of point mutations in the genes encoding the HA

and the NA surface glycoproteins. As HA represents the major target of neutralizing antibodies, the accumulation of amino acid changes in key antigenic sites can promote escape from neutralising antibodies raised to previous vaccination and/or infection. This process is referred to as *antigenic drift*. IAV, but not IBV, also undergo a second major mechanism of antigenic variation known as *antigenic shift*. This refers to the emergence of a novel IAV in the human population that expresses a HA and/or NA that is immunologically distinct to those of recently circulating IAV strains. As there is limited pre-existing immunity in the human population, the new virus has the potential to spread and cause a global pandemic. IAV with pandemic potential can result from direct transmission of an avian/mammalian virus into humans, or as a result of reassortment between virus strains from different hosts due to the segmented nature of the viral genome (reviewed in [7]). In the last 100 years, only the H1N1, H2N2 and H3N2 subtypes have been associated with sustained human-to-human transmission, resulting in pandemics in 1918, 1957, 1968 and 2009 [8]. Although direct transmission of highly pathogenic avian influenza (HPAI) H5N1 and other subtypes of avian viruses often results in severe disease in humans, little evidence of effective human-to-human transmission has been reported to date (reviewed in [9]).

Vaccination and antiviral drug treatment represent the most common strategies used to limit the impact of influenza disease in the human population. Vaccination represents the most effective method for the prevention and control of influenza, reducing illness and the severity of infection in high-risk groups, including children and the elderly (reviewed in [10]). Influenza vaccines require annual evaluation and reformulation to ensure a good match between the vaccine and recently circulating strains, which

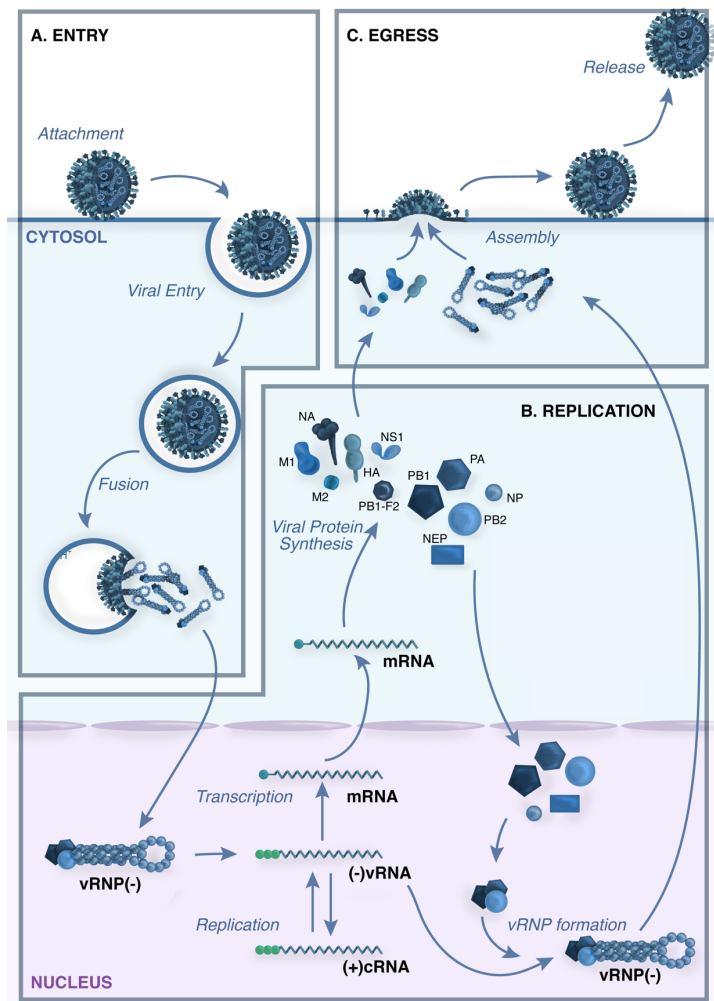
continually change as a result of antigenic drift [11]. Inhibitors of the viral M2 ion channel (adamantanes), the NA (NA inhibitors, NAI) and the polymerase complex represent the antiviral drugs currently approved for use in humans, although most circulating human IAV are now resistant to adamantanes [12]. NAI are widely used, although their effectiveness is currently not fully understood (reviewed in [13]). Drugs targeting the polymerase complex are the newest antiviral compounds approved, and their use and effectiveness are still being evaluated (reviewed in [14]).

## **1.2. Infection of host cells**

Airway epithelial cells (AEC) represent the primary targets of influenza virus infection, although immune cells such as airway macrophages (AM $\phi$ ) and dendritic cells (DC) are also susceptible. While infection of AEC by seasonal influenza viruses results in productive replication and amplification of virus, this process is generally considered abortive in AM $\phi$  and DC, and infectious progeny are not released (reviewed in [15]). For the purposes of this thesis, we have focussed on influenza virus infection of epithelial cells. **Figure 1.2** shows an overview of the influenza virus replicative cycle.

### *1.2.1. Early stages of influenza virus infection*

Irrespective of cell type, influenza virus infection is initiated following attachment of the viral HA glycoprotein to terminal sialic acid (SIA) expressed by glycoproteins and glycolipids on the surface of target cells. SIA is generally attached to underlying galactose residues by an  $\alpha$ 2-3 or  $\alpha$ 2-6 linkage and the nature of this linkage is an important



**Figure 1.2.** Influenza A virus replicative cycle. **(A) ENTRY.** Influenza virus HA attaches to host cell receptors containing sialic acid moieties. Virus is internalized by receptor-mediated endocytosis. Within the endosome the low pH induces the fusion between the viral envelope and the endosomal membrane releasing the vRNP complexes to the cytosol. **(B) REPLICATION.** After the vRNP are imported to the nucleus, vRNA is transcribed and replicated by the viral polymerase complex. Viral proteins are synthesized by host ribosomal complexes in the cytosol. HA, NA and M2 are transported to the plasma membrane through the secretory pathway. The polymerase complex, M1 and NP are imported to the nucleus for the formation of new vRNPs. Newly-formed vRNPs are exported to the site of virus assembly. **(C) EGRESS.** HA and NA accumulates in lipids rafts at the cell surface. M1 interacts with HA, NA and vRNP at the plasma membrane for the assembly and budding of newly-synthesized virions. After M2-mediated scission of viral particles, NA cleaves cell surface sialic acids for the final virus release. Figure adapted from [16].

determinant of virus tropism. For example, the HA of avian influenza viruses binds preferentially to  $\alpha$ 2-3-linked SIA and this is the predominant linkage expressed in the gastrointestinal and respiratory tracts of birds. In contrast, the HA of most human strains shows preference for  $\alpha$ 2-6 linked SIA, which is the predominant linkage in the human respiratory tract [17].

Following virus attachment, entry generally occurs via receptor-mediated endocytosis, although the specific entry receptors for influenza virus remain poorly defined, particularly for AEC. Clathrin- or caveolae-mediated endocytosis is the dominant entry route for IAV into AEC, and also in M $\phi$ , where C-type lectin receptors have been defined as attachment and entry receptors that can facilitate this process (reviewed in [18]). However, IAV entry pathways also appear to vary depending on the cell type, viral strains and culture conditions utilised, highlighted by studies implicating viral entry can also occur through macropinocytosis [19-21]. After internalization, virions are trafficked to late endosomes where the viral HA is activated following induction of a pH-dependent conformational change, exposing the fusion peptide, which binds to the endosomal membrane to facilitate fusion. Together with this, the interior of the virus particle is acidified by the M2 ion channel, allowing the depolymerization of M1 and subsequent dissociation from the vRNPs. As a result, the vRNPs are released through the fusion pore into the cytosol and then imported into the nucleus, where genomic transcription and replication occurs (reviewed in [22]). Inside the nucleus, each viral (v)RNA is processed by the polymerase complex into messenger (m)RNA for protein synthesis and complementary (c)RNA that is used as template to produce the new vRNAs that will be incorporated into new virions (reviewed in [23]). After translation of the mRNA encoding

for PB1, PB2, PA, NP, M1, NS1 and NEP in cytoplasmic ribosomes, each protein is imported into the nucleus, to form the new vRNPs. Translation of the HA, NA and M2 occurs in endoplasmic reticulum-associated ribosomes, where HA and NA proteins undergo glycosylation during transport to the plasma membrane (reviewed in [24]).

#### *1.2.2. Late stages of influenza virus infection*

Influenza virus assembly and budding occurs at the cell surface. Thus, HA, NA and M2 are transported to the plasma membrane through the trans-Golgi network and are the first viral proteins to reach the virus assembly point. During transport, the viral HA must undergo proteolytic cleavage by host proteases in order to acquire the fusogenic capacity that is essential during virus entry [25]. Additionally, M2 prevents the premature activation of HA during its trafficking through the acidic secretory pathway, equilibrating the pH between the Golgi lumen and the cytoplasm [26, 27]. Once at the plasma membrane, HA and NA accumulate in cholesterol- and sphingolipid-enriched domains or “lipid rafts”, while M2 is localized more uniformly at the cell surface [28]. Meanwhile, newly-assembled vRNPs are exported from the nucleus and transported to the virus assembly site together with M1, in a Rab11-dependent manner [29]. At the plasma membrane, M1 interacts with the cytoplasmic tails (CTs) of HA and NA, allowing the recruitment of vRNPs to the virus assembly site [30]. Moreover, M1 also binds to the plasma membrane where, after undergoing oligomerization, it mediates the mechanical stress required to initiate virus budding [31]. M1 also recruits M2 to the periphery of the budzone, where M2 induces membrane scission of the newly-formed virions, which occurs independently of the endosomal sorting complex required for transport (ESCRT) machinery [32]. Finally, the enzymatic activity of the viral NA allows

for cleavage of SIA, preventing virus aggregation at the cell surface and promoting the release of newly-synthesised virions [33].

## **2. *Pneumoviridae* family**

### **2.1. General features**

Respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) are important pathogens associated with lower respiratory tract infections in children, including croup, bronchiolitis and pneumonia (reviewed in [34, 35]). Both viruses belong to the *Pneumoviridae* family with each representing a different genus: *Metapneumovirus* (hMPV) and *Orthopneumovirus* (RSV) [36]. Pneumoviruses are enveloped viruses with a genome comprised of non-segmented, negative sense, single-stranded RNA which encodes 9-11 proteins. The RSV genome is slightly larger than that of hMPV and contains two additional genes that encode for the non-structural NS1 and NS2 proteins which are present in orthopneumoviruses, but not in metapneumoviruses. Three transmembrane proteins are expressed within the viral envelope: the glycoprotein (G), responsible for attachment of virions to cell surface receptors; the fusion protein (F), which contributes to virus attachment and promotes fusion of viral and host cell membranes, and the small hydrophobic protein (SH), which is thought to act as a viroporin and/or inhibit innate immune responses [37, 38]. Multiple copies of the matrix protein (M) interact with cytoplasmic domains of the F protein to form a layer beneath the viral envelope. The vRNA is coated by the nucleoprotein (N) and forms a long helical nucleocapsid, which serves as the template for RNA synthesis by the RNA-dependent RNA polymerase. The

nucleocapsid also contains copies of the viral phosphoprotein (P), which acts as a polymerase cofactor, as well as the large RNA-dependent RNA polymerase (L) (reviewed in [39, 40]) (**Figure 1.3**). The viral M2-1 protein also interacts with the viral nucleocapsids and, at least for RSV, forms a layer underneath M, acting as a bridge between M and the nucleocapsid complex [41, 42].

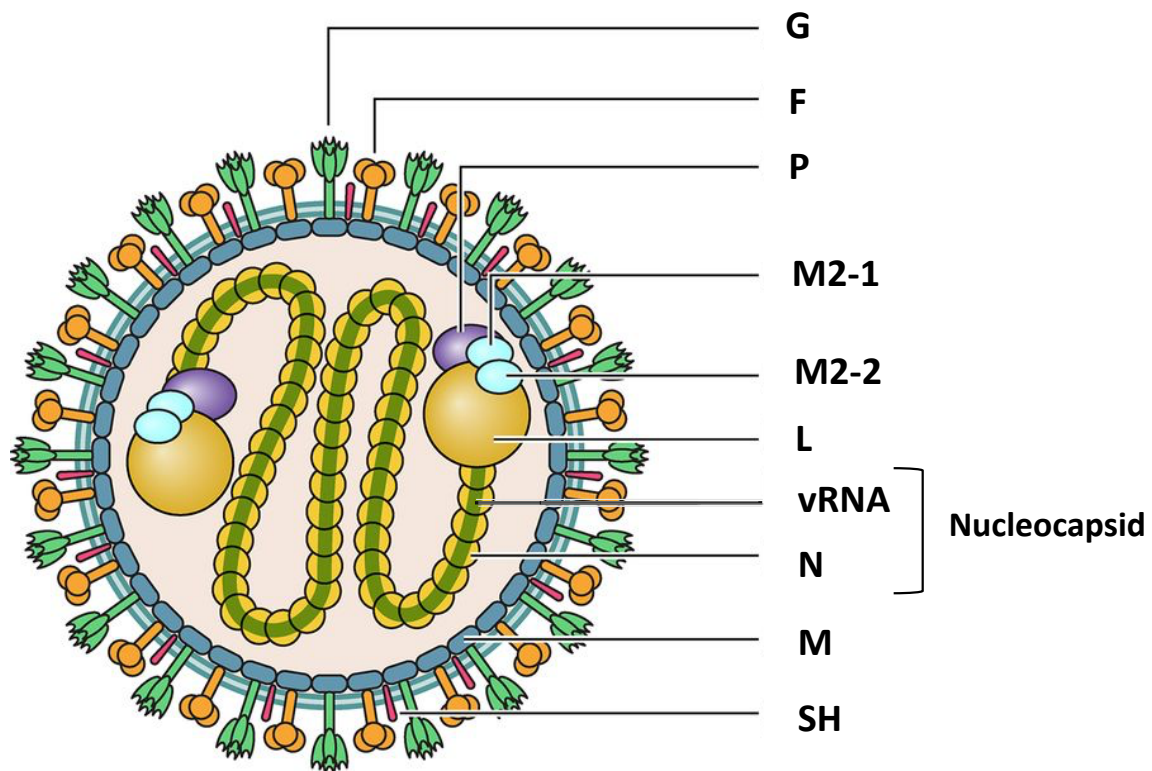
In terms of classification, RSV can be divided in two antigenic subgroups (A and B) that differ in the hypervariable region of the G protein gene (reviewed in [43]). hMPV can be also divided into A and B lineages, however each can be further divided into the sub-lineages A1, A2, B1 and B2 [44].

Compared to influenza virus, less is known about RSV and hMPV in terms of pathogenesis and disease burden. Moreover, while some antiviral drugs and vaccines (particularly for RSV) are currently under evaluation in clinical trials, none are licensed for use in humans [45]. Currently, a humanized monoclonal antibody against the F glycoprotein of RSV (Palivizumab) is the only US Food and Drug Administration (FDA)-approved therapy for use in high-risk infants, however its efficacy and cost efficiency remain to be fully elucidated [46].

## **2.2. Infection of host cells**

Like influenza viruses, RSV and hMPV have been reported to replicate productively in AEC. Interestingly, both pneumoviruses are able to infect alveolar macrophages,





**Figure 1.3. Schematic diagram of pneumovirus virion.** The transmembrane proteins small hydrophobic (SH), glycoprotein (G) and fusion protein (F) are expressed within the host-derived viral envelope. The matrix protein (M) forms a layer beneath the viral envelope. The viral (v)RNA is encapsidated by multiple copies of the nucleoprotein (N), forming the helical nucleocapsid. The nucleocapsid also contain copies of the phosphoprotein (P) and the large RNA-dependent RNA-polymerase (L). Matrix 2 protein 1 and 2 (M2-1 and M2-2) are also incorporated into the virion. Figure adapted from [34].

however only hMPV appears to replicate productively in these cells after infection [47, 48].

#### 2.2.1. Early stages of pneumovirus replication

As for influenza viruses, attachment and entry represent the first steps of pneumovirus infection of host cells. The F protein is sufficient to mediate both steps, however the G protein also plays an important role during attachment, particularly *in vivo*. Different cell-surface molecules have been implicated as attachment factors, including glycosaminoglycans (GAGs) such as heparan sulfate. However, whether sufficient levels of heparan sulfate are expressed on the apical surface of airway epithelial cells, the main target of pneumovirus infection *in vivo*, remains to be elucidated [49]. CX3C-chemokine receptor 1 (CX3CR1) and nucleolin have also been proposed as attachment factors and/or receptors for RSV. In this context, it has been shown that RSV-G can bind CX3CR1 [50, 51], while RSV-F also interacts with nucleolin [52]. Nonpermissive cells transfected to express integrin  $\alpha\text{v}\beta 1$  resulted in susceptibility to infection by hMPV, but not RSV, and this was mediated by interactions with the hMPV-F protein [53], suggesting that  $\alpha\text{v}\beta 1$  integrins represent functional receptors for hMPV infection.

While influenza viruses enter target cells via receptor-mediated endocytosis and pH-dependent fusion, it is widely accepted that pneumovirus entry is mediated by pH-independent fusion at the plasma membrane and only involves the F protein. However, recent reports indicate that RSV can also use macropinocytosis, followed by fusion in endosomal compartments to infect AEC and other cell types [54]. Additionally, hMPV can be internalized via clathrin-mediated endocytosis in a dynamin-dependent manner,

followed by pH-independent fusion with endosomal membranes [55]. Of interest, the F protein of some hMPV strains from the lineage A displayed enhanced fusogenic activity under acidic conditions in cell-cell fusion assays [56]. Altogether, these findings suggest that pneumovirus entry into host cells may be more complex than first thought and virus strain and/or cell type may be important factors in determining the specific pathway utilised for infection.

Following fusion and release of viral genome into the cytoplasm, both RSV and hMPV induce formation of inclusion bodies (IB) to facilitate efficient transcription and replication of vRNA [57, 58]. Transcription of the viral genome is mediated by the L protein, commencing at the 3' end of the vRNA. Each gene is flanked by its own promotor and terminator, referred to as gene start (GS) and gene end (GE) sequences, respectively. Following a sequential stop-start mechanism, the L protein is able to synthesise individual mRNAs, that are capped and polyadenylated, and then translated by the host ribosomal complexes. Although the “switch” mechanism is still unclear, the L protein is also responsible for replication of the genome, through the formation of a positive-sense copy of the full genome, known as the antigenome (reviewed in [35]).

### *2.2.2. Late stages of pneumovirus replication*

Although the assembly and budding processes of pneumoviruses are still largely unknown, important steps have been made towards understanding these processes in the context of RSV and hMPV infections. Similar to influenza virus, pneumoviruses assemble and bud from the plasma membrane of infected cells, requiring all viral components to co-localize at the site of assembly. Interestingly, while hMPV-F is cleaved

at a single site to gain fusogenic capacity [59], proteolytic cleavage of RSV-F occurs at two sites, generating F1 and F2 subunits, as well as a small glycosylated peptide of 27 amino acids, whose function remains unknown [60]. Once at the cell surface, F and G proteins are able to induce mechanical stress and produce short filament-like structures at the plasma membrane, however self-assembly and polymerization of the M protein upon lipid binding is essential for the formation of elongated filaments at the site of assembly. M is also important as a coordinator of the assembly process, since its absence during RSV infection has been associated with retention of nucleocapsid complexes in inclusion bodies, suggesting that M is also required for transport of nucleocapsid to the site of virus assembly [61-63]. Although the mechanism by which nucleocapsid is transported to the assembly sites is unknown, evidence to date supports a role for actin in this process [64].

In terms of localization, Chang *et al.* demonstrated that lipid rafts are required for the release of infectious RSV particles from virus-infected cells [65]. Consistent with these findings, RSV-F and other viral proteins were reported to localize to lipid rafts, and this localization was critical for the formation of infectious RSV particles [66]. Similar to influenza viruses, budding of newly-assembled RSV and hMPV is independent of the ESCRT-machinery, however the mechanism of scission currently remains unknown [67, 68].

In addition to budding and release of virions, direct cell-to-cell spreading also appears to be an important mechanism for dissemination of at least some members of the *Pneumoviridae* family [69]. One form of cell-to-cell spreading often seen *in vitro* for RSV and hMPV is cell-cell fusion [70, 71]. As a result of the accumulation at the plasma

membrane of infected cells, multiple F proteins then bind to the membrane of uninfected neighbouring cells and undergo a series of conformational changes to induce membrane fusion. After enlargement of the fusion pore, both membranes fuse completely, generating enlarged multinucleated cells called syncytia. Ultimately, the cytosolic content is mixed and IBs and nucleocapsids are transfer from one cell to another. Another form of direct cell-to-cell spreading is the formation of intercellular extensions or filopodia. RSV-F is able to induce filopodia at the cell surface of infected cells, thereby shuttling RSV particles to neighbouring cells [72]. Similar structures are also formed at the surface of hMPV-infected cells. Interestingly, it was proposed that hMPV filopodia could facilitate a direct connection between the cytoplasm of neighbouring cells, potentially transporting vRNA and IBs between infected and non-infected target cells [73].

### **3. Paramyxoviruses.**

Another important family of viruses responsible for respiratory infections in humans is the *Paramyxoviridae* family. After a recent update in virus classification, this family can be divided in 7 genera, of which only 4 contain mammalian paramyxoviruses: *Morbillivirus* (measles virus, MeV), *Respirovirus* (human parainfluenza viruses 3, PIV-3), *Rubulavirus* (mumps virus, MuV) and *Henipavirus* (Hendra virus, HeV) (reviewed in [74]). Paramyxoviruses are enveloped viruses containing a single stranded, non-segmented, negative-sense RNA genome coding at least 6-8 viral proteins following the canonical gene arrangement: 3' **N-P/V/C-M-F-RBP-L** 5', where **N** corresponds to the RNA-binding

nucleocapsid protein, **P** corresponds to a polymerase associated phosphoprotein, **M** corresponds to the matrix protein, **F** corresponds to the fusion protein, **RBP** corresponds to a attachment/receptor binding protein and **L** corresponds to the RNA-directed RNA-polymerase with cap and cap-methylation activities. Depending on the virus, RBP can be called **HN**, if the protein exhibits hemagglutinin and neuraminidase activities, **H**, if it exhibits only hemagglutinin activity or **G**, if the protein does not exhibit either of these activities. Some viruses express additional non-structural proteins known as C and V, both of which are derived from the P locus (reviewed in [75]).

Pneumoviruses and paramyxoviruses share similar features in their replicative cycles, however there are important differences, especially regarding the fusion protein. While it is known that the fusion proteins of RSV and hMPV can exert their fusogenic activity independent of the attachment protein, the F protein of paramyxoviruses strictly require the presence of the receptor binding protein (HN, H or G). Similar to pneumoviruses, the fusogenic activity of the F protein of paramyxoviruses is not triggered by low pH, however its activation depends on the activity of its specific attachment protein partner [76]. Two different models have been proposed to explain the attachment protein-mediated fusion activation: the association model and the dissociation model. The first model proposes that binding of the attachment protein to its specific receptor triggers conformational changes in the attachment protein that allow its interaction with the F protein. This interaction, in turn, induces activation of the F protein and subsequent membrane fusion. Conversely, the dissociation model proposes that the attachment and fusion proteins are already interacting at the surface of the virion. Upon binding receptor, conformation changes in the attachment protein

allow the dissociation and/or release of the F protein, allowing its activation (reviewed in [77]). To date, experimental evidence supports both models, suggesting that different viruses could use different mechanisms to induce the activation of the fusion protein (reviewed in [78]).

#### **4. Type I interferons and antiviral immunity**

##### **4.1. Induction of type I interferons.**

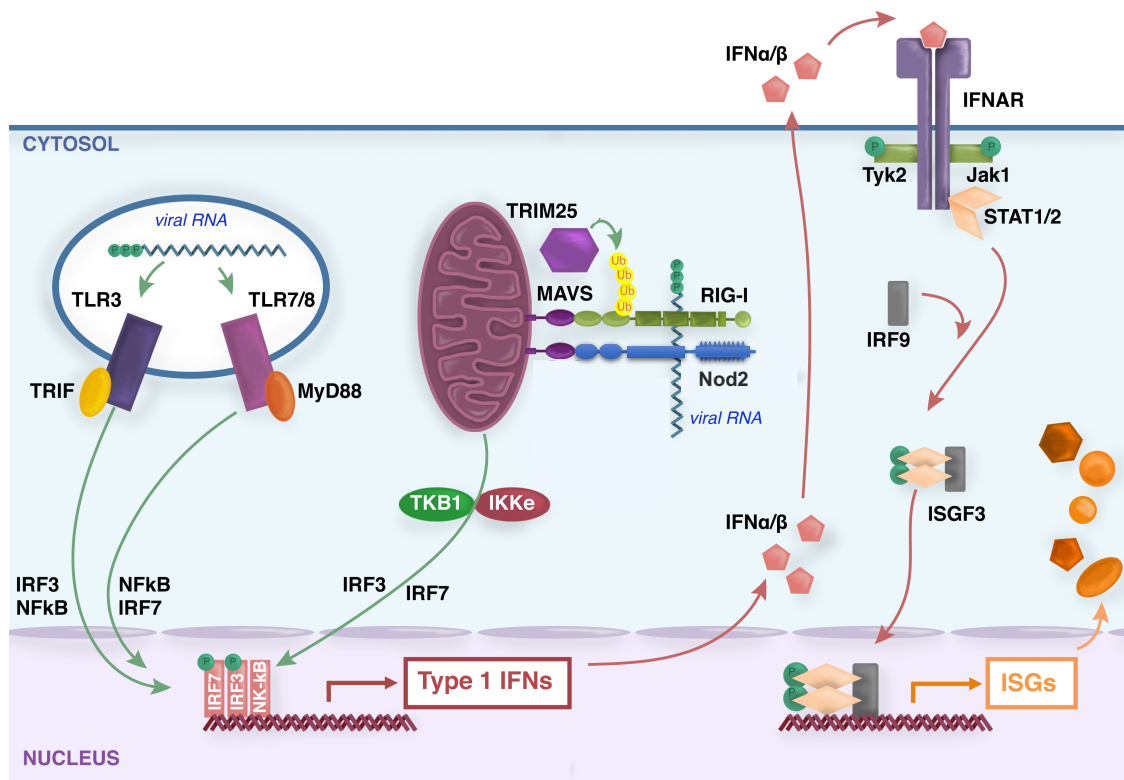
It is well established that infection of host cells by viruses, including a range of respiratory viruses, induces the secretion of type I interferon (IFN), a major arm of the innate antiviral response. Depending of the nature of the virus and/or the target cell, different pathways can be activated in order to induce type I IFNs. Induction of type I IFNs is dependent on a range of cellular pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) expressed by the virus and/or generated during the viral infection.

RNA viruses can be detected by at least three different classes of PRRs: Toll-like receptors (**TLR**), retinoic acid inducible gene (RIG)-I-like receptors (**RLR**) and nucleotide-binding oligomerization domain (NOD)-like receptors (**NLR**). TLR3 is expressed in endosomal compartments of a range of different cell types relevant to respiratory virus infection including AEC and DC (reviewed in [79]). Recognition of double-stranded (ds)RNA by TLR3 specific TRIF-dependent signalling pathways culminates in the nuclear translocation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) and IFN-regulatory factor 3 (IRF3) thereby

activating the transcription of type I IFNs (reviewed in [80]). Human TLR8 and mouse TLR7, also expressed within endosomal membranes, can sense ssRNA to promote the transcription of IFNs via MyD88-dependent activation of IFN-regulatory factor 7 (IRF7). In addition to endosomal PRRs, most cells in the body express cytosolic PRRs of the RLR family, including RIG-I and melanoma differentiation associated gene (MDA)-5, which recognize cytoplasmic viral RNA. RIG-I tends to recognize short RNA ligands with 5'-triphosphate caps that are generated during viral replication while MDA-5 tends to recognizes long kilobase-scale genomic viral RNA and replication intermediates (reviewed in [81]). Following recognition of ligand, RLRs bind ATP to facilitate interactions with the mitochondrial antiviral signalling protein (MAVS). This interaction promotes the aggregation of MAVS into microfibrils through a prion-like mechanism, resulting in the recruitment of signalling proteins from the cytosol. These proteins then enable the activation and translocation of IRF3, IRF7 and NF- $\kappa$ B to the nucleus to induce IFN production [82, 83]. Another class of PRRs expressed in immune and epithelial cells that can recognize ssRNA is Nod2. After sensing viral RNA, Nod2 can also associate with MAVS, inducing the production of IFN- $\beta$  through the activation of IRF3 [84].

Following intracellular sensing of viral infection by PRRs, type I IFNs secreted from virus-infected cells bind to the ubiquitously expressed interferon  $\alpha/\beta$  receptor (IFNAR). Signalling via the IFNAR activates the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, leading to transcription of IFN-stimulated genes (ISGs) as well as further amplification of IFN production (reviewed in [85]). This results in induction of an “antiviral state” in cells, which limits further virus replication and spread. A summary of the induction of type I IFN after viral infection is shown in **Figure 1.4**.





**Figure 1.4. Induction of type I interferons (IFNs) by respiratory viruses.** Depending on the entry route of the respiratory virus, different pattern recognition receptors (PRRs) can sense viral RNA (vRNA). Within the endosomes, vRNA can be exposed to toll-like receptors (TLR)3/7/8. In the cytosol, vRNA and potentially dsRNA by-products can be recognized by the ubiquitously expressed cytoplasmic sensor retinoic acid inducible gene (RIG)-I and/or nucleotide-binding oligomerization domain (Nod)2. TLR signaling by the adaptors TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF) or myeloid differentiation factor (MyD)88, and RIG-I or Nod2 signaling by the adaptor mitochondrial antiviral signaling (MAVS) protein, trigger signal transduction cascades that result in activation of IFN regulatory factor (IRF)3/7 and NF- $\kappa$ B factors that translocate to the nucleus to induce synthesis of type I IFN mRNAs. Secreted type I IFNs signal through the IFN- $\alpha/\beta$  receptor complex (IFNAR) via activation of the intracellular kinases Jak1 and Tyk2, which phosphorylate the signal transducer and activator of transcription (STAT) transcription factors that, together with IRF-9, form the interferon-stimulated gene factor 3 (ISGF3) that translocates to the nucleus and activates the transcription of IFN-stimulated genes (ISGs). The green circle represents a phosphate group. Figure adapted from [16].

## **4.2 Viral strategies to counteract antiviral immune responses mediated by interferons.**

Many viral proteins, both structural and non-structural, can antagonise the host IFN response. For some viruses there are specific viral genes that encode non-structural proteins, however non-structural viral proteins can also arise as the result of alternative open reading frames (ORFs) or alternative splicing of genes that normally express structural proteins [86]. Influenza viruses encode at least 2 non-structural proteins with known capacity to block IFN production: NS1 and PB1-F2. NS1 is encoded by the NS gene, the smallest segment of the IAV genome, and is highly expressed in infected cells. Its main role is to antagonize the RIG-I-dependent signalling pathway to block the production of IFNs in a number of different ways. NS1 binds dsRNA to prevent recognition and subsequent activation of RIG-I [87] and can bind RIG-I directly, in a strain dependent manner, to inhibit activation of IRF3 and therefore the induction of IFN- $\beta$  [88, 89]. NS1 can also modulate activation of RIG-I by binding to (i) protein activator of the interferon-induced protein kinase (PACT), a well-known cofactor that enhances the RIG-I-mediated IFN-production [90], or (ii) the E3 ligases Tripartite Motif Containing 25 (TRIM25) or Riplet [91] to inhibit ubiquitin-mediated activation of RIG-I. Moreover, NS1 can block other pathways associated with the production of IFN, including the NF- $\kappa$ B signalling pathway [92]. Finally, NS1 also blocks the activity of certain ISGs including 2'-5' oligoadenylate synthetase (OAS) [93] and protein kinase R (PKR) [94], amongst others.

PB1-F2 is translated from an alternative ORF in segment 2, the PB1 gene, of the IAV genome and localizes to the mitochondria. This non-structural protein affects the RIG-I signalling pathway by interacting with the adaptor protein MAVS, thereby inhibiting IFN production [95].

Structural proteins of IAV have also been implicated as antagonists of IFN responses. For example, the subunit PB2 of the polymerase complex has been shown to bind to MAVS, thereby inhibiting expression of IFN- $\beta$  [96]. Additionally, the M2 protein channel can target the ISG BST-2 (tetherin, CD317, and HM1.24), inducing its downregulation and subsequent proteasomal degradation [97].

Pneumoviruses have also evolved strategies to inhibit or antagonise IFN responses. The NS1 and NS2 proteins of RSV target similar immune molecules to those of IAV NS1, including RIG-I, TRIM25 and MAVS [98-100]. Interestingly, RSV NS1 and NS2 not only bind to target proteins to block the formation of signalling complexes, but also reduce their expression. It has been proposed that RSV NS1 and NS2 form a degradative complex with a number of additional host proteins, including MAVS. This complex, called the NS degradasome (NSD), is thought to exhibit proteasome-like activity, due to the presence of a subunit of the 20S core proteasome [101]. Supporting the idea of proteasomal-mediated degradation of immune proteins, NS1 has been identified as a putative E3 ligase responsible for degradation of STAT2 [102]. Other immune molecules known to be degraded after RSV infection include RIG-I and IRF7 [101]. In contrast, hMPV does not encode non-structural proteins. Instead, the structural proteins G, M2-2 and SH have been identified as modulators of IFN-mediated antiviral responses. In this context, hMPV G interacts with RIG-I, blocking the activation of downstream signalling molecules [103], whereas hMPV M2-2 interacts with MAVS, inhibiting the production of IFN- $\beta$  [104] or with IRF7, inhibiting the production of IFN- $\alpha$  [105]. In addition, hMPV SH can target STAT1 by blocking its phosphorylation [106] or NF- $\kappa$ B by altering its phosphorylation and/or acetylation [107]. Interestingly, the phosphoprotein

(P) of hMPV sub-lineage B1 has also been associated with an antagonistic effect against IFNs by preventing RIG-I-mediated sensing of ssRNA during infection [108].

Paramyxoviruses also encode non-structural proteins with IFN-antagonistic capacities. Depending on the virus, the V and/or C accessory proteins, both expressed from the P gene, have been identified as inhibitors of IFN-induced responses. Interestingly, paramyxoviruses can target proteins to block IFN production, such as MDA-5, as well as proteins involved in the IFN-signalling pathway, including STAT1 and STAT2 (reviewed in [109]).

## **5. Cellular restriction factors that mediate antiviral activity against respiratory viruses**

Type I IFNs are potent mediators of antiviral activity against a broad range of viruses, including respiratory viruses. While activation of the IFN signalling pathway results in induction of hundreds of ISGs within host cells, functional and biochemical analyses of only a small subset of ISG proteins have been described. Among them, some ISG proteins exhibit broad antiviral activity, whereas others appear to be specific for one or more virus families. Moreover, there are still many ISGs that have not been assessed for antiviral activity against different virus families. For most ISG proteins, there is currently little information regarding their spectrum of antiviral activity, or the underlying mechanisms by which they may limit or block viral infections. In addition to ISGs, other host proteins regulated independently of type I IFNs can also exhibit antiviral activity [110]. ISGs and other cellular proteins with antiviral activity (hereafter referred to as

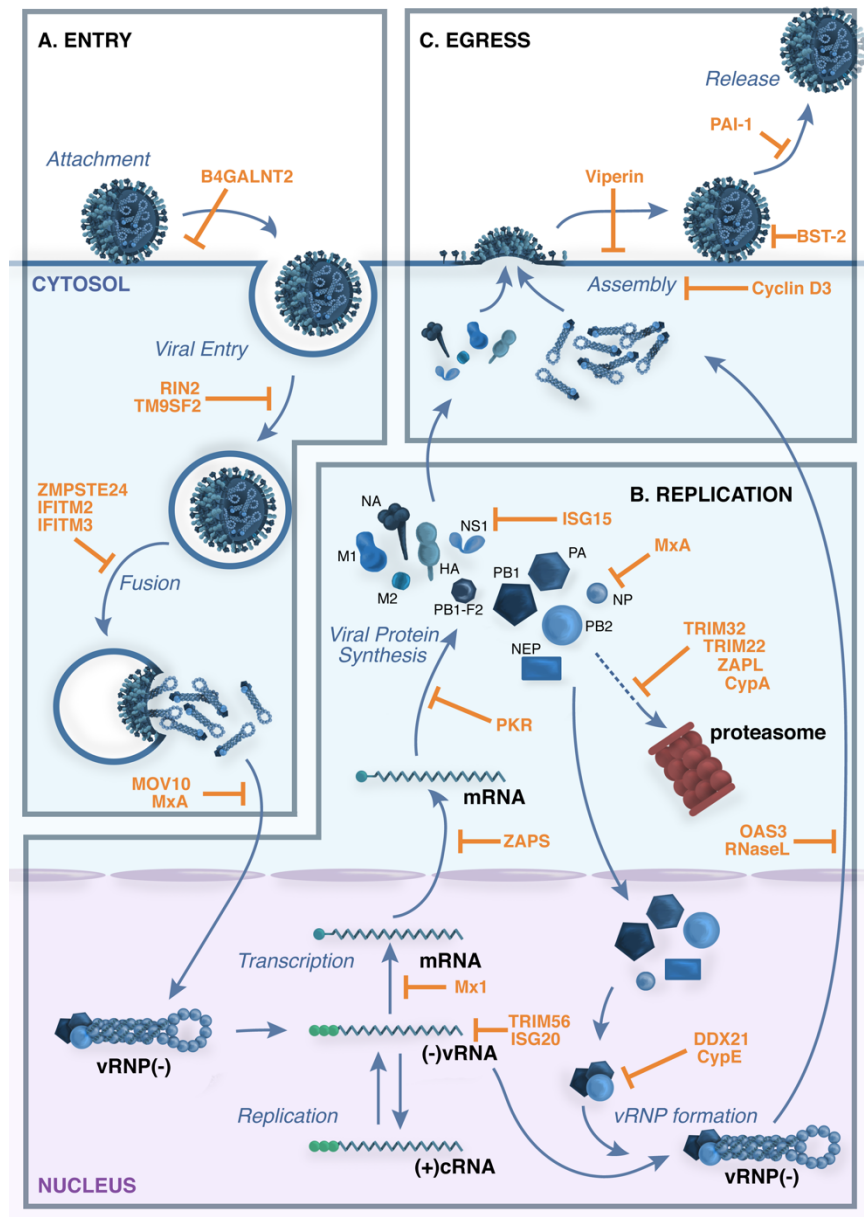
“restriction factors”) may be expressed constitutively at different levels in particular cell types and levels may be modulated by virus infection, type I IFNs and/or other factors.

### **5.1. Cellular restriction factors that act against influenza virus.**

Of the respiratory viruses, the majority of studies have focussed on identifying ISG proteins and cellular restriction factors that mediate antiviral activity against influenza viruses. Different antiviral factors have been shown to target different steps in the IAV replicative cycle, including virus entry, replication and exit. An overview of influenza virus replication and associated restriction factors is shown in **Figure 1.5**.

#### *5.1.1. Restriction factors that target virus attachment and entry*

One of the best studied cellular restriction factors that acts against influenza viruses is IFITM3, which localises to late endosomes/lysosomal compartments within host cells. IFITM3 does not inhibit influenza virus attachment and internalization but instead traps virions in endocytic compartments, blocking release of the viral genome into the cytoplasm [111]. Currently, the mechanism by which IFITM3 inhibits influenza virus infection is not fully understood, although accumulating evidence indicates it may block formation of a fusion pore after the hemifusion of the virus envelope with the endolysosomal membrane [112]. Experimental evidence also supports a role for IFITM3 in modulating influenza virus *in vivo*, as IAV infection of IFITM3 knockout mice was associated with exacerbated disease and mortality [113]. Moreover, previous reports have identified an enrichment of the rs12252-C allele of IFITM3 in patients hospitalized



**Figure 1.5. Host restriction factors that modulate influenza virus infection.** Influenza viruses can be targeted by multiple host restriction factors at each step of the virus replicative cycle. Examples of host restriction factors targeting influenza virus entry (A), replication (B) and egress (C) are shown in orange. Figure from [16].

during the H1N1 IAV pandemic in 2009 [114] and the correlation between this allele and influenza severity has been validated in some, but not all, subsequent human cohort studies. Currently, the role of other IFITM-family proteins (e.g. IFITM-1/2) in recognition and response to influenza viruses is less clear.

In addition to IFITMs, other host restriction factors have been implicated in blocking IAV attachment and/or entry. For example, B4GALNT2 was reported to inhibit IAV binding by modifying the subterminal sialic acid moiety of glycans present in the cell surface, while RIN2 and TM9SF2 have been reported to modulate IAV internalization by altering endocytosis and endosomal maturation, respectively [115]. Zinc metallopeptidase STE24 (ZMPSTE24) also appears to inhibit cytosolic entry of IAV and other viruses [116], although the specific mechanism/s underlying its antiviral activity are yet to be elucidated.

#### *5.1.2. Restriction factors that target genomic transcription and replication.*

The majority of the restriction factors identified to date target genomic replication and/or translation during IAV replication. Certain restriction factors target particular viral proteins (e.g., NP, M1, polymerase basic (PB)1 and PB2), to promote their degradation and/or alter cellular localization, whilst other restriction factors target the viral genome itself. Amongst the well-studied ISGs which target these steps in IAV replication are 2'-5' oligoadenylate synthetase (OAS)-directed RNase L pathway family proteins, protein kinase R (PKR) and the myxovirus resistance (Mx) family of GTPases. Human OAS 1-3 act as PRRs that recognize dsRNA. As a result, OAS proteins synthesize 2'-5'-oligoadenylate which, in turn, activate RNase L resulting in degradation of viral RNA

and induction of cellular apoptosis (reviewed in [117]). PKR is a kinase that, upon recognition of dsRNA, acts to phosphorylate the eukaryotic initiation factor 2 $\alpha$ -subunit (eIF-2 $\alpha$ ), thereby inhibiting viral protein synthesis. Additionally, PKR can phosphorylate I $\kappa$ B promoting activation of NF- $\kappa$ B and subsequent IFN production (reviewed in [118]). Human MxA, a member of the dynamin-like GTPase family, is a cytoplasmic ISG protein that blocks two distinct steps in the replicative cycle of IAV: (i) following membrane fusion, MxA prevents transport of incoming vRNPs to the nucleus [119] and (ii) during virus replication, MxA blocks the amplification of vRNA from cRNA copies (secondary transcription) [120]. In contrast, mouse Mx1 is localized to the nucleus and blocks IAV replication by inhibiting the synthesis of viral mRNA (primary transcription) [120].

A growing number of restriction factors target specific structural viral proteins to interfere with genomic transcription and/or virus replication. For example, Moloney leukemia virus 10 (MOV10) interacts with NP blocking its transportation to the nucleus [121] while Interferon-stimulated gene 20 (ISG20) also interacts with NP to inhibit IAV transcription and replication [122]. Cyclophilin E (CypE) is another restriction factor that interacts with NP to block the formation of vRNPs [123]. Other restriction factors such as the DDX21 RNA helicase interact with the viral PB1, inhibiting assembly of the polymerase complex [124]. Plakophilin 2 (PKP2) also binds to PB1, altering the formation of the polymerase complex and therefore inhibiting polymerase activity [125]. Two isoforms of the zinc finger antiviral protein (ZAP), ZAPL and ZAPS, restrict IAV infection. ZAPL binds to PA and PB2 to promote their proteasomal degradation, while ZAPS decreases the expression of PA, PB2 and NA by reducing their mRNA levels [126]. The isomerase cyclophilin A (CypA) interacts with M1 and induces its degradation through



the ubiquitin/proteasome system [127]. Of interest, restriction factors also target IAV non-structural proteins. Interferon-stimulated gene 15 (ISG15) is an ubiquitin-like molecule that can be bound covalently to target proteins changing their properties. While NS1 antagonises a number of antiviral responses within the cell, conjugation of ISG15 to NS1 disrupts its activity, resulting in suppression of virus replication [128].

Tripartite motif (TRIM) proteins are key components of the host response to viral infections, functioning as direct antiviral restriction factors or modulating signalling cascades that lead to proinflammatory cytokine induction. Of the >80 TRIM family proteins in humans, the majority are E3 ubiquitin ligases and some have been identified as restriction factors against IAV. For example, TRIM14 interacts with the viral NP to alter the formation of vRNPs [129], while TRIM56 reduces the synthesis of vRNA by a mechanism independent of its E3 ligase activity [130]. TRIM22 and TRIM32 ubiquitinate NP and PB1 respectively, promoting their proteasomal degradation and thereby reducing virus replication [131, 132].

#### *5.1.3. Restriction factors that target late stages of IAV infection*

Cellular restriction factors that inhibit the late stages of influenza virus have been also identified. Human guanylate-binding protein (GBP)2 and GBP5 can inhibit the proteolytical maturation of HA during its transport through the secretory pathway, by reducing the activity of the endoprotease furin [133]. Later during infection, cyclin D3 can bind to M2, altering the interaction between M1 and M2 required for IAV assembly [134], while viperin has been reported to inhibit the release of newly-synthesized virions from infected cells by decreasing the biosynthesis of isoprenoids, thereby affecting the

formation of lipids rafts [135]. BST-2 (tetherin, CD317, HM1.24) restricts virus release by tethering a range of enveloped viruses to the cell surface. Currently its role as an antiviral factor against influenza virus remains somewhat controversial, with some studies supporting a role in inhibiting virus release, but others arguing against this [97, 136, 137]. At the latest stage of IAV replication, plasminogen activator inhibitor 1 (PAI-1) can reduce the infectivity of progeny viruses by inhibiting extracellular proteases involved in the maturation of HA [138].

## **5.2. Cellular restriction factors that act against other respiratory viruses.**

Much less is known about cellular restriction factors that block the replication of respiratory viruses of the *Pneumoviridae* family. IFITM1 and IFITM3 have been reported to inhibit RSV infection at an early stage after virus entry, however the molecular mechanisms by which this occurs are yet to be defined [139]. Similarly, the ubiquitin-like ISG15 protein can inhibit RSV replication, likely through its ability to conjugate to specific viral (or host) proteins, although the detailed mechanism was not reported [140]. Viperin can block late stages of RSV infection, mediating antiviral activity by altering the formation of virus filaments and reducing cell-to-cell transmission [141]. Recently, tudor domain-containing 7 (TDRD7) was also identified as a novel restriction factor against RSV, with antiviral activity related to its ability to impair activation of the AMP-dependent kinase (AMPK). By blocking AMPK, TDRD7 inhibited initiation of autophagy, a cellular process required for RSV replication [142].

Currently, very little is known regarding cellular restriction factors that are active against hMPV. IFITM3 has been reported to restrict early stages of hMPV infection. Interestingly, the antiviral effect observed was similar to that observed using endocytosis inhibitors, consistent with recent findings implicating endocytosis as an alternative entry route used by hMPV to infect certain cell types [143]. In the same study, IFITM1 and, to a lesser extent, IFITM2 also restricted HMPV infection to some degree.

Given the diversity of viruses in the *Paramyxoviridae* family, it is not surprising that multiple restriction factors have been reported to block some, but not all, family members. Examples of restriction factors reported to restrict paramyxoviruses include IFITM1 (PIV-3), MxA (MeV), IFIT1 (PIV-3, MuV, PIV2, PIV5), BST-2 (MuV, NiV) and viperin (PIV-3, MeV) [144-148].

## **6. Approaches to study specific cellular restriction factors**

Most studies investigating cellular restriction factors have utilised overexpression studies alone or in combination with deletion and/or depletion of endogenous proteins. A range of methods can be used to generate cell lines that transiently or stably overexpress a particular protein of interest. Transfection of plasmids, as well as the use of retroviruses or lentiviruses to transduce cells, are the methods most widely used. Many host restriction factors have been identified using these methodologies, however a number of issues should be considered. First, the importance of using a cell line relevant to infection by the virus of interest. Other issues include an appreciation of the

levels of endogenous protein expression, the effect of overexpression on the cellular distribution of the protein of interest, and finally, the importance of species compatibility between the host cell and the protein of interest. For example, some restriction factors may require interactions with other cellular proteins expressed only in cells of the same species. In general terms, overexpression approaches are particularly useful to investigate the mechanisms by which particular ISGs or putative restriction factors (often with low levels of endogenous expression) act. Overexpression approaches also allow for the expression of mutants to study and identify particular domains, and even residues, involved in specific antiviral activity.

Alternatively, transient knockdown of a particular endogenous protein in host cells can be achieved using short interfering (si)RNAs or small hairpin (sh)RNAs. These RNA interference (RNAi) approaches have been used to screen large knockdown libraries to identify specific cellular host factors, including ISG proteins, with antiviral or proviral effects. Recently, a new gene editing technology, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) system, has been widely used to study the effects of knockdown/deletion of different cellular proteins in the context of viral infection. Unlike RNAi, the CRISPR/Cas9 system is bestowed with the ability to introduce heritable precision insertions and/or deletions into the eukaryotic genome.

Ultimately, approaches that utilise overexpression systems in combination with validation through knockdown of endogenous protein are ideal for the characterization of specific cellular factors for their ability to exert antiviral activity. For example, studies investigating the role of BST-2 during influenza virus infection have utilized cell lines

engineered to either overexpress or lack expression of this cellular protein. Differences in BST-2 expression levels in engineered cells and/or expression patterns of BST-2 in heterologous cell lines may have contributed to some of the discrepancies reported in the ability of BST-2 to restrict release of influenza virus from infected cells. In the case of BST-2 and influenza, complementary *in vivo* studies (where appropriate murine models are available) using BST-2 deficient mice have been helpful to resolve such discrepancies in the literature. Knockout mice lacking endogenous BST-2 showed no difference in their susceptibility to influenza infection [136], providing evidence that BST-2 does not afford potent protection against this virus. Moreover, homology between human and murine cellular restriction factors has facilitated the validation of other putative antiviral factors in studies using mice deficient in the expression of these proteins. For example, mice lacking IFITM3 [114] or Mx1 [149] show enhanced susceptibility to influenza virus infection, confirming their role as cellular restriction factors with potent antiviral activity towards this virus.

## **7. Identifying ISGs and other antiviral factors with antiviral activity against influenza and/or other respiratory viruses.**

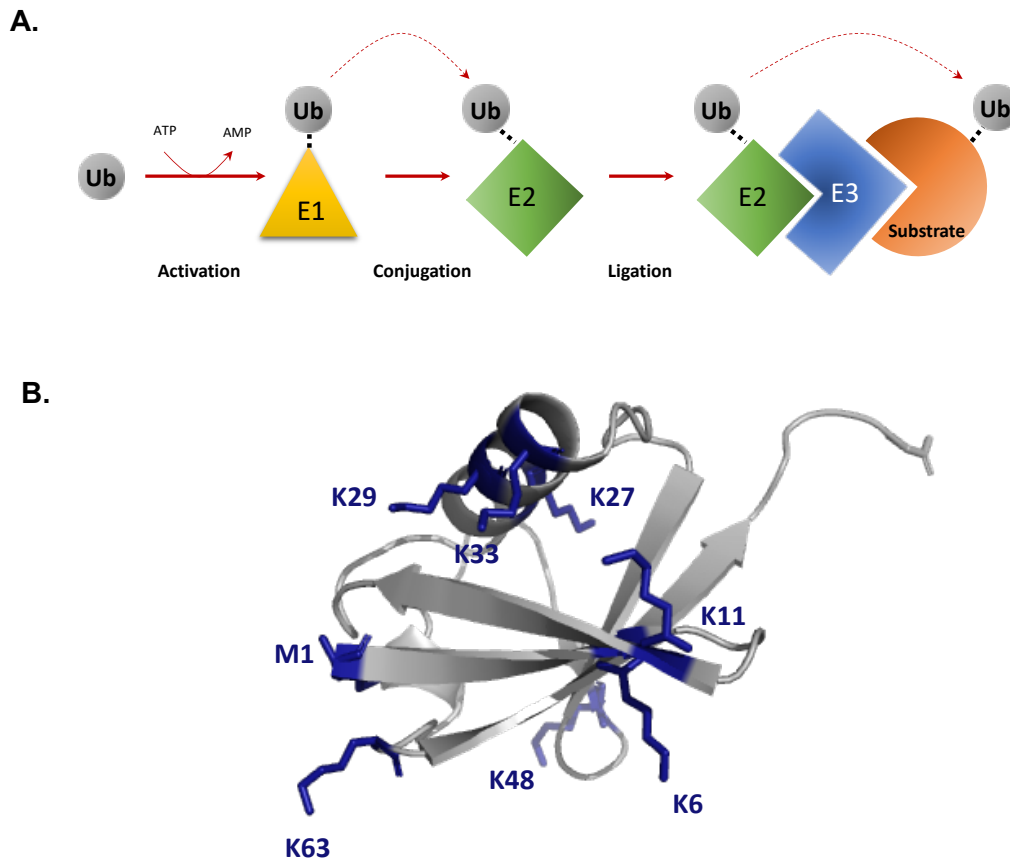
Growing evidence indicates that distinct cell types exhibit unique innate immune signatures, including their ability to express antiviral genes. A recent study performed in our laboratory examined transcriptional responses in purified AEC and AM $\phi$  from mock- or influenza virus-infected mice using RNA sequencing (RNA- seq) [150]. While both AM $\phi$ s and AEC constitutively expressed a range of ISGs, these genes were generally

expressed at higher levels in AM $\phi$ . Moreover, AM $\phi$  expressed a larger number of unique ISGs, most of which (to our knowledge) have not been assessed for anti-IAV activity. Following IAV infection, AEC upregulated a greater number of ISGs, including 99 unique genes, while many ISGs maintained high levels of expression in IAV-infected AM $\phi$ . These findings suggest that AM $\phi$  might be primed to recognize and block respiratory virus infection while AEC must sense infection before upregulation of antiviral genes is triggered.

RNA-seq data identified a number of families of cellular proteins where some family members (i) have been implicated in mediating antiviral activity, and (ii) showed differential expression in AEC vs AM $\phi$  and may therefore contribute to the different outcomes of respiratory virus infection in these distinct cell types (ie: productive infection vs abortive infection, respectively). Supporting this hypothesis, we found several family proteins that have been previously described as restriction factors against influenza and other respiratory viruses, including IFITMs, TRIMs and GBPs. Moreover, proteins that have not been described as restriction factors against respiratory viruses were also identified in this study. A protein family of particular interest is the membrane-associated RING-CH (MARCH) proteins. Functionally, these proteins are E3 ubiquitin ligases involved in the final step of the ubiquitination process. As the role of MARCH family-proteins in modulating respiratory virus infection is the focus of this thesis, subsequent sections of this literature review will focus on understanding ubiquitination, as well as MARCH-family proteins and their role in regulating immunity and infection.

## 8. Ubiquitination.

Ubiquitination is an enzymatic process that involves the covalent attachment of a ubiquitin protein to a substrate. Ubiquitin (Ub) is a small protein of 76 amino acids that is highly conserved across higher and lower eukaryotes, including animals, plants and fungi [151]. The successful ubiquitination of a substrate requires the coordinated action of three different enzymes: Ub-activating enzymes (E1), Ub-conjugating enzymes (E2) and Ub-ligases (E3) (**Figure 1.6A**) (reviewed in [152]). First, the terminal glycine residue (G-76) of Ub is activated by the E1 enzyme, allowing the formation of a thioester linkage between Ub-G76 and a cysteine residue of E1 in an adenosine triphosphate (ATP)-dependent manner. In humans, only two E1 enzymes able to activate Ub have been described to date: Ube1 and Uba6 [153]. The activated Ub is then transferred to a cysteine residue in the catalytic site of the E2 protein via a transthioesterification reaction. E2 Ub-conjugating enzymes are the central component of the ubiquitination process, interacting with the E1 and the E3 enzymes. They can bind to one or more E3 ligases and can add the activated Ub to a substrate, producing a monoubiquitination, or to a Ub molecule that was previously attached to a substrate, generating a polyubiquitination. Additionally, E2s differ in their ability to perform specific Ub-linkages, determining the topology of the assembled Ub-chain and therefore the fate of the ubiquitinated substrate [154, 155]. Lastly, the E3 ligases are responsible for the substrate-specificity of the ubiquitination process, interacting with specific substrates and with the E2-Ub, allowing the transference of Ub to the target protein, generally through the formation



**Figure 1.6. Ubiquitination process.** (A) Ubiquitination is an enzymatic process that ultimately leads to the attachment of a ubiquitin molecule to a target protein. The process involves 3 steps: first, the ubiquitin (Ub) molecule is activated by a Ub-activating enzyme or E1. Then, the activated Ub is transferred to the E2 Ub-conjugating enzyme. Finally, the E3 Ub-ligase interacts with the E2 and the specific substrate, allowing the transference of Ub to the target protein. (B) Crystal structure of Ub (PDB code 1UBQ, [156]). Each potential Ub-binding site is shown in blue (M1, K6, K11, K27, K29, K33, K48 and K63).



of an isopeptide bond between the  $\epsilon$ -amino group of a lysine (K) residue in the substrate and the C-terminal carboxyl group of Ub [157].

### **8.1. Chain topology and ubiquitin signaling.**

As mentioned, Ub can be added to a unique site in the target protein (monoubiquitination) or to multiple sites (multi-monoubiquitination). Additionally, Ub can be also added to Ub itself producing polyubiquitin chains. The Ub molecule contains 8 different  $\epsilon$ -amino groups that can be targeted: 1 in the methionine at position 1 (M1) and 1 in each K residue: K6, K11, K27, K29, K33, K48 and K63 (reviewed in [154]) (**Figure 1.6B**). Although ubiquitination is often associated with protein degradation, it can be involved in multiple cellular processes depending on the topology of the polyubiquitin chains (Ub-chains). Roles for ubiquitination have been described in the DNA damage response, cell cycle control, immune responses and protein trafficking (reviewed in [158]). The most common Ub-chain topologies are K48-linkages, the 'canonical' signal for proteasomal degradation [159, 160], and K63-linkages, which induce lysosomal degradation by modulating the endocytic trafficking of ubiquitinated substrates through interactions with the ESCRT machinery [161]. More recently, important contributions to the role of the "less common" Ub-chains have been ascribed. M1-linkages have been associated with the modulation of the NF- $\kappa$ B signaling pathway, K6-linkages associated with DNA damage responses and mitophagy, K11-linkages with the regulation of cell cycle processes, K27-linkages as modulators of the innate immune responses, K29-linkages involved in the Wnt signaling pathway and K33-linkages have been associated

with post-Golgi trafficking (reviewed in [162, 163]). Thus, although the “ubiquitin code” is not fully understood yet, it is clear that Ub-chain topology can certainly impact the fate of the targeted substrates.

## **8.2. Ubiquitination specificity and E3 ubiquitin ligases.**

E3 ligases are the largest group of enzymes involved in the ubiquitination process. This diverse group of proteins are responsible for the specificity of the ubiquitination process and can be divided in three groups: Really Interesting New Gene (RING), Homologous to E6-AP Carboxyl Terminus (HECT) and RING-Between-RING (RBR), according to their structure and mechanism of action (reviewed in [164]). RING E3 ligases contain a RING or U-box domain responsible for interaction with the E2 Ub-conjugating enzyme, promoting direct Ub transfer from the E2 to the substrate. HECT E3 ligases transfer Ub following a two-step mechanism: first, Ub is transferred from the E2 to a cysteine in the HECT domain before subsequent transfer to a lysine on the specific substrate. RBR E3 ligases utilize a hybrid mechanism combining the properties of the RING and HECT E3 ligases (reviewed in [157]). RING E3 ligases are the most abundant, with approximate 600 members encoded in the human genome. Their RING finger domains adopt a cross-braced arrangement of 8 different residues, generally cysteine (C) and histidine (H) residues, that coordinate two  $\text{Zn}^{+2}$  ions. Commonly, these residues follow a C3HC4 linear arrangement (RING-HC), however some RING ligases contain variations of this finger domain following a C3H2C3 (RING-H2) or C4HC3 lineal arrangement (RING-CH) (reviewed in [165])

## **9. Membrane-Associated RING-CH (MARCH) proteins.**

### **9.1. Viral MARCH proteins.**

The first RING-CH E3 ligases described were the Kaposi's Sarcoma associated Herpesvirus (KSHV) proteins K3 and K5. Initially, both viral proteins were reported to downregulate Major Histocompatibility Complex (MHC)-I from the surface of KSHV-infected cells, allowing escape of these virus-infected cells from immunosurveillance by CD8<sup>+</sup> cytotoxic T cells [166]. In later studies, other proteins of the immune system were also identified as targets, including Intercellular adhesion molecule 1 (ICAM-1), cluster of differentiation 86 (CD86), cluster of differentiation 1d (CD1d) and IFN- $\gamma$  receptor 1 (IFN- $\gamma$ R1) [167-169]. Additionally, other viral proteins exhibiting similar immunomodulatory activity through E3 ligase activity were identified. These include mK3 from murine gamma-herpesvirus 68 [170] and M153R, a viral protein expressed by certain members of the *Poxviridae* family [171]. Although differences in mechanism of action were noted, these viral proteins shared a similar structure comprising an N-terminal RING-CH domain responsible for E3 ligase activity, followed by two transmembrane domains (reviewed in [172]).

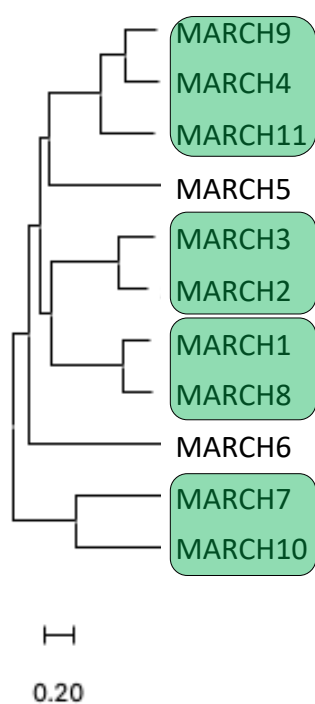
Interestingly, lysine (K) residues are not the only acceptor sites used by viral RING-CH E3 ligases for ubiquitination. Studies using MHC-I mutants carrying arginine (R) instead of K residues in the cytoplasmic tail (CT) demonstrated that KSHV-K3 could also ubiquitinate cysteine (C) residues [173]. Similarly, KSHV-K5 was also capable of ubiquitinating C residues, although only if they were located closer to the transmembrane domain [174]. The mK3 protein was shown to be more promiscuous,

ubiquitinating serine (S) and threonine (T) residues in the CT of the heavy chain of MHC-I molecules [175].

## **9.2. Mammalian MARCH proteins: General features**

Viral homologues of host genes involved in the immune response are found in a number of large DNA viruses, suggesting that viruses have “stolen” genes from the host for subsequent incorporation into the viral genome. Considering the variety of viruses encoding RING-CH E3 ligases, it was not surprising that genes encoding RING-CH E3 ligases were also identified in eukaryotic hosts. Initially, bioinformatic and experimental approaches identified c-MIR (now called MARCH8) as the first human homolog of KSHV-K3 and -K5, demonstrating its ability to downregulate CD86 [176]. Subsequently, 10 additional MARCH proteins (MARCH1-11) were identified, all sharing the RING-CH domain [177-179]. Despite conservation of the RING-CH domain, MARCH-family proteins can show marked differences in overall structure. Most members are characterized by a cytoplasmic N-terminal RING-CH domain, followed by 2 transmembrane domains (TM) that are connected by a luminal loop. However, MARCH5 has 4 predicted TM domains, while MARCH6 has 14 putative TM domains. Additionally, MARCH7 and MARCH10 lack TM domains and their RING-CH domain is localized proximal to the C-terminal end of the protein [177, 180].

Based on phylogenetic analysis, some members of the family can be further classified in subgroups: MARCH1/8, MARCH2/3, MARCH4/9/11, MARCH7/10 (**Figure 1.7**). These subgroups shared a high sequence conservation, especially within the RING-TM1-TM2



**Figure 1.7. Phylogenetic analysis of human MARCH proteins.** Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) X software. Human MARCH protein sequences were obtained from The Uniprot Consortium database. MARCH protein subgroups are highlighted in green.

domains of MARCH1/8 (89%), MARCH2/3: (76%), MARCH4/9 (91%), MARCH4/11 (75%), and MARCH9/11 (72%) (reviewed in [181]).

In general, MARCH proteins are expressed endogenously in cells at low levels, and therefore qPCR of mRNA has been the predominant approach used to detect their expression and establish tissue distribution. Furthermore, information regarding the subcellular localization of these proteins within cellular compartments has generally been determined through overexpression of tagged proteins. A summary of tissue distribution and subcellular localization for each MARCH protein is shown in **Table 1.1**.

Interestingly, the subcellular localization of some MARCH proteins can be modulated by other host proteins. For example, the chaperone Bap31 is involved in intracellular transport of MARCH4 and MARCH8 [182]. Additionally, mutations in the PDZ-binding motifs of MARCH2 and MARCH3 alter their cellular distribution [183, 184], suggesting that PDZ proteins modulate intracellular trafficking of these proteins.

Expression of MARCH proteins appears to be highly regulated and this is likely an important factor contributing to the low levels of endogenous protein expression in cells. For example, some MARCH proteins are regulated by autoubiquitination, including MARCH1/5/6/7/8/10 [180, 185-189]. Moreover, certain stimuli modulate the expression of particular MARCH proteins. For example, MARCH1 is regulated by IL-10 and LPS [190], MARCH2 (but not the other 10 family members) is upregulated in 293T cells after HIV-1 infection [191] and LPS stimulation has been reported to upregulate MARCH3 [192].

**Table 1.1. Tissue distribution and cellular localization of the mammalian MARCH proteins**

| <b>Family member</b> | <b>Expression</b>                                               | <b>Subcellular localization</b>          | <b>REF</b> |
|----------------------|-----------------------------------------------------------------|------------------------------------------|------------|
| MARCH1               | Secondary lymphoid tissue (spleen and lymph nodes)              | Endosomes, lysosomes and plasma membrane | [177]      |
| MARCH2               | Broad expression. Higher levels in the heart                    | Endosomes, lysosomes and plasma membrane | [177, 183] |
| MARCH3               | Broad expression. Higher levels in spleen, colon and lungs      | Endosomes, lysosomes and plasma membrane | [184]      |
| MARCH4               | Mainly expressed in brain and placenta. Also expressed in lungs | Golgi apparatus                          | [177]      |
| MARCH5               | Broad expression.                                               | Mitochondria (outer membrane)            | [186]      |
| MARCH6               | Broad expression.                                               | Endoplasmic reticulum (ER)               | [187]      |
| MARCH7               | Broad expression.                                               | Mainly nuclear, but also in cytoplasm    | [188]      |
| MARCH8               | Broad expression. High levels in lungs                          | Endosomes, lysosomes and plasma membrane | [177]      |
| MARCH9               | Broad expression. High levels in lymph nodes and lungs          | Lysosomes and trans-Golgi network (TGN)  | [177]      |
| MARCH10              | Mainly in testis                                                | Cytoplasm                                | [180]      |
| MARCH11              | Mainly in testis                                                | TGN and MVB                              | [179]      |

Of note, MARCH proteins show some overlapping substrate specificity, however distinct expression patterns (both in terms of tissues, as well as subcellular distribution) suggest that even if different MARCH proteins ubiquitinate the same substrate, this process might occur in different cellular compartments and/or with different ubiquitination patterns. This, in turn, could result in differential sorting of target proteins into distinct cellular pathways.

### **9.3. Mammalian MARCH proteins: Regulation of immune responses.**

Since the discovery of MARCH8, the first identified mammalian homologue of KSHV-K3 and -K5, the role of the MARCH proteins in immune regulation has been studied intensely. Cumulative evidence supports an important role for both MARCH1 and MARCH8 in modulating MHC-II expression *in vitro* and *in vivo*. MHC-II is predominantly expressed by professional antigen presenting cells (APC) and plays an important role in facilitating presentation of antigens to CD4<sup>+</sup> helper T cells (reviewed in [193]). Interestingly, due to the differential expression of MARCH1/8, it has been proposed that MARCH1 regulates MHC-II expression in APC of hematopoietic origin, while MARCH8 controls regulation in non-hematopoietic derived APC, such as thymic epithelial cells [194].

Like KSHV-K3 and -K5, MARCH4 and MARCH9 can also downregulate expression of MHC-I. *In vitro* studies demonstrated that overexpression of MARCH4 induced internalization of MHC-I via endocytosis, following monoubiquitination of K residues in its cytoplasmic tail [177]. Similarly, overexpression of MARCH9 decreased levels of cell-surface MHC-I,



while knockdown of MARCH9 reduced access of pMHC-I complexes from the trans Golgi network (TGN) to endosomes, suggesting that MARCH9 could play a role in antigen cross-presentation [195].

Together with MHC molecules, co-stimulatory molecules also play important roles during the antigen presentation process. CD86, a co-stimulatory molecule important for CD4<sup>+</sup> T cell activation, can be targeted by MARCH1/2/8 [176, 177, 196] for downregulation. Similarly, CD83, which plays a critical role during CD4<sup>+</sup> T cell selection in the thymus, can be downregulated by MARCH1/8 [194, 197]. Moreover, MARCH4/9 have the ability to reduce the surface expression of the T cell co-receptor CD4 [177].

While the role of MARCH proteins in modulating antigen presentation has been particularly well studied, MARCH proteins also target other immune receptors. MARCH3 and MARCH8 have been associated with the modulation of interleukin-1 beta (IL-1 $\beta$ )-mediated inflammatory responses by targeting the IL-1 receptor I (IL-1RI) and IL-1 receptor accessory protein (IL-1RAP), respectively [198, 199]. MARCH3 also targets the inhibitory Fc gamma receptor IIb (Fc $\gamma$ RIIb) and has been reported to positively regulate antibody-mediated responses in monocytes and macrophages [192]. MARCH4/9 can downregulate expression of Mult1, a ligand recognized by the receptor NKG2D which is expressed by natural killer (NK) cells and certain subsets of T cells [200]. MARCH8 can also attenuate the apoptosis of tumor cells by polyubiquitinating the tumor necrosis factor-related apoptosis inducing ligand receptor 1 (TRAIL-R1), although their relevance in modulation of different cancers is yet to be fully explored [201].

Interestingly, pathogenic bacteria have developed strategies to exploit immune regulation exhibited by some MARCH-family proteins. In this context, different bacteria including *Francisella tularensis*, *Salmonella enterica* and *Streptococcus suis* reduce expression of MHC-II from the surface of APC by modulating the expression and/or functionality of MARCH1 or MARCH8 [202-204].

#### **9.4. Mammalian MARCH proteins: Role as modulators of viral infections.**

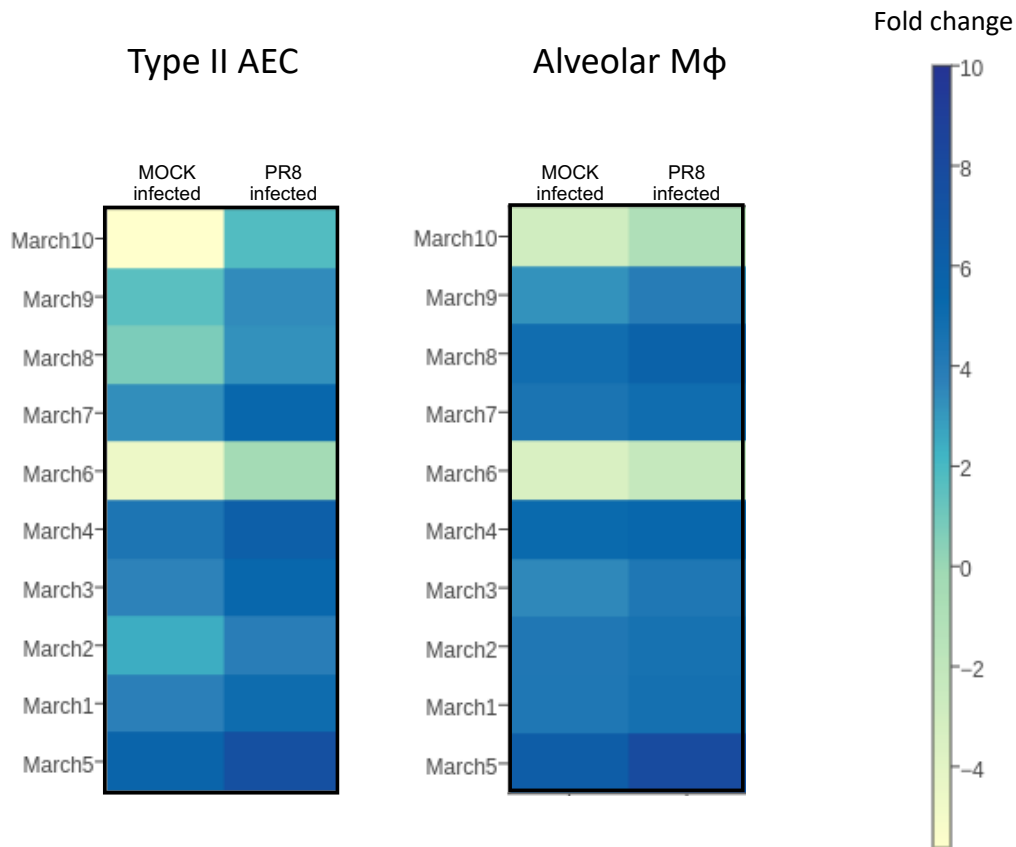
Following infection, a number of cellular pattern recognition receptors (PRR) can detect viral infections and initiate intracellular antiviral responses (Section 4.1). Mitochondria serve as an important signalling platform relevant to PRR signalling during viral infections (reviewed in [205]), and the localization of MARCH5 to the outer membrane of mitochondria is consistent with its reported role in modulating antiviral immunity. For example, MARCH5 can regulate TLR7-mediated antiviral signalling by polyubiquitinating the TRAF family member Associated NF- $\kappa$ B Activator (TANK) protein [206]. TANK is a negative regulator of TLR-mediated NF- $\kappa$ B signalling pathways that control the induction of proinflammatory cytokines and ISGs [207]. MARCH5 has also been associated with RIG-I-mediated antiviral responses and it has been proposed that MARCH5 can control type-I IFN production by targeting MAVS protein aggregates and promoting their degradation [208]. Similarly, MARCH5 can recognise the active form of RIG-I and target this protein for proteasomal degradation [209]. Thus, by ubiquitinating either MAVS or RIG-I, it has been proposed that MARCH5 acts to limit exacerbated proinflammatory responses following viral infections.

MARCH proteins have also been reported to directly target a number of viral proteins. In a study by Tada *et al.*, overexpression of MARCH8 in 293T cells was reported to downregulate the HIV-1 Env and VSV-G proteins from the surface of infected cells [210]. By targeting these viral surface glycoproteins, MARCH8 reduced their incorporation into nascent virions, thereby reducing virus infectivity. Mechanistically, direct interaction of MARCH8 with the HIV-1 Env and VSV-G proteins was demonstrated using co-immunoprecipitation while site-directed mutagenesis was used to confirm that the E3 ligase activity of MARCH8 was critical for its antiviral effect. However, the fate of each viral protein in the presence of MARCH8 was different. HIV-1 Env accumulated in intracellular compartments following downregulation from the cell-surface, whereas intracellular VSV-G was detected only after cells were treated with lysosomal protease inhibitors, suggesting that each viral protein was internalized by different pathways. The same study also used qPCR to report that MARCH8 was highly expressed in human monocyte-derived macrophages (hMDMs) and dendritic cells (hMDDCs). Knockout or knockdown of MARCH expression in these cells resulted in a significant increase in the infectivity of virions released from infected cells. Subsequent studies demonstrated that overexpression of MARCH1 and MARCH2 also resulted in downregulation of HIV-1 Env and VSV-G from the surface of infected cells, following similar mechanisms to those exhibited by MARCH8. However, in contrast to MARCH8, expression of MARCH1 and MARCH2 were reported to be upregulated in hMDMs after stimulation with type I IFNs [211]. In a separate study, only MARCH2 expression was reported to be upregulated in a variety of cell types after HIV-1 infection [191].

Recently, MARCH8 was identified as a proviral factor for flaviviruses. Using siRNA approaches, it was shown that MARCH8 is required for efficient infection of Huh7 cells with hepatitis C virus (HCV), dengue 2 virus (DENV-2) and Zika virus (ZIKV). Additionally, MARCH8 was recognized as the E3 ligase responsible for the polyubiquitination of the HCV non-structural 2 (NS2) protein [212] critical during viral envelopment. HCV envelopment is an ESCRT-dependent process, however HCV lacks the late-domain motifs required for the recruitment of this machinery to the site of envelopment. Instead, the polyubiquitination of NS2 mediates the binding of hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), one of the ESCRT-0 components, allowing the recruitment of ESCRT and therefore HCV envelopment [213].

## **10. Aims of this thesis.**

Consistent with growing evidence supporting the importance of the MARCH proteins in modulating the infection of different viruses, our preliminary RNA-seq studies suggested that different MARCH proteins are expressed in cell types relevant to respiratory virus infection, at least in the murine lung. As seen in **Figure 1.8**, various MARCH proteins showed different expression levels within one particular cell type. For example, MARCH1/3/4/5/7 are more highly expressed in type II AEC when compared to MARCH2/8/9, and particularly to MARCH6/10, which were expressed at lower levels. Of interest, several members of the MARCH family proteins (MARCH2/7/8 and, to a lesser extent, MARCH4/9) were constitutively expressed at higher levels in AM $\phi$  when compared to type II AEC, suggesting that the varying MARCH expression levels could



**Figure 1.8. Expression levels of MARCH proteins in AEC and AMΦ from mice after IAV infection.** C57/BL6 mice were inoculated via the intranasal route with 50  $\mu$ L of phosphate-buffered saline (PBS) containing  $10^6$  PFU/mouse of IAV strain A/Puerto Rico/8/34 (PR8). Mock-infected mice received an equivalent volume of diluted allatonic fluid that did not contain virus (MOCK). At 8-10 hr p.i. mice were killed, lung lobes were harvested and used to prepare single cell populations. Fluorescent-labeled antibodies and cell sorting were used to isolate alveolar type II epithelial cells (ATII) and alveolar macrophages (AMΦ), after which RNA was extracted and RNA-seq performed. Data was aligned to the mouse transcriptome, aligned fragments were summarized into genes and raw counts were transformed to  $\text{Log}_2$  counts per million. Normalization and differential expression testing were conducted using edgeR. Genes that are  $\text{Log}_2$  fold change (FC)  $\geq \pm 1$  were considered to be differentially expressed. The dark color (blue) corresponds to high expression whereas the light color (yellow) corresponds to low expression. For the heatmaps, average  $\text{Log}_2$  count per million of 4 independent biological replicates were plotted. Heatmaps were generated using Plotly.

play a role in the differential outcome observed in these cell types following infection with seasonal IAV (abortive vs productive IAV infection, respectively). Additionally, expression of the majority of the MARCH family members was upregulated, at least to some degree, after IAV infection of type II AEC.

Given this background, the **overall aim of this thesis** was to investigate the ability of different MARCH-family proteins to mediate antiviral activity against influenza virus, as well as respiratory viruses of the *Pneumoviridae* and *Paramyxoviridae* family.

**The specific aims of this thesis were:**

1. To develop experimental approaches to express different MARCH-family proteins in mammalian cells, therefore allowing for assessment of their ability to modulate infection by influenza virus and other human respiratory viruses.
2. To characterize the antiviral activity of MARCH8 against influenza viruses, including studies to understand features of MARCH8 and of influenza viruses that contributed to MARCH8-mediated restriction.
3. To investigate the role of endogenous MARCH8 in antiviral defense against influenza virus infection *in vitro* and *in vivo*.



## Chapter 2

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# **Materials and Methods**

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## 2.1. Cell lines.

Madin-Darby Canine kidney (**MDCK**) cells (American Type Culture Collection (ATCC) CCL-34) were maintained and passaged in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, ThermoFisher Scientific, MA, USA), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco). **A549** cells (ATCC CCL-185) were maintained and passaged in Ham's F-12K (Kaighn's) medium (Gibco) supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 1 mM sodium pyruvate. **293T** cells (ATCC CRL-3216) and **HeLa** cells (ATCC CCL-2) were maintained and passaged in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 1 mM sodium pyruvate. Human epithelial type 2 (**HEp-2**) cells (ATCC CCL-23) were maintained and passaged in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 1 mM sodium pyruvate. **LLC-MK2** cells (ATCC CCL-7) were maintained and passaged in Opti-MEM I medium (Gibco) supplemented with 5% (v/v) FBS. 293T, A549 and HeLa cell lines engineered for doxycycline (DOX) inducible protein expression (Section 2.10) were cultured with Tet System Approved FBS (Takara Bio USA, CA, USA), to eliminate the possibility of low level DOX in standard FBS. All cell lines were cultured at 37°C in a humidified atmosphere with 5% (v/v) CO<sub>2</sub>.

## 2.2. Viruses.

**Influenza A viruses** (IAV) and **influenza B viruses** (IBV) viruses have been used in studies described in this thesis. IAV strains were: **(i) H3N2**: A/Memphis/1/71, A/Udorn/307/72, A/Port Chalmers/1/73, A/Beijing/353/89, A/Guangdong/25/93, A/Shanghai/11/95,

A/South Australia/4/00, A/New York/55/04 and A/Perth/16/09. **(ii) H1N1:** A/Brazil/11/78, A/New Caledonia/20/99 and A/Solomon Islands/3/06. **(iii) H1N1pdm09:** A/Tasmania/2004/09 and A/Fiji/2/16. Additionally, we used two reassortant IAV strains, HKx31 (H3N2) and RG-PR8-Viet04 (H5N1), which are high-yielding reassortants of A/PR/8/34 with A/Aichi/2/68 (H3N2) and A/Vietnam/1203/04 (H5N1), respectively. It should be noted that the HA of RG-PR8-Viet04 (also known as vaccine strain SJRG-161052) has had the multibasic cleavage site removed. The IBV strain used was B/Brisbane/1/07 (B/Yamagata lineage). Viruses were obtained from the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia. All viruses were grown in the allantoic cavity of 10-11 day embryonated hen's eggs by standard procedures [214] and viral titres were determined by plaque assay in MDCK cells (Section 2.5.1).

**Respiratory syncytial virus (RSV)** strain A2 (ATCC VR-1540) and **parainfluenza virus 3 (PIV-3)** strain C243 (ATCC VR-93) were propagated in HEp-2 cells at 37°C by standard procedures [215] and titres of infectious virus were determined by plaque assay in HEp-2 cells (Section 2.5.3).

**Human metapneumovirus (hMPV)** strain CAN-1993/87 (a kind gift from Professor Kirsten Spann, Queensland University of Technology, Australia) was propagated in LLC-MK2 cells at 32°C in the presence of 5 µg/mL L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemical, NJ, USA), based on methods reported previously [216]. Titres of infectious virus were determined by ViroSpot assay in LLC-MK2 cells (Section 2.5.4).

## 2.3. Primers

**Table 2.3.1. Primers used for cloning into pTRE-tight plasmid**

| Primer Name   | Sequence                                  |
|---------------|-------------------------------------------|
| HpaI-MARCH FW | 5'-GGTTAACATGGATTACAAGGATGACGATGACAAGG-3' |
| Geneblock RV  | 5'-CGTATGTTGTGTGGAATTGTGAG-3'             |
| BamHI-cOVA FW | 5'-ATGGATCCAGACAACCTCAGAGTTCACCATGG-3'    |
| EcoRI-cOVA RV | 5'-AATATTGAATTCTTTTAAGGGGAAACACATCTGC-3'  |
| pcDNA3 seq RV | 5'-GGCAACTAGAAGGCACAGTCGAGG-3'            |

**Table 2.3.2. Primers and probe used for vRNA quantification**

| Name             | Sequence                                   |
|------------------|--------------------------------------------|
| IAV Matrix FW    | 5'-GACCRATCCTGTACCTCTGAC-3'                |
| IAV Matrix RV    | 5'-GGGCATTYTGGACAAKCGTCTACG-3'             |
| IAV Matrix Probe | 6FAM-5'-TGCAGTCCTCGCTCACTGGGCACG-3'-MGBNFQ |

**Table 2.3.3. Degenerate primers used to generate recombinant IAV**

| Primer Name | Sequence                                  |
|-------------|-------------------------------------------|
| HA_K557R FW | 5'-ATGTGGGCCTGCCAGAGAGGCAACATTAGGTGC-3'   |
| HA_K557R RV | 5'-GCACCTAATGTTGCCTCTCTGGCAGGCCACATG-3'   |
| NA_K6R FW   | 5'-GAATCCAAATCAAAGGATAATAACAATTGGC-3'     |
| NA_K6R RV   | 5'-GCCAATTGTTATTATCCTTTGATTTGGATTG-3'     |
| M2_K49R FW  | 5'-GATCGTCTTTTTTTCAGATGCATCTATCGATTG-3'   |
| M2_K49R RV  | 5'-GAATCGATAGATGCATCTGAAAAAAGACGATC-3'    |
| M2_K60R FW  | 5'-CTTTGAACACGGTCTGAGAAGAGGGCCTTCTACG-3'  |
| M2_K60R RV  | 5'-CGTAGAAGGCCCTCTTCTCAGACCGTGTTCAAAG-3'  |
| M2_K78R FW  | 5'-GGGAAGAATATCGAAGGGAACAGCAGAGTGC-3'     |
| M2_K78R RV  | 5'-GCACTCTGCTGTTCCCTTCGATATTCTTCCC-3'     |
| M1_K21R FW  | 5'-GTCAGGCCCCCTCAGAGCCGAGATCGCG-3'        |
| M1_K21R RV  | 5'-CGCGATCTCGGCTCTGAGGGGGCCTGAC-3'        |
| M1_K35R FW  | 5'-GATGTCTTTGCTGGAAGGAACACAGATCTTGAGG-3'  |
| M1_K35R RV  | 5'-CCTCAAGATCTGTGTTCTTCCAGCAAAGACATC-3'   |
| M1_K47R FW  | 5'-CTCATGGAATGGCTAAGGACAAGACCAATCCTGTC-3' |
| M1_K47R RV  | 5'-GACAGGATTGGTCTTGTCCTTAGCCATTCCATGAG-3' |

|             |                                              |
|-------------|----------------------------------------------|
| M1_K57R FW  | 5'-GTCACCTCTGACTAGAGGGATTTTGGGATTTG-3'       |
| M1_K57R RV  | 5'-CAAATCCCAAAATCCCTCTAGTCAGAGGTGAC-3'       |
| M1_K98R FW  | 5'-CATGGACAGAGCAGTTAGACTGTATAGAAAAC-3'       |
| M1_K98R RV  | 5'-GTTTTCTATACAGTCTAACTGCTCTGTCCATG-3'       |
| M1_K102R FW | 5'-GCAGTTAACTGTATAGAAGACTTAAGAGGGGAGATAAC-3' |
| M1_K102R RV | 5'-GTTATCTCCCTCTTAAGTCTTCTATACAGTTTAACTGC-3' |
| M1_K104R FW | 5'-CTGTATAGAAAACCTTAGGAGGGGAGATAACATTCC-3'   |
| M1_K104R RV | 5'-GGAATGTTATCTCCCTCCTAAGTTTTCTATACAG-3'     |
| M1_K113R FW | 5'-CATTCCATGGGGCCAGAGAAATAGCACTCAG-3'        |
| M1_K113R RV | 5'-CTGAGTGCTATTTCTCTGGCCCCATGGAATG-3'        |
| M1_K187R FW | 5'-GCCAGCACTACAGCTAGGGCTATGGAGCAAATG-3'      |
| M1_K187R RV | 5'-CATTTGCTCCATAGCCCTAGCTGTAGTGCTGGC-3'      |
| M1_K230R FW | 5'-CCAGTGCTGGTCTAAGAGATGATCTTCTTGAAAATTTG-3' |
| M1_K230R RV | 5'-CAAATTTTCAAGAAGATCATCTCTTAGACCAGCACTGG-3' |
| M1_K242R FW | 5'-GCAGGCCTATCAGAGACGAATGGGGGTGCAG-3'        |
| M1_K242R RV | 5'-CTGCACCCCCATTCTGTCTCTGATAGGCCTGC-3'       |
| M1_K252R FW | 5'-GATGCAACGATTCAGGTGACCCTCTTGTTG-3'         |
| M1_K252R RV | 5'-CAACAAGAGGGTCACCTGAATCGTTGCATC-3'         |

**Table 2.3.4. Primers used to determine relative expression of MARCH8**

| Primer Name | Sequence                        |
|-------------|---------------------------------|
| MARCH8 FW   | 5'-AGCCACTGAGAAAAATGGGAGAAG-3'  |
| MARCH8 RV   | 5'-TGTCAGTGAACACATGATCTTCC-3'   |
| GAPDH FW    | 5'-TGAAGGTCTGGAGTCAACGG-3'      |
| GAPDH RV    | 5'-GGCAACAATATCCACTTTACCAGAG-3' |
| RPL13a FW   | 5'-GCCCCTGTTTCAAGGGATAA-3'      |
| RPL13a RV   | 5'-CCTCGACCATCAAGCACCA-3'       |
| TBP FW      | 5'-GCACTGATTTTCAGTTCTGG-3'      |
| TBP RV      | 5'-GCTGGAAAACCCAACTTCTGT-3'     |

**Table 2.3.5. Primers used to generate MARCH8-MARCH1 chimeric mutants.**

| Primer Name     | Sequence                              |
|-----------------|---------------------------------------|
| MARCH8 S149T FW | 5'-GCTGCAGATGACCACCAGCGAGCGGCGGAAG-3' |
| MARCH8 S149T RV | 5'-CCGCCGCTCGCTGGTGGTCATCTGCAGC-3'    |
| MARCH8 M156F FW | 5'-GCGGCGGAAGATCTTCTGCAGCGTGACC-3'    |

|                   |                                                            |
|-------------------|------------------------------------------------------------|
| MARCH8 M156F RV   | 5'-GGTCACGCTGCAGAAGATCTTCCGCCGC-3'                         |
| MARCH8_M1 loop FW | 5'-GGAAATCAAGCAGGGAAACGACAACGGAGTGCTGGAATG<br>GCCC-3'      |
| MARCH8_M1 loop RV | 5'-CCAGAAGGGCCATTCCAGCACTCCGTTGTCGTTTCCCTGCT<br>TGATTTC-3' |
| MARCH8 L213V FW   | 5'-CCGGCGGACTGGTGTATGTACG-3'                               |
| MARCH8 L213V RV   | 5'-GCACGTACATAAACACCAGTCCGCCGG-3'                          |

## 2.4 Antibodies

**Table 2.4.1. Antibodies used to detect virus-infected cells by flow cytometry.**

| Antibody       | Conjugate | Clone      | Supplier             |
|----------------|-----------|------------|----------------------|
| Anti-IAV NP    | FITC      | 431        | Abcam                |
| Anti-RSV NP    | FITC      | 0671       | Sapphire Biosciences |
| Anti-hMPV NP   | AF-488    | hMPV123    | In Vitro Tech        |
| Anti-PIV3 HN   | -         | M02122321  | Abcam                |
| Anti-mouse IgG | AF-488    | Polyclonal | Invitrogen           |

**Table 2.4.2. Antibodies used to detect IAV viral proteins by western blot**

| Antibody           | Clone      | Supplier      |
|--------------------|------------|---------------|
| (mouse) Anti-NP    | A1 & A3    | Bio-Rad       |
| (goat) Anti-HA     | Polyclonal | BEI resources |
| (goat) Anti-NA     | Polyclonal | BEI resources |
| (mouse) Anti-M1    | GA2B       | Bio-Rad       |
| (mouse) Anti-M2    | 14C2       | Abcam         |
| Anti-mouse IgG HRP | Polyclonal | Agilent Dako  |
| Anti-goat IgG HRP  | Polyclonal | Abcam         |

**Table 2.4.3. Antibodies used to detect immune cell populations by flow cytometry**

| Antibody    | Conjugate    | Clone    | Supplier  |
|-------------|--------------|----------|-----------|
| Anti-CD45.2 | Pacific blue | 104      | BioLegend |
| Anti-NKp46  | BV650        | 29A1.4   | BioLegend |
| Anti-CD3    | PerCP-Cy5.5  | 145-2C11 | BioLegend |
| Anti-CD4    | AF700        | GK1.5    | BioLegend |
| Anti-CD8    | PE-Cy7       | 53-6.7   | BioLegend |

|                  |        |             |               |
|------------------|--------|-------------|---------------|
| Anti-TCR-β       | BV711  | H57-5       | BD Horizon    |
| Anti-Ly6G        | PE     | 1A8         | BD Pharmingen |
| Anti-MHC-II      | AF700  | M5/114.15.2 | BioLegend     |
| Anti-CD11b       | BV605  | M1/70       | BioLegend     |
| Anti-CD11c       | BV785  | N418        | BioLegend     |
| Anti-SiglecF     | PE     | E50-2440    | BD Horizon    |
| Anti-CD24        | PE-Cy7 | M1/69       | BioLegend     |
| Anti-CD64        | APC    | X54-5/7.1   | BioLegend     |
| Anti-CD16/CD32   | -      | 93          | BioLegend     |
| Anti-Ly49H       | APC    | 3D10        | eBioscience   |
| Anti-Ly49C/F/I/H | FITC   | 14B11       | eBioscience   |
| Anti-Ly49D       | AF647  | 4E5         | BioLegend     |
| Anti-CD49b       | FITC   | DX5         | BD Pharmingen |
| Anti-NK1.1       | BV605  | PK136       | BioLegend     |

## 2.5 Determination of titres of infectious virus

### 2.5.1. *Plaque assay for influenza viruses.*

MDCK cells were seeded into 6-well tissue culture plates (Costar Cell Culture, Corning, NY, USA) and cultured overnight. Confluent monolayers were washed with serum-free RPMI 1640 and inoculated with 10-fold serial dilutions of virus in serum-free media for 45 min at 37°C in 5% CO<sub>2</sub>. After incubation, cell monolayers were overlayed with 3 mL of Lebovitz L-15 medium (Gibco, Thermo Fisher Scientific) containing 0.9% (w/v) agarose type I low EEO (Sigma-Aldrich, MO, USA) and supplemented with 0.8 mM HEPES (Gibco, ThermoFisher Scientific), 0.056% (w/v) NaHCO<sub>3</sub> (Sigma-Aldrich), 100 units/mL of penicillin, 100 µg/mL streptomycin and 2 µg/mL TPCK-treated trypsin. After 2-3 days incubation at 37°C, wells containing <100 plaques were counted, and titres of infectious virus were expressed as plaque-forming units (PFU) per mL of original sample.

### ***2.5.2. ViroSpot assay for influenza viruses.***

MDCK cells were seeded into flat-bottomed 96-well tissue culture plates (Costar Cell Culture, Corning) and cultured overnight. Confluent monolayers were washed with serum-free RPMI 1640 and inoculated with 10-fold serial dilutions of virus in serum-free media for 1 hr at 37°C, 5% CO<sub>2</sub>. After incubation, cell-monolayers were overlaid with 100 µL of 2x FBS-free DMEM media containing 6.4% (w/v) carboxymethyl cellulose (CMC, Sigma-Aldrich) and 4 µg/mL TPCK-treated trypsin. After 24 hr incubation at 37°C, cells were washed, fixed with 80% (v/v) ice-cold acetone in water and then stained with mAb MP3.10g2.1C7 (WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia), which is specific for the NP of IAV. After incubation for 1 hr at RT, cells were washed and stained with rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP; Agilent Dako, CA, USA). After incubation, cells were washed and 50 µL TrueBlue peroxidase substrate (SeraCare Life Sciences, MA, USA) was added to allow for colour development before the reaction was stopped with 200 µL H<sub>2</sub>O. Plates then were washed and dried, and spots counted using a CTL-Immunospot S6 Macro analyzer with CTL Switchboard 2.6.0 (x86). Wells containing <200 spots were counted using ImageJ software and counts were used to determine titres of infectious virus, expressed as virospots (VS) per mL of original sample.

### ***2.5.3. Plaque assay for RSV and PIV-3.***

HEp-2 cells were seeded in 12-well tissue culture plates (Costar Cell Culture, Corning) and cultured overnight. Confluent monolayers were washed with serum-free DMEM and inoculated with 10-fold serial dilutions of virus in serum-free media for 2 hr at 37°C

in 5% CO<sub>2</sub>. After incubation, virus inoculum was removed, and cells were overlaid with 1.5 mL of DMEM containing 2% (v/v) FBS and 0.3% (w/v) agarose type I low EEO. After incubation for 4 (PIV-3) or 7 (RSV) days at 37°C, cells were fixed with 1% (v/v) formaldehyde in phosphate-buffered saline (PBS) for 2-3 hr. After fixation, agarose overlays were removed, cells were washed and stained with 0.05% (v/v) neutral red (Sigma-Aldrich) in water for 1 hr and then washed and air dried. Wells containing <100 plaques were counted and titres of infectious virus were expressed as PFU per mL of original sample.

#### **2.5.4. ViroSpot assay for hMPV.**

LLC-MK2 cells were seeded into flat-bottomed 96-well tissue culture plates (Costar Cell Culture, Corning) and cultured overnight. Confluent monolayers were washed with serum-free medium and inoculated with 10-fold serial dilutions of virus serum-free Opti-MEM I medium for 1 hr at 37°C in 5% CO<sub>2</sub>. After incubation, cell monolayers were overlaid with 100 µL of 2x serum-free Opti-MEM I containing 6.4% (w/v) carboxymethyl cellulose (CMC, Sigma-Aldrich) and 4 µg/mL TPCK-treated trypsin. After incubation for 48 hr at 37°C, cells were washed, fixed with 80% (v/v) ice-cold acetone in water and stained with anti-hMPV-NP mAb (Clone 36, Merck Millipore) followed by rabbit anti-mouse IgG conjugated to HRP. Plates were developed with TrueBlue peroxidase substrate (SeraCare Life Sciences) and then counted as described in 2.5.2. Wells containing <200 spots were counted using ImageJ software and titres of infectious virus were expressed as virospots (VS) per mL of original sample.



## **2.6. Molecular cloning.**

Genes encoding different human MARCH proteins were cloned into pcDNA3 for transfection of 293T or A549 cells, or into pTRE-tight and pFUV1mCherry plasmids for generation of lentiviral vectors.

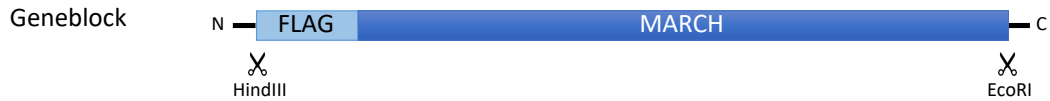
### ***2.6.1. Cloning of MARCH1/8 in pcDNA3.***

Coding sequences of human MARCH1 and MARCH8 containing an N-terminal FLAG tag (peptide sequence DYKDDDK) and flanked with restriction sites for HindIII and EcoRI were synthesized as geneblocks (GeneArt Gene Synthesis, Thermo Fisher Scientific) (**Figure 2.1A**). Geneblocks and pcDNA3 plasmids (Invitrogen, CA, USA) were digested with appropriate enzymes followed by ligation using the T4 DNA ligase (Promega, WI, USA) according to manufacturer's instructions. Plasmids carrying genes of interest were identified by digestion with appropriate restriction enzymes following standard procedures. Each plasmid was sequenced (Australian Genome Research Facility, AGRF) to confirm the cloned gene of interest.

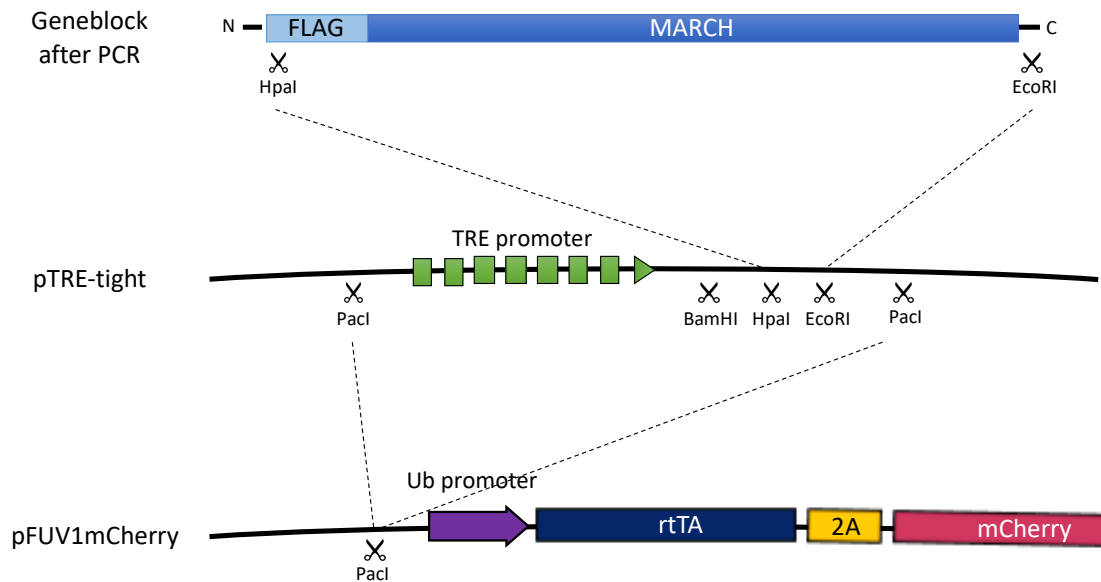
### ***2.6.2. Cloning of MARCH1/2/3/5/6/7/8/9 into pTRE-tight and FUV1mCherry plasmids.***

To produce lentiviruses for the generation of stable cell lines with doxycycline (DOX)-inducible expression of MARCH proteins (Tet-ON system), a two-step cloning strategy was required (**Figure 2.1B**). First, the coding sequence of each MARCH protein was cloned into pTRE-tight, which carries a tight TRE promoter containing seven tet operator sequences that confer DOX-inducibility. Second, fragments containing the TRE promoter and gene of interest were released from the pTRE-tight vector and then cloned into the

**A.**



**B.**



**Figure 2.1. Cloning strategy to generate pFUV1mCherry plasmids for the generation of lentiviral particles.** (A) Geneblocks were synthesised to contain coding sequences of each MARCH protein, including a N-terminal FLAG and flanked by HindIII and EcoRI restriction sites. (B) pFUVmCherry vectors carrying each MARCH protein were generated following a two-step cloning strategy. First, the HindIII restriction site at the N-terminal end of each geneblock was replaced with a HpaI restriction site via PCR and then sequences encoding FLAG-tagged MARCH were cloned into pTRE-tight using HpaI/EcoRI restriction sites. Next, FLAG-tagged MARCH under the control of the TRE promoter were cloned into pFUV1mCherry using PacI restriction sites.

pFUV1mCherry lentivirus transfer plasmid, which also expresses the gene for mCherry under the control of the constitutive ubiquitin promoter. Both plasmids were kindly provided by Professor Marco Herold (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia)

As for MARCH1/8, MARCH2/3/5/6/7/9 were synthesized as geneblocks and then amplified by PCR using the Phusion High-fidelity DNA polymerase (New England Biolabs, NEB, MA, USA) according to manufacturer's instructions. Due to incompatibility of the restriction sites at the N-terminal end of each geneblock, forward primers carrying a HpaI restriction site and reverse primers specific for the C-terminal end of the geneblocks (HpaI-MARCH and geneblock RV, **Table 2.3.1**) were used to replace the HindIII restriction site at the N-terminal end of each geneblock. A similar strategy was used to introduce compatible restriction sites flanking the coding sequence of a cytoplasmic version of hen egg ovalbumin (cOVA) lacking the sequence for cell-surface trafficking [217], with the aim of generating cell lines expressing an irrelevant control protein. Sequences of the primers used (cOVA FW and RV) are shown in **Table 2.3.1**. Purified PCR products and pTRE-tight plasmid were digested with HpaI and EcoRI (NEB) and then ligated using the T4 DNA ligase (Promega) according to manufacturer's instructions. Next, pTRE-tight plasmids carrying the sequence of interest were digested with PacI (NEB), gel purified and ligated into PacI digested pFUV1mCherry using the T4 DNA ligase (Promega) according to manufacturer's instructions. pFUV1mCherry plasmids carrying sequences of interest were identified by digestion with appropriate restriction enzymes following standard procedures. Each plasmid was then sequenced (AGRF) to confirm each gene of interest.

### **2.6.3. Transformation and plasmid purification.**

At various steps in the cloning process, ligation reactions or final plasmid product were used to transform competent NOVA XG *Escherichia coli*. For transformation, 3 µL of each ligation or 100 ng of plasmid were mixed with 50 µL competent bacteria and incubated on ice. After 30 min, each mixture was incubated at 42°C for 90 sec and then on ice for 2 min. After incubation, 1 mL of Luria-Bertani (LB) broth (Media Preparation Unit (MPU), Department of Microbiology and Immunology, The University of Melbourne) was added before further incubation at 37°C with shaking (180 rpm) for 1 hr. Each culture was then plated onto LB agar plates containing 100 µg/mL of ampicillin (MPU, Department of Microbiology and Immunology, The University of Melbourne) and incubated overnight at 37°C. Single colonies were then picked, inoculated in 10 mL of LB media with 100 µg/mL of ampicillin and cultured at 37°C with shaking overnight. Plasmid DNA was purified using the AccuPrep Plasmid Mini Extraction Kit (Bioneer, South Korea) according to manufacturer's instructions. DNA concentration was determined using a NanoDrop spectrophotometer (ThermoFisher Scientific).

### **2.7. Transient transfection of 293T cells with pcDNA3 plasmids.**

293T cells were seeded in 12-well plates (Costar Cell Culture, Corning) and cultured overnight. Cells at 80-90% confluency were then transiently transfected with 1 µg of pcDNA3 containing FLAG-tagged MARCH1/8 using Lipofectamine 2000 (Life Technologies, CA, USA), following manufacturer's instructions. As a negative control,

cells were transfected with pcDNA3 encoding a cOVA lacking a FLAG tag. At 48 hr post-transfection, protein expression was analysed by flow cytometry and/or western blot.

## **2.8. Analysis of FLAG expression by flow cytometry.**

Adherent monolayers of 293T, A549 or HeLa cells expressing FLAG-tagged proteins were washed and detached and then transferred to wells of round-bottom 96-well plates (Costar Cell Culture, Corning). Cells were stained with fixable viability dye eFlour 780 (eBioscience, CA, USA) for 15 min on ice, washed with FACS buffer (PBS supplemented with 2% FBS and 1 mM Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich)) and fixed with 2% (v/v) paraformaldehyde in PBS. After fixation, cells were permeabilized in PBS containing 0.5% (v/v) Triton X-100 (Sigma-Aldrich) for 10 min at room temperature (RT). Cells were then stained with anti-FLAG-Allophycocyanin (APC) mAb (Clone L5, Biolegend, CA, USA) diluted in FACS permeabilization buffer (PBS supplemented with 2% FBS, 0.25% (v/v) Triton X-100 and 1 mM EDTA) for 30 min on ice, washed and analysed by flow cytometry. Samples were acquired on a FACSCanto II (BD Biosciences, CA, USA) or a LSRFortessa flow cytometer (BD Bioscience) and analyzed using FlowJo analysis software version 10.6.2.

## **2.9. Analysis of FLAG expression by western blot.**

### ***2.9.1. Preparation of cell lysates.***

293T cells transiently expressing FLAG-tagged proteins were washed, detached and transferred to 1.5 mL microcentrifuge tubes (Axygen, Corning). Cells were lysed with ice-

cold lysis buffer (50 mM Tris- HCl, 0.5% (v/v) Triton X-100, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>) supplemented with protease inhibitors (cOmplete™ Mini Protease Inhibitor Cocktail, Roche, Switzerland) for 20 min on ice and centrifuged (13000 rpm, 15 min, 4°C) to remove cell debris. Clarified supernatants were transferred to fresh microcentrifuge tubes for subsequent analysis. Protein concentration of cell lysates was determined using a BCA Protein Assay kit (ThermoFisher Scientific) according to manufacturer's instructions.

### **2.9.2. Western blot.**

Cell lysates (50 µg of protein) were mixed with reducing sample buffer (50mM Tris, 2% (v/v) sodium dodecyl sulfate (SDS), 0.05% (w/v) bromophenol blue, 10% (v/v) glycerol and 20mM dithiothreitol (DTT) in water) and incubated at 95°C for 10 min. Samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% (v/v) acrylamide/bis-acrylamide gel. Polyvinylidene difluoride (PVDF) membranes (Immobilon-P PVDF Membrane, Merck Millipore, MA, USA) were activated with methanol (Chem-Supply, SA, Australia) before protein transfer to activated membranes using a Mini Trans-Blot cell system (Bio-Rad, CA, USA) according to manufacturer's instructions. After transfer, membranes were incubated with blocking buffer (5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich), 0.1% Tween-20 (Sigma-Aldrich) in PBS) for 1 hr at RT and then incubated overnight on a shaker at 4°C with anti-FLAG-APC and anti-β-actin mAb (clone sc-47778, Santa Cruz Biotechnology, TX, USA). Membranes were washed and then incubated with chicken anti-mouse IgG conjugated to Alexa Fluor 488 (Invitrogen) for 1 hr at RT. After washing, membranes were then imaged using a Molecular Imager PharosFX System (Bio-Rad).

## **2.10. Stable cell lines with DOX-inducible expression of MARCH proteins.**

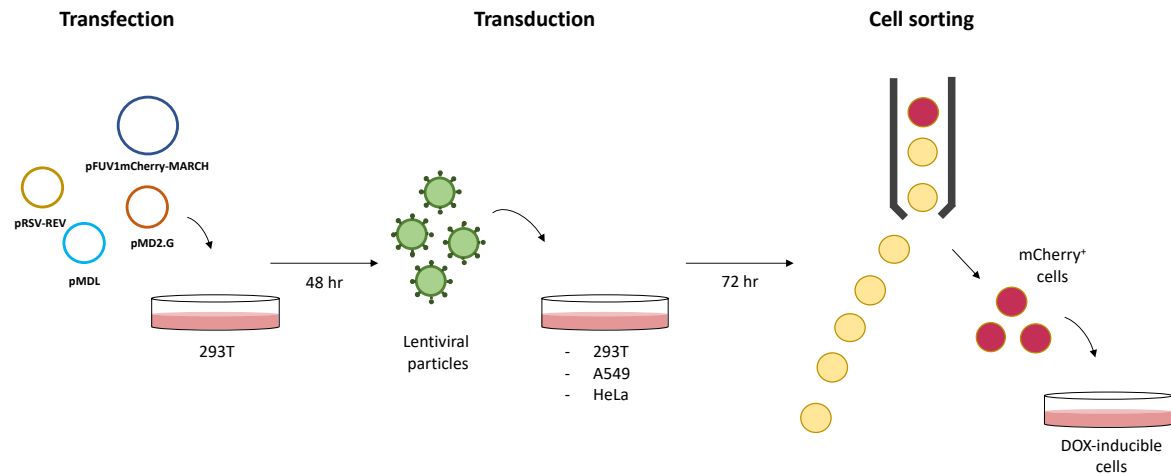
To generate stable cell lines with DOX-inducible expression of FLAG-tagged MARCH proteins, cell lines were transduced with different lentivirus stocks, followed by cell-sorting to enrich for transduced mCherry-positive cells (**Figure 2.2**).

### ***2.10.1. Generation of lentiviral particles.***

To generate lentivirus particles using pFUV1mCherry containing different MARCH or control (cOVA) proteins, 293T cells seeded into 12-well plates (Costar Cell Culture, Corning) were incubated overnight at 37°C in 5% CO<sub>2</sub>. Cells at 80-90% confluency were then transiently transfected with a 2:1:1.2:4 ratio of the packaging plasmids pMDL, pRSV-REV, pMD2.G, along with the pFUV1mCherry transfer plasmid (1 µg of total plasmids) using Lipofectamine 2000, following manufacturer's instructions. pMDL, pRSV-REV and pMD2.G plasmids were kindly provided by Professor Marco Herold (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). At 48 hr post-transfection, supernatants containing lentivirus particles were harvested and filtered through a 0.45 µm filter for subsequent transduction.

### ***2.10.2. Transduction and cell sorting.***

293T, A549 or HeLa cells were seeded into 6-well plates (Costar Cell Culture, Corning) and cultured overnight. Cells at 50-60% confluency were then transduced with lentiviruses expressing individual MARCH proteins or cOVA (CONTROL) using spinoculation (1800 rpm, 45 min, 32°C) and then incubated overnight at 37°C in 5% CO<sub>2</sub>. At 24 and 48 hr post-transduction, cell supernatants were removed and replaced with



**Figure 2.2. Generation of stable cells lines with DOX-inducible expression of MARCH proteins.** First, 293T cells were transfected with plasmids to generate lentivirus particles. At 48 hr post-transfection, supernatants containing lentiviruses were harvested and used to transduce 293T, A549 or HeLa cells. At 72 hr post-transduction, cell sorting was performed to enrich for mCherry-positive cells with DOX-inducible expression of MARCH proteins.



fresh culture media. At 72 hr post-transduction, cells were detached, stained with fixable viability dye eFlour 780 for 15 min on ice, washed and filtered through a BD Falcon Tube with a 35  $\mu$ m cell strainer cap (BD Bioscience). Cell sorting was then performed using a BD FACSAria III Cell Sorter (BD Biosciences) to recover mCherry-positive viable cells, which were then seeded into tissue culture flasks. After expansion of cells in culture at 37°C in 5% CO<sub>2</sub>, flow cytometry was used to determine mCherry and intracellular expression of FLAG-tagged proteins.

### ***2.10.3. DOX-induction of stably transduced cell lines.***

After cell sorting and expansion in culture, cells were seeded in 24-well tissue culture plates (Costar Cell Culture, Corning) and cultured overnight. To induce expression of proteins of interest, cells were incubated in media containing 1  $\mu$ g/mL of DOX (Sigma-Aldrich) for 24 hr at 37°C. In some experiments, 10  $\mu$ M of the proteasomal inhibitor MG132 (Sigma-Aldrich) was also added 20 hr after DOX induction to avoid intracellular protein degradation. At 24 hr post-DOX induction, intracellular FLAG expression was determined by flow cytometry as described in section 2.8. For long-term storage, cell stocks were frozen in freezing media (90% (v/v) FBS, 10% (v/v) dimethyl sulfoxide (DMSO, Sigma-Aldrich)) and stored in liquid N<sub>2</sub>.

### **2.11. Detection of cell-surface CD86 by flow cytometry.**

To evaluate the functionality of MARCH proteins after DOX-induction, flow cytometry was used to monitor cell-surface expression of CD86. In these experiments, DOX-

inducible 293T cells were seeded into 12-well tissue culture plates and cultured overnight. Cells at 80% confluency were then transiently transfected with 1 µg of pHAGE-CD86-ZsGreen using Lipofectamine 2000, according to manufacturer's instructions. pHAGE-CD86-ZsGreen (kindly provided by Dr. Melissa Call, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) contains an internal ribosome entry site (IRES) allowing for simultaneous expression of CD86 and the fluorescent protein ZsGreen. At 12 hr post-transfection, cells were incubated in media alone (uninduced) or in media supplemented with 1 µg/mL DOX to induce protein expression. At 48 hr post-transfection, cells were washed, detached and transferred to individual wells of round bottom 96-well plates. Cells were then stained with fixable viability dye eFlour 780 and anti-CD86 conjugated to Brilliant Violet (BV)711 mAb (Clone 2331 (FUN-1), BD Biosciences) for 30 min on ice. After incubation, cell suspensions were washed, resuspended in FACS buffer and levels of cell-surface CD86 were determined by flow cytometry using a BD LSRFortessa flow cytometer (BD Biosciences).

## **2.12. Detection of virus-infected cells by flow cytometry.**

Flow cytometry has been used to determine the susceptibility of different cell lines to infection with a number of human respiratory viruses, including influenza viruses, RSV, hMPV and PIV-3. In some experiments, 293T, A549 and MDCK cells were seeded into 24-well tissue culture plates and cultured overnight. In other experiments to determine the impact of DOX-inducible MARCH proteins on respiratory viral infections, DOX-inducible cell lines (293T, A549 or HeLa) seeded into 24-well tissue culture plates were

incubated in media alone or in media supplemented with 1 µg/mL of DOX for 24 hr prior to infection. After overnight culture, cells were washed and inoculated with human respiratory viruses at the indicated multiplicity of infection (MOI), which were determined by viable cell counts in the presence of trypan blue (Gibco, ThermoFisher Scientific) just prior to virus inoculation. After incubation with virus dilutions for 1 hr at 37°C in 5% CO<sub>2</sub>, cells were washed to remove residual inoculum and incubated in serum-free media for an additional 7 hr (IAV, IBV) or 17 hr (RSV, hMPV, PIV-3) at 37°C. Note that for experiments using cell lines with DOX-inducible expression of MARCH proteins, serum-free media was supplemented with 1 µg/mL of DOX during this incubation period. After incubation, virus-infected cells were washed, detached and transferred to individual wells of round bottom 96-well plates. Cells were stained with fixable viability dye eFlour 780 for 15 min on ice, washed in FACS buffer and then fixed with 2% (v/v) PFA in PBS. After fixation, cell suspensions were permeabilized with PBS containing 0.5% (v/v) Triton X-100 for 10 min at RT and then incubated with antibodies for detection of specific viral proteins (**Table 2.4.1**) for 30 min on ice. While most mAb to viral proteins were directly conjugated to fluorescent tags, detection of PIV-3-infected cells, required incubation with a secondary chicken anti-mouse IgG conjugated to Alexa Fluor 488 (Invitrogen). Cells were then washed and resuspended in FACS buffer for subsequent analysis by flow cytometry. Samples were acquired on a FACSCanto II (BD Biosciences) or a LSRFortessa flow cytometer (BD Bioscience) analyzed using FlowJo analysis software version 10.6.2.

### **2.13. Assessment of productive replication of respiratory viruses in mammalian cell lines.**

We have assessed the ability of different cell lines to support productive virus growth following infection with a number of human respiratory viruses, including influenza viruses, RSV, hMPV and PIV-3. In some experiments, 293T, A549 and MDCK cells were seeded into 24-well tissue culture plates and cultured overnight. In other experiments, to determine the impact of DOX-inducible MARCH proteins on respiratory viral infections, DOX-inducible cell lines (293T, A549 or HeLa) seeded into 24-well tissue culture plates were incubated in media alone or in media supplemented with 1 µg/mL of DOX for 24 hr prior to infection. After overnight culture, cells were washed and then inoculated with human respiratory viruses at the indicated MOI (calculated as described in section 2.12). After incubation with virus inoculum for 1 hr at 37°C in 5% CO<sub>2</sub>, cells were washed to remove residual inoculum and then incubated in serum-free media. Note that (i) for experiments using cell lines with DOX-inducible expression of MARCH proteins, serum-free media was supplemented with 1 µg/mL of DOX during this incubation period and (ii) for multicycle growth curves with IAV, serum-free media was supplemented with 0.5 µg/ml TPCK-treated trypsin. For IAV and IBV, cell supernatants harvested at different time points after infection (as indicated) were clarified by centrifugation (3000 rpm, 5 min, 4°C) and titres of infectious virus determined by plaque assay (Section 2.5.1) or ViroSpot assay (Section 2.5.2). For RSV, hMPV and PIV-3, plates containing virus-infected cells were frozen overnight at -80°C. After thawing, samples were transferred to 1.5 mL microcentrifuge tubes, vortexed for 2 min and clarified by centrifugation (3000 rpm, 5 min, 4°C). Titres of infectious RSV and PIV-3 were then

determined by plaque assay (Section 2.5.3), and titres of hMPV were determined by ViroSpot assay (Section 2.5.4).

## **2.14 Generation of DOX-inducible cell lines expressing MARCH8 RING-CH mutants.**

### ***2.14.1. Molecular cloning.***

Based on previous studies, we generated the MARCH8 RING-CH mutants MARCH8-CS [218] and MARCH8-W114A [199]. For the MARCH8-CS mutant, where cysteines residues (C) in the positions 80, 83, 97, 99, 110, 123 and 126 were replaced by serine residues (S), a geneblock was synthesised where the codon TGT (C) was replaced by the codon AGC (S) at the appropriate positions. A pFUV1mCherry-MARCH8-CS plasmid was then generated as described (Section 2.6.2). For the MARCH8-W114A mutant, where the tryptophan (W) at position 114 was replaced by alanine (A), site-directed mutagenesis was performed using the pcDNA3-MARCH8 plasmid and degenerate primers (Forward primer: 5'-GCCTGTCTGCAGCAGGCGATCAAGAGCAGCGACAC-3', Reverse primer: 5'-GTGTCGCTGCTCTTGATCGCCTGCTGCAGACAGGC-3') carrying the W114A mutation (underlined).

#### ***2.14.1a. Site-directed mutagenesis.***

Site-directed mutagenesis was performed using the pcDNA3-MARCH8 plasmid and the *Pfu* DNA polymerase (Agilent Technologies) following the manufacturer's instructions. After 18 PCR cycles, PCR products were treated with the restriction enzyme DpnI (NEB) to digest template plasmid DNA that does not carry the specific mutation required.

Digested PCR products were transformed into *E. coli* NOVA XG as described (section 2.6.3) and mutagenesis of the purified plasmid (pcDNA3-MARCH8-W114) was confirmed via sequencing (AGRF).

#### *2.14.1b. Cloning of MARCH8-W114 into pTRE-tight and FUV1-mCherry plasmids.*

First, PCR was used to amplify MARCH8-W114 from pcDNA3-MARCH8-W114, using the Phusion High-fidelity DNA polymerase (NEB) according to manufacturer's instructions. Due to the incompatibility of the restriction sites, PCR was performed using the same forward primer described previously (HpaI-MARCH FW) in combination with a reverse primer that binds outside the multiple cloning site (MCS) of pcDNA3 (pcDNA3 RV, **Table 2.3.1**). The following cloning steps used to generate the plasmid pFUV1mCherry-MARCH8-W114A were similar to those described (section 2.6.2).

#### **2.14.2. DOX-inducible 293T cells.**

293T cells with DOX inducible expression of MARCH8-CS and MARCH8-W114A were generated as described (Section 2.10).

### **2.15. Quantification of vRNA in supernatant from infected cells and calculation of infectivity per particle.**

293T cells with DOX-inducible expression of MARCH1 and MARCH8 were seeded in 24-well tissue culture plates and cultured overnight. To induce expression of MARCH1/8, cells were incubated in media alone (uninduced) or media containing 1 µg/mL of DOX

(induced). At 24 hr post-DOX induction, cells were washed and inoculated with IAV strain Beij89 (H3N2) at MOI 5 PFU/cell in serum free media. After 1 hr incubation with virus inoculum, cells were washed to remove residual inoculum and incubated with serum-free media for an additional 23 hr at 37°C. For cells with DOX-inducible expression of MARCH proteins, serum-free media was supplemented with 1 µg/mL of DOX during this incubation period. At 24 hr post-infection, supernatants were harvested and each supernatant was transferred to two 1.5 mL microcentrifuge tubes and clarified by centrifugation (3000 rpm, 5 min, 4°C). Of the two aliquots, one was used to determine titres of infectious virus (Section 2.5.1) and the other was used to determine the number of copies of vRNA by qPCR. To determine viral copy number, vRNA was extracted from the supernatant of infected cells using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to manufacturer's instructions. To calculate the number of copies of the segment coding for the matrix (M) proteins, 4 µL of vRNA were amplified using specific primers and probe (**Table 2.3.2**) in combination with the SensiFAST Probe No-ROX One-Step Kit (Bioline, TN, USA) following manufacturer's instructions. The qPCR reaction was performed using the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, CA, USA). The copy numbers of the M segment were calculated by generating a standard curve using 10-fold serial dilutions of plasmid containing DNA encoding the IAV M gene from A/California/7/2009 (H1N1pdm09). To determine the infectivity per particle, the ratio between the titre of infectious virus and the number of copies of the M segment was calculated, as described previously by Bedi *et al.* [219].

## **2.16. Cell-surface expression of IAV glycoproteins by flow cytometry.**

To determine cell-surface expression of the HA, NA and M2 viral proteins in IAV-infected cells, 293T cells with DOX-inducible expression of MARCH8 were seeded in 12-well tissue culture plates and cultured overnight. To induce expression of MARCH1/8, cells were incubated in media alone (uninduced) or in media containing 1 µg/mL of DOX. At 24 hr post-DOX induction, cells were washed and infected with IAV strain Beij89 at MOI 1 PFU/cell in serum-free media. After 1 hr incubation with virus inoculum, cells were washed to remove residual inoculum and incubated with serum-free media at 37°C. For cells with DOX-inducible expression of MARCH proteins, serum-free media was supplemented with 1 µg/mL of DOX during this incubation period. At 8 hr or 24 hr post-infection, cells were washed, detached and transferred to individual wells of round bottom 96-well plates. Cells were then stained with a mAb against IAV-M2 (clone 14C2, Abcam, UK), a biotinylated mAb against IAV HA (clone C1/1, kindly provided by Professor Lorena Brown, The University of Melbourne) or a goat polyclonal antisera (pAbs) against IAV-NA (NR-3137, Biodefense and Emerging Infections Research Resources Repository (BEI Resources, USA)) in combination with fixable viability dye eFlour 780 for 30 min on ice. After incubation, cells were washed and incubated with either anti-mouse IgG phycoerythrin (PE) (BD Bioscience) to detect viral M2, V450-conjugated streptavidin (BD Bioscience) to detect viral HA or anti-goat IgG Alexa Fluor 647 (Invitrogen) to detect viral NA for 30 min on ice. Cells were then washed with FACS buffer, fixed with 2% (v/v) PFA and then permeabilized in PBS containing 0.5% (v/v) Triton X-100 for 10 min at RT. Finally, cell suspensions were incubated with anti-IAV NP Fluorescein (FITC) mAb (Clone 431, Abcam) in FACS permeabilization buffer for 30 min on ice and then washed and



resuspended in FACS buffer for subsequent flow cytometry analysis. Samples were acquired on a BD LSRFortessa flow cytometer (BD Bioscience) and analysed using FlowJo analysis software version 10.6.2.

## **2.17. Analysis of viral glycoproteins in concentrated supernatants and cell lysates from infected cells by western blot.**

### ***2.17.1 Preparation of samples.***

293T cells with DOX-inducible expression of MARCH8 were seeded into 10 cm tissue culture dishes (Corning) and cultured overnight. To induce expression of MARCH1/8, cells were incubated in media alone (uninduced) or media containing 1 µg/mL of DOX. At 24 hr post-DOX induction, cells were washed and infected with IAV strain Beij89 at MOI 5 PFU/cell in serum-free media. After 1 hr incubation with virus inoculum, cells were washed to remove residual inoculum and incubated with serum-free media for 23 hr at 37°C. For cells with DOX-inducible expression of MARCH proteins, serum-free media was supplemented with 1 µg/mL of DOX during this incubation period. At 24 hr post-infection, supernatants from 15 tissue culture dishes were harvested and clarified by centrifugation (3000 rpm, 15 min, 4°C). To prepare samples of infected-cell supernatants containing concentrated IAV particles, 8 mL of 30% (w/v) sucrose were added to 38.5 mL Ultra-Clear tubes (Beckman Coulter, CA, USA) and clarified supernatants were loaded on top of the 30% cushion sucrose. Ultracentrifugation was performed at 120,000 xg for 2 hr at 4°C in an Optima XPN-100 ultracentrifuge using a SW32 Ti rotor (Beckman Coulter). After ultracentrifugation, pelleted virus was

resuspended in PBS. Cell lysates were also prepared from the virus-infected cells as described (Section 2.9.1).

#### **2.17.2 Protein quantification and western blot.**

The protein concentration of cell lysates and samples containing concentrated viral particles were determined as described (Section 2.9.1). Next, 1 µg of cell lysate or concentrated viral particles were mixed with reducing sample buffer and subjected to SDS-PAGE, followed by transfer to activated PVDF membranes as described (Section 2.9.2). The primary and secondary antibodies used for western blot are described in **Table 2.4.2**. Detection of bound antibodies was performed by chemiluminescence, using the SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher scientific) according to manufacturer's instructions. Images were acquired using the Amersham Imager 600 (GE Healthcare Life Sciences, MA, USA). The densitometry of the different bands obtained by western blot was measured using ImageJ software.

### **2.18 Assessment of hemagglutination and neuraminidase activity in supernatants from virus-infected cells.**

#### **2.18.1. Preparation of samples.**

293T cells with DOX-inducible expression of MARCH8 were seeded in 24-well tissue culture plates and cultured overnight. To induce expression of MARCH1/8, cells were incubated in media alone (uninduced) or media containing 1 µg/mL of DOX. At 24 hr post-DOX induction, cells were washed and infected with IAV strain Beij89 or Braz78 at

MOI 5 PFU/cell in serum-free media. After 1 hr incubation with virus inoculum, cells were washed to remove residual inoculum and incubated with serum-free media for 23 hr at 37°C. For cells with DOX-inducible expression of MARCH proteins, serum-free media was supplemented with 1 µg/mL of DOX during this incubation period. At 24 hr post-infection, supernatants were harvested and each supernatant was transferred to two 1.5 mL microcentrifuge tubes and clarified by centrifugation (3000 rpm, 5 min, 4°C). Of the two aliquots, one was used in hemagglutination assay and the other in a neuraminidase activity assay, as described below.

#### ***2.18.2. Hemagglutination assay.***

Serial 2-fold dilutions of clarified IAV-infected cell supernatants were performed in PBS using round-bottom 96-well plates (25 µL per well). Control wells received 25 µL of PBS alone. After addition of 1% turkey erythrocytes in PBS (25 µL per well), plates were shaken and incubated for 30 min at RT. HA titres were recorded as the reciprocal of the highest dilution of supernatant that induced complete agglutination of erythrocytes.

#### ***2.18.3. Enzymatic activity of the viral neuraminidase.***

Enzymatic activity of the IAV neuraminidase activity was determined using a fluorometric based assay that measures the amount of fluorescent 4-MU cleaved after incubation of virus-containing samples with the MUNANA substrate [220]. In these experiments, serial 2-fold dilutions of clarified IAV-infected cell supernatants diluted in NA assay buffer (33.3 mM 2-(N-morpholino) ethanesulfonic acid (MES) and 4 mM CaCl<sub>2</sub>, pH 6.5) containing 0.1% (v/v) NP-40 (ThermoFisher scientific) were prepared in round-bottom 96-well plates (50 µL per well). Control wells received 50 µL of NA assay buffer

containing 0.1% (v/v) NP-40 alone. Next, 50  $\mu$ L of 300  $\mu$ M MUNANA (Sigma-Aldrich) in NA assay buffer was added to each well and plates were incubated for 1 hr at 37°C. After incubation, 100  $\mu$ L of stop solution (138 mM NaOH in absolute ethanol) was added to stop the enzymatic reaction. Fluorescence measurements were acquired using a Fluoroskan Ascent fluorometer (ThermoFisher scientific) and NA activity was recorded as relative fluorescence units (RFU).

## **2.19 Analysis of IAV particles by transmission electron microscopy.**

### ***2.19.1 Preparation of the samples.***

293T cells with DOX-inducible expression of MARCH8 were seeded into 10 cm tissue culture dishes and cultured overnight. To induce expression of MARCH1/8, cells were incubated in media alone (uninduced) or media containing 1  $\mu$ g/mL of DOX. At 24 hr post-DOX induction, cells were washed and infected with IAV strain Braz78 at MOI 5 PFU/cell in serum-free media. After 1 hr incubation with virus inoculum, cells were washed to remove residual inoculum and incubated with serum-free media for 23 hr at 37°C. For cells with DOX-inducible expression of MARCH proteins, serum-free media was supplemented with 1  $\mu$ g/mL of DOX during this incubation period. At 24 hr post-infection, supernatants were harvested and clarified by centrifugation (3000 rpm, 15 min, 4°C). Virus was then purified from clarified supernatants by ultracentrifugation using a three-step protocol. First, cell-supernatants were concentrated through a 30% (w/v) cushion sucrose as described (Section 2.17.1). Next, concentrated supernatants were loaded on top of a sucrose step gradient (from 55% to 25% (w/v) sucrose, with 5%

increments) prepared in 13.2 mL Ultra-Clear tubes (Beckman Coulter). Ultracentrifugation was performed at 210,000 g for 1.5 hr at 4°C, without break, in an Optima XPN-100 ultracentrifuge using a SW41 Ti rotor (Beckman Coulter). After ultracentrifugation, the fraction containing 40% (w/v) sucrose was collected, diluted in PBS in 38.5 mL Ultra-Clear tubes (Beckman Coulter) and ultracentrifuged at 120,000 g for 1 hr at 4°C in an Optima XPN-100 ultracentrifuge using a SW32 Ti rotor (Beckman Coulter). After ultracentrifugation, purified viruses were resuspended in PBS for subsequent transmission electron microscopy.

### ***2.19.2 Transmission electron microscopy.***

Purified samples were fixed with 2% (v/v) PFA for 1 hr at 4°C. Fixed samples were placed onto carbon-coated copper grids (ProSciTech, USA) treated with 1% Alcian blue for 5 min and then blotted to remove liquid excess. Each grid was then washed with PBS and distilled water. Samples were negatively stained with 1% uranyl acetate for 30 sec, blotted to remove excess liquid and then dried overnight. Data acquisition was performed using a Phillips CM120 transmission electron microscope.

### **2.20. Generation of recombinant IAV by reverse genetics.**

For the generation of recombinant viruses by reverse genetics [221] we used pHW2000 plasmids containing each of the 8 genes of A/Udorn/307/1972 (H3N2). All plasmids were kindly provided by Dr. Robert Webster (St. Jude Children's Research Hospital, Memphis, TN). Site-directed mutagenesis was used to introduce appropriate mutations into genes

encoding the viral HA, NA, M1 and M2 proteins as described (Section 2.14.1a). The specific primers used to generate these mutants are listed in **Table 2.3.3**. To generate recombinant viruses by reverse genetics, co-cultures of MDCK and 293T cells at a 1:8 ratio were seeded into 6-well plates (Costar Cell Culture, Corning) and cultured overnight. Cells were transiently transfected with 1 µg of each of the 8 pHW2000 plasmids expressing different IAV genes (8 ug in total) using lipofectamine 2000 according to manufacturer's instructions. At 8 hr post-transfection, media was removed and replaced with serum-free media. At 24 hr post-transfection, media was supplemented with 2 µg/mL TPCK-treated trypsin to promote multicycle virus replication. At 72 hr post-transfection, cell supernatants were harvested, clarified by centrifugation (3000 rpm, 10 min, 4°C) and inoculated into 10-day embryonated eggs and incubated at 35°C for 2 days to facilitate virus amplification. After incubation, allantoic fluid containing virus was harvested, clarified and stored at -80°C. When site-directed mutagenesis had been performed using pHW2000 plasmids, vRNA sequencing of the relevant gene was performed on the resultant virus stock to confirm incorporation of the mutation of interest.

### **2.21. Knockdown of endogenous MARCH8 using siRNA.**

293T cells were seeded into 24-well tissue culture plates and incubated overnight. Cells were transfected with 1 µM siRNAs specific for MARCH8 or non-targeting control (NTC) (Accell SMART pool, Dharmacon, CO, USA) using Lipofectamine RNAiMAX (ThermoFisher Scientific), according to manufacturer's instructions. At 72 hr post-siRNA

treatment, cells were infected with IAV strain Beji89 at MOI 0.01 and multicycle virus growth was determined as described (Section 2.13). At 2, 24 and 48 hr post-infection, supernatants were harvested, clarified and titres of infectious virus were determined by plaque assay on MDCK cells (Section 2.5.1). At the same time points, infected cells were also collected, and qPCR was used to determine the efficiency of siRNA knockdown during IAV infection through quantitation of *MARCH8* mRNA. First, total RNA was extracted from virus-infected cells using a RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Next, RNA was converted to cDNA using a SensiFAST cDNA Synthesis Kit (Bioline) following manufacturer's instructions. SYBR green-based qPCR was used to analyse the expression of *MARCH8* relative to 3 housekeeping genes: *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase), *RPL13A* (ribosomal protein L13a) and *TBP* (TATA-binding protein) using the SensiFAST SYBR Lo-ROX Kit according to manufacturer's instructions. Specific primers used are listed in **Table 2.3.4**. Data acquisition was performed using the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems).

## **2.22. Generation of MARCH8/MARCH1 chimeric proteins.**

Ten different MARCH8/MARCH1 chimeric proteins were generated, each with different regions/domains of MARCH1 substituted into the MARCH8 "backbone". For chimeric mutants requiring up to 3 point mutations (MARCH8\_M1-DIRT, MARCH8\_M1-TM1, MARCH8\_M1-loop, MARCH8\_M1-TM2, MARCH8\_M1-TM1-TM2 and MARCH8\_M1-TM1-loop-TM2), site-directed mutagenesis was used, as described for generation of

MARCH8-W114A (Section 2.14.1). Degenerate primers sequences are listed in **Table 2.3.5**. The remaining mutants (MARCH8\_M1-C-CT, MARCH8\_M1-N-CT, MARCH8\_M1-RING, MARCH8\_M1-ACD) were synthesized as geneblocks, following a similar strategy to that described for the generation of MARCH8-CS (Section 2.14.1). Details relevant to cloning into pFUV1mCherry and generation of 293T cells with DOX-inducible expression of chimeric proteins are described in Sections 2.6.2 and 2.10, respectively.

## **2.23. IAV infection in mice**

C57BL/6 (wild-type, WT) and C57BL/6 mice carrying a mutation in *March8* (MARCH8 KO) were bred and maintained at the Bio21 Institute animal facility (The University of Melbourne). Mice were imported to our animal house (Department of Microbiology and Immunology, The University of Melbourne) at least 2-3 days prior the start of the experiment, to allow for adjustment. Experiments were conducted using male and female mice aged 6-10 weeks, in accordance with The University of Melbourne's Animal Experimentation and Ethics guidelines.

### ***2.23.1. Total and upper respiratory tract infections.***

Age matched WT and MARCH8 KO mice have been used in studies described in this thesis. MARCH8 KO mice were generated by crossing chimeric mice carrying the MARCH8 mutation (generated by injecting MARCH8 mutant R1 stem cells from a 129 background into blast cysts from C57BL/6 mice) with CAG-Cre mice (C57BL/6 background) and then backcrossing to C57BL/6 [222]. For total respiratory tract (TRT)



infections, mice were anaesthetised with isoflurane and infected with different doses (in PFU per mouse, as indicated) by the intranasal route in a total volume of 50  $\mu$ L of PBS. For upper respiratory tract (URT) infections, anaesthetised mice received virus inoculum in a total volume of 5  $\mu$ L, inoculated onto the nares. Mice were weighed daily and monitored for a range of clinical signs indicative of viral respiratory disease. Animals displaying evidence of pneumonia or having lost  $\geq 20\%$  of their original body weight were culled. All research complied with the University of Melbourne's Animal Experimentation Ethics guidelines and policies.

### ***2.23.2. Viral titres in tissue samples.***

At various times after infection, mice were culled and the lung and nasal tissues were removed and homogenized in PBS using a Polytron PT 2100 homogenizer (Kinematica AG, Switzerland). Tissue homogenates were clarified by centrifugation (3000 rpm, 15 min, 4°C) and titres of infectious virus in clarified supernatants were determined by standard plaque assay on MDCK cells (Section 2.5.1).

### ***2.23.3. Detection of inflammatory mediators and cells in bronchoalveolar lavage fluids.***

At various times after infection, mice were culled and bronchoalveolar lavage (BAL) was performed by inserting a catheter into the trachea and flushing the lungs 3 times with 1 mL of PBS. After centrifugation of BAL (1800 rpm, 5 min, 4°C), clarified supernatants were assessed for levels of inflammatory mediators while leukocytes present in cell pellets were analysed by flow cytometry.

### *2.23.3a. Detection of inflammatory mediators in cell-free BAL fluid.*

Levels of cytokines and chemokines in cell-free BAL fluids were determined by cytometry bead array (CBA) using the LEGENDplex Mouse Anti-Virus Response Panel Standard (Biolegend), according to manufacturer's instructions. Data was acquired using a BD FACSCanto II flow cytometer (BD Bioscience) and analysed using LEGENDplex software provided with the kit.

### *2.23.3b. Analysis of immune cell populations in BAL fluid*

Single-cell suspensions prepared from the BAL of IAV-infected mice were treated with Red Blood Cell Lysing Buffer Hybri-Max (Sigma-Aldrich) to lyse erythrocytes. Cell suspensions were then resuspended in FACS buffer and stained for 30 min on ice with appropriate combinations of fluorescent-conjugated antibodies (**Table 2.4.3**) in conjunction with fixable viability dye eFlour 780, to exclude dead cells from the analysis. The antibody cocktail also included a Fc receptor binding inhibitory antibody (anti-CD16/CD32), to avoid non-specific antibody binding. In some experiments, cells were stained with PE- or APC-labelled IAV-specific tetramers H2-D<sup>b</sup>NP<sub>366</sub> (nucleoprotein; ASNENMETM) and H2-D<sup>b</sup>PA<sub>224</sub> (acid polymerase; SSLENFRAYV) for 1 hr at RT prior to incubation with antibodies. Tetramers were kindly prepared by Dr. Jie Lin (The University of Melbourne, Melbourne, Australia). Cells were washed and resuspended in FACS buffer containing a predetermined number of blank calibration beads (Sphero blank calibration beads (BD Bioscience), to allow for calculation of absolute cell numbers in each sample. Data acquisition was performed on a BD LSRFortessa flow cytometer (BD Bioscience). Immune cell populations (CD45.2<sup>+</sup>) identified using FlowJo analysis

software version 10.6.2. were alveolar macrophages (Siglec-F<sup>+</sup>, CD11b<sup>+</sup>, CD11c<sup>+</sup>, CD64<sup>+</sup>), eosinophils (Siglec-F<sup>+</sup>, CD11b<sup>+</sup>, CD11c<sup>-</sup>, CD64<sup>+</sup>), neutrophils (Siglec-F<sup>-</sup>, CD11b<sup>+</sup>, Ly6G<sup>+</sup>), pan-macrophages (Siglec-F<sup>-</sup>, CD11b<sup>+</sup>, CD64<sup>+</sup>), pan-dendritic cells (Siglec-F<sup>-</sup>, CD11b<sup>+</sup>, CD11c<sup>+</sup>, Ly6G<sup>-</sup>, CD64<sup>-</sup>, MHC-II<sup>+</sup>), NK cells (CD3<sup>-</sup>, NKp46<sup>+</sup>), CD4<sup>+</sup> T cells (CD3<sup>+</sup>, CD4<sup>+</sup>) and CD8<sup>+</sup> T cells (CD3<sup>+</sup>, CD8<sup>+</sup>).

#### ***2.23.4. Isolation and infection of murine peritoneal macrophages.***

Peritoneal cells were obtained by lavage of the peritoneal cavity with 5 mL of ice-cold RPMI 1640 supplemented with 5 mM EDTA. Recovered cells were washed, treated with Red Blood Cell Lysing Buffer Hybri-Max (Sigma-Aldrich), seeded into 8-well glass chamberslides (LabTek, Nunc) and incubated at 37°C for 4 hr. After incubation, chamberslides were washed to remove the non-adherent cells and adherent peritoneal exudate cell macrophages (PEC Mφ) were cultured overnight at 37°C in 5% CO<sub>2</sub>.

##### ***2.23.4a. Immunofluorescence to detect IAV infection of PEC Mφ.***

After overnight incubation, PEC Mφ monolayers were washed and then infected with IAV strain Beij89 (MOI 10 PFU/cell) or with RG-PR8-Viet04 (MOI 5 PFU/cell) for 1 hr at 37°C. After washing, PEC Mφ were incubated in serum-free media for an additional 7 hr. IAV-infected PEC Mφ were then washed, fixed with 2% (v/v) PFA and permeabilized with PBS containing 0.5% Triton X-100. Cells were then stained with anti-NP FITC mAb (Clone 431, Abcam) for 30 min on ice, washed and mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector laboratories, CA, USA). Images were acquired with a Leica DMI4000 B inverted microscope and manually counted using ImageJ software.

The percentage of fluorescent cells was determined in at least 3 random fields and a minimum of 150 cells was counted for each sample.

#### *2.23.4b. Growth of IAV in PEC M $\phi$ .*

After overnight incubation, PEC M $\phi$  monolayers were washed and then infected with IAV strain Beij89 or with RG-PR8-Viet04 (both at MOI 0.01 PFU/cell) for 1 hr at 37°C. After washing, PEC M $\phi$  were incubated in serum-free media supplemented with 0.5  $\mu$ g/mL of TPCK-treated trypsin to allow for multicycle virus growth. Supernatants collected at 2, 24 and 48 hr post-infection were clarified by centrifugation (3000 rpm, 5 min, 4°C) and titres of infectious virus determined by plaque assay on MDCK cells (Section 2.5.1).

#### **2.24. Data analysis.**

Flow cytometry data was analysed using FlowJo software for Mac, version 10.6.2 (Becton, Dickinson and Company, NJ, USA). Fluorescence microscopy and western blot images were analysed using ImageJ software, version 2.0.0-rc-68/1.52f [223]. Graphs and statistical analysis (as indicated in the figure legends) were performed using GraphPad Prism (GraphPad software, CA, USA).



## Chapter 3

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**Investigating MARCH-family proteins as putative restriction factors against influenza and other respiratory viruses.**

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### 3.1. Introduction.

The majority of studies investigating mammalian MARCH proteins have focused on their role as regulators of the immune response, due to their initial discovery as homologues of viral MARCH E3 proteins. Early reports that expression of MARCH E3 ligases by  $\gamma 2$  group  $\gamma$ -herpesviruses and poxviruses resulted in downregulation of MHC I molecules from the surface of virus-infected cells led to subsequent studies showing mammalian homologues could also regulate expression of immune molecules. Mammalian MARCH E3 ligases have now been reported to downregulate MHC Class II, cluster of differentiation 86 (CD86), intercellular adhesion molecule 1 (ICAM-1), Mult1, Fc $\gamma$  Receptor IIb (Fc $\gamma$ RIIb) and TNF-related apoptosis inducing ligand receptor 1 (TRAIL-R1), amongst others (reviewed in [181]). Recently, some MARCH-family proteins have also been identified as modulators of viral replication processes. They have been shown to act as antiviral factors against human immunodeficiency virus 1 (HIV-1) and vesicular stomatitis virus (VSV), due to their ability to downregulate viral envelope glycoproteins from the surface of infected cells [210]. Interestingly, they have been also identified as proviral factors required for infection with some flaviviruses. More specifically, MARCH8 has been reported to promote viral assembly and envelopment of hepatitis C virus (HCV) at the late stages of viral replication [212].

Currently, little is known regarding the role of MARCH proteins in the context of a respiratory virus infection. Important respiratory viruses relevant to human health include influenza A viruses (IAV), members of the *Pneumoviridae* family (respiratory syncytial virus (RSV), human metapneumovirus (hMPV)) and also the *Paramyxoviridae* family (parainfluenza viruses (PIV)). Of interest, multiple viral proteins and/or host

restriction factors have been identified as substrates of ubiquitination, suggesting a potential role for the MARCH proteins in modulating respiratory virus infections. In addition, the differential localization of particular MARCH proteins within cells is consistent with the possibility that MARCH proteins may encounter respiratory viruses such as IAV, RSV, hMPV and PIV-3 at different stages of their replicative cycle within host cells.

In this chapter we aimed to investigate the ability of different MARCH-family proteins to modulate infection of host cells by IAV, RSV, hMPV and PIV-3. We have focused on MARCH1/2/3/5/6/7/8 and 9 proteins, as these tend to be expressed broadly in tissues, including the respiratory tract. We did not include MARCH4 or MARCH10/11 in our studies, as these are expressed predominantly in the brain and male reproductive tissues, respectively (**Table 1.1**). To assess the ability of MARCH family proteins to modulate respiratory virus infections: (i) different experimental approaches were used to generate stable human cell lines with ectopic expression of particular human MARCH proteins, (ii) where possible, the functionality of expressed MARCH proteins was confirmed using a known target of MARCH protein-mediated ubiquitination (i.e. CD86) and (iii) the antiviral activity of individual MARCH proteins to block early and/or late stages of respiratory virus infection was determined.

Herein, we demonstrate that of the MARCH proteins studied, only MARCH8 could restrict seasonal IAV, blocking the late stages of viral infection. Similar to IAV, only MARCH8 displayed antiviral activity against RSV. However, in this case, MARCH8 was found to block an early stage of RSV infection. In contrast, none of the MARCH proteins examined were found to modulate hMPV infection.



### **3.2. Results.**

#### **3.2.1. Establishing parameters to study putative cellular restriction factors for respiratory virus infections.**

The main target of human respiratory viruses are the airway epithelial cells in the upper and lower respiratory tract. These cells express the appropriate receptors and cellular machinery required for viruses such as IAV, RSV and hMPV to infect, replicate and produce new viral progeny from infected cells. In initial studies, we assessed the ability of three cell lines to support the infection and growth of different respiratory viruses, in order to define the most suitable cell line for future studies: (i) Madin-Darby canine kidney (MDCK) cells, a canine cell line widely used to study influenza viruses (IAV and IBV) as they are highly susceptible to infection and support high levels of virus growth, (ii) A549 cells, a human epithelial cell line derived from a lung adenocarcinoma, known to be susceptible to infection by influenza and other respiratory viruses, and (iii) 293T cells, a human embryonic kidney epithelial cell line, widely used in ectopic expression studies due to its high susceptibility to transfection and its enhanced capacity to express foreign proteins.

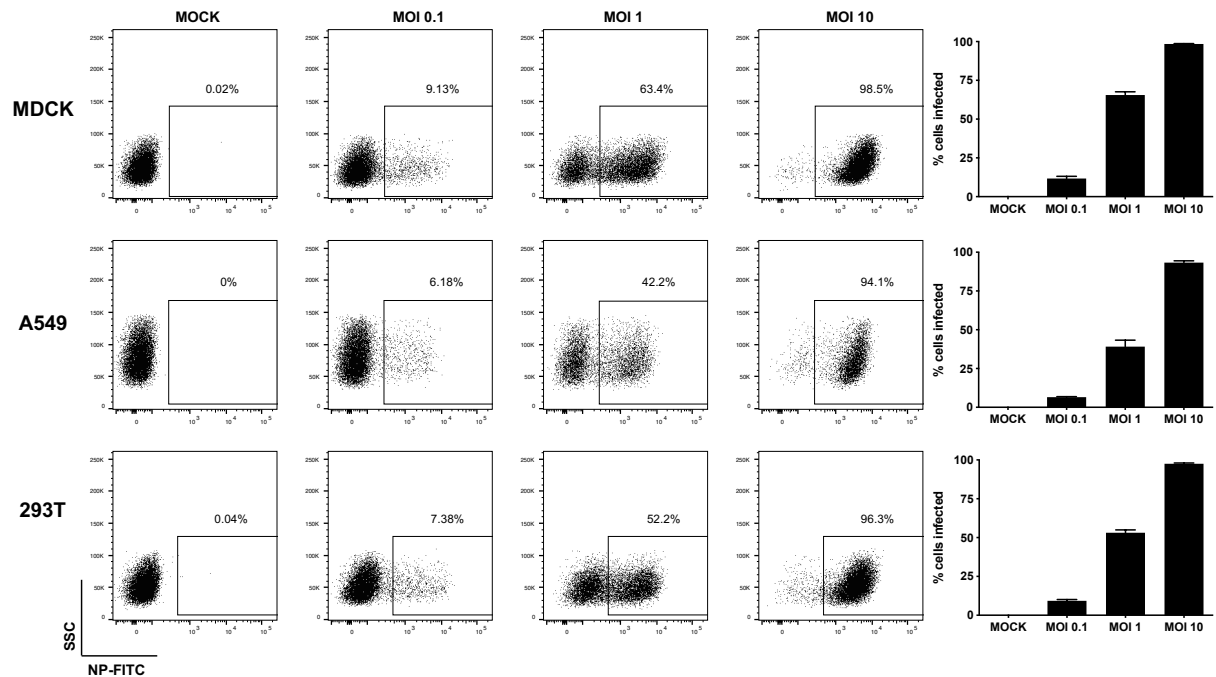
To assess the susceptibility of each cell line to infection, cells were incubated for 1 hr with increasing doses of IAV (strain A/Beijing/353/1989, Beij/89), RSV (strain A2), hMPV (strain CAN-1993/87) or PIV-3 (strain 243), washed and then cultured an additional 7 hr (IAV) or 17 hr (RSV, hMPV or PIV-3), as described in Materials and Methods. Cells were then fixed, permeabilized, stained for expression of newly-synthesized viral proteins and the percentage of infected cells determined using flow cytometry. As expected, all cell

lines tested were susceptible to IAV infection and a higher percentage of infected cells was observed as an increasing MOI was used to infect cells (**Figure 3.1(i)A**). While A549 and 293T cells showed similar susceptibility to RSV and hMPV infection, MDCK cells were markedly less sensitive, particularly to infection by RSV (**Figure 3.1(i)B**). Similarly, A549 and 293T cells were highly susceptible to PIV-3 infection, however the percentage of MDCK cells infected even at the highest MOI used (MOI 10) was <25% (**Figure 3.1(ii)D**).

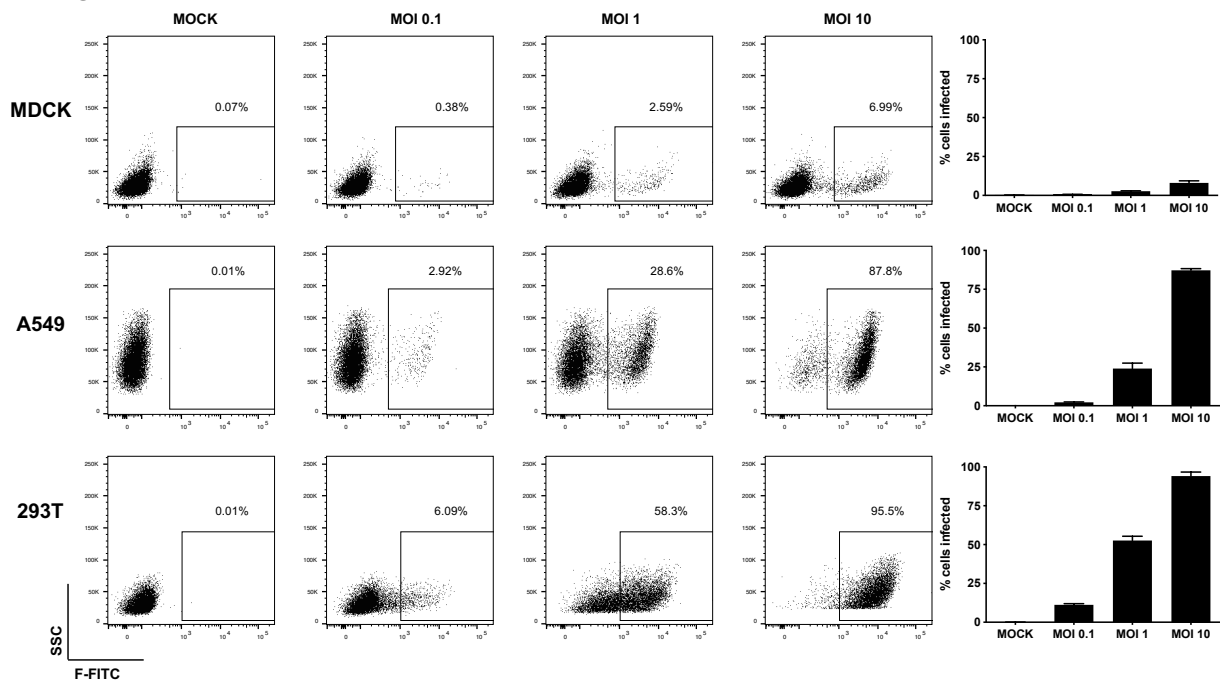
Next, we compared the ability of the different cell lines to support virus growth. In these experiments, a low MOI (0.01 PFU/cell) was used to examine multicycle virus replication over a 48 hr or 72 hr incubation period. For IAV, serum-free media was supplemented with exogenous trypsin, to allow for the activation of the viral hemagglutinin which is required to facilitate multicycle virus replication. Supernatants were collected at different time points, clarified and titres of infectious IAV were determined by plaque assay on MDCK cells. As seen in **Figure 3.2A**, IAV grew to high titres on each cell line. As expected, peak viral titres were markedly higher in MDCK cells. Peak viral titres were similar in A549 and 293T cells at 48 hr post-infection, although virus titres recovered from 293T cells were higher at earlier time points.

Considering the low sensitivity of MDCK cells to infection by pneumoviruses (RSV and hMPV) (**Figure 3.1(i)B/(ii)C**) and paramyxoviruses (PIV-3) (**Figure 3.1(ii)D**), virus growth kinetics were assessed only in A549 and 293T cells. Compared to orthomyxoviruses like IAV, a distinct feature of pneumoviruses and paramyxoviruses is the amount of nascent viral particles that remain associated with virus-infected cells. Therefore, following virus infection and incubation, tissue culture plates (i.e. containing both cell-associated virus and virus in supernatants, referred to as “total virus”) were frozen, thawed, vortexed

## A. IAV

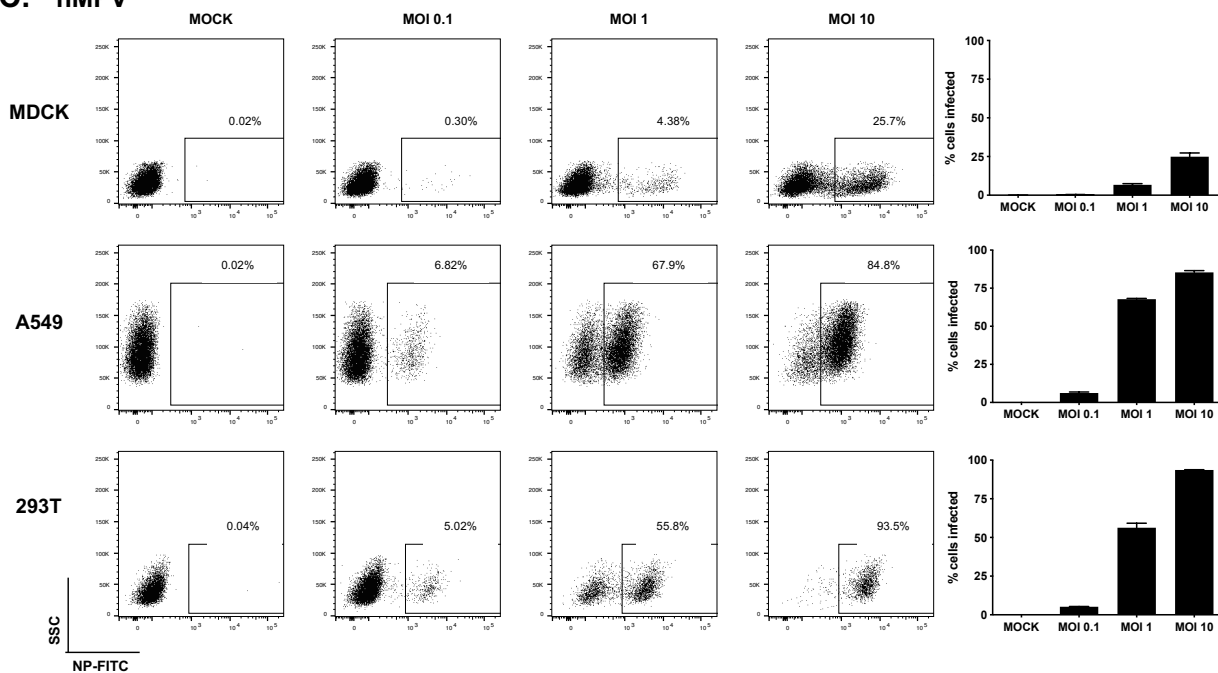


## B. RSV

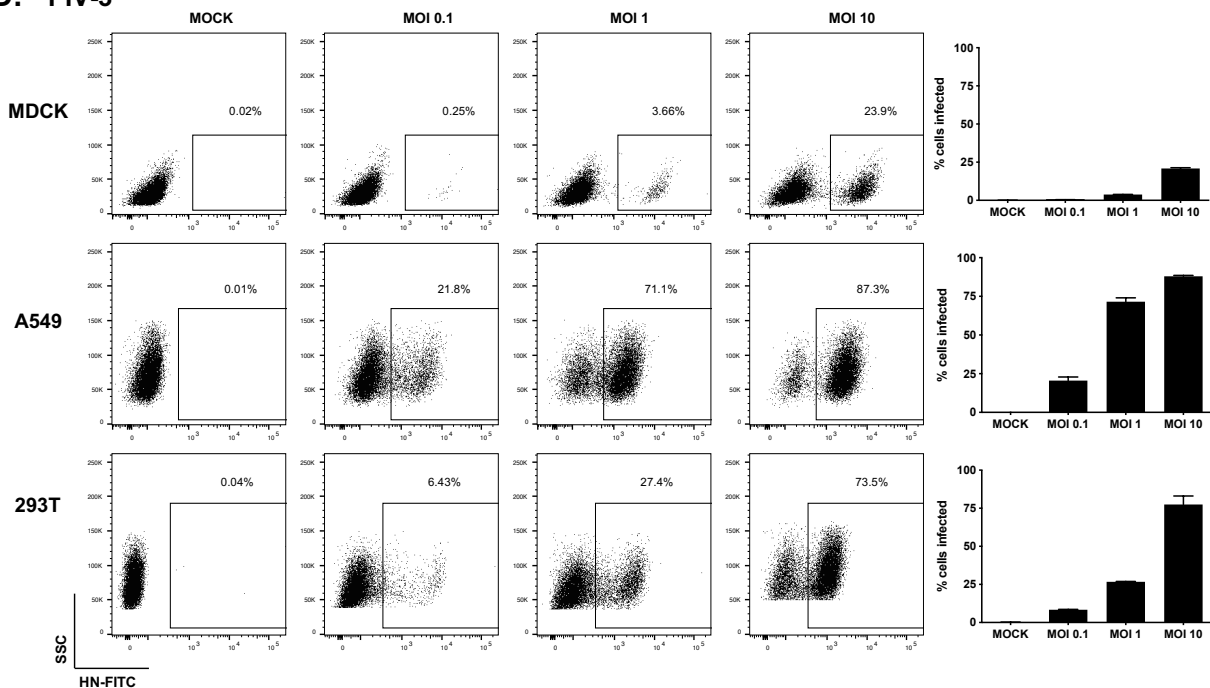


**Figure 3.1(i). Susceptibility of MDCK, A549 and 293T cells to infection by IAV, RSV, hMPV and PIV-3.** MDCK, A549 and 293T cells were seeded into 12-well tissue culture plates as described in Materials and Methods. After overnight culture, cells were infected for 1 hr at 37°C with IAV Bejj/89 (A), RSV-A2 (B), hMPV CAN-1993-87 (C) or PIV-3 strain 243 (D) at MOI = 0.1, 1 and 10 PFU/cell, washed and cultured an additional 7 (IAV) or 17 hr (RSV, hMPV, PIV-3). Cells were then washed, fixed, permeabilized and stained with mAbs to detect IAV NP (A), RSV F (B) hMPV NP (C) or PIV-3 HN (D) proteins. The percentage of virus-infected cells was then determined using flow cytometry. Representative FACs plots are shown. Bar graphs show mean  $\pm$  SD from triplicate samples.

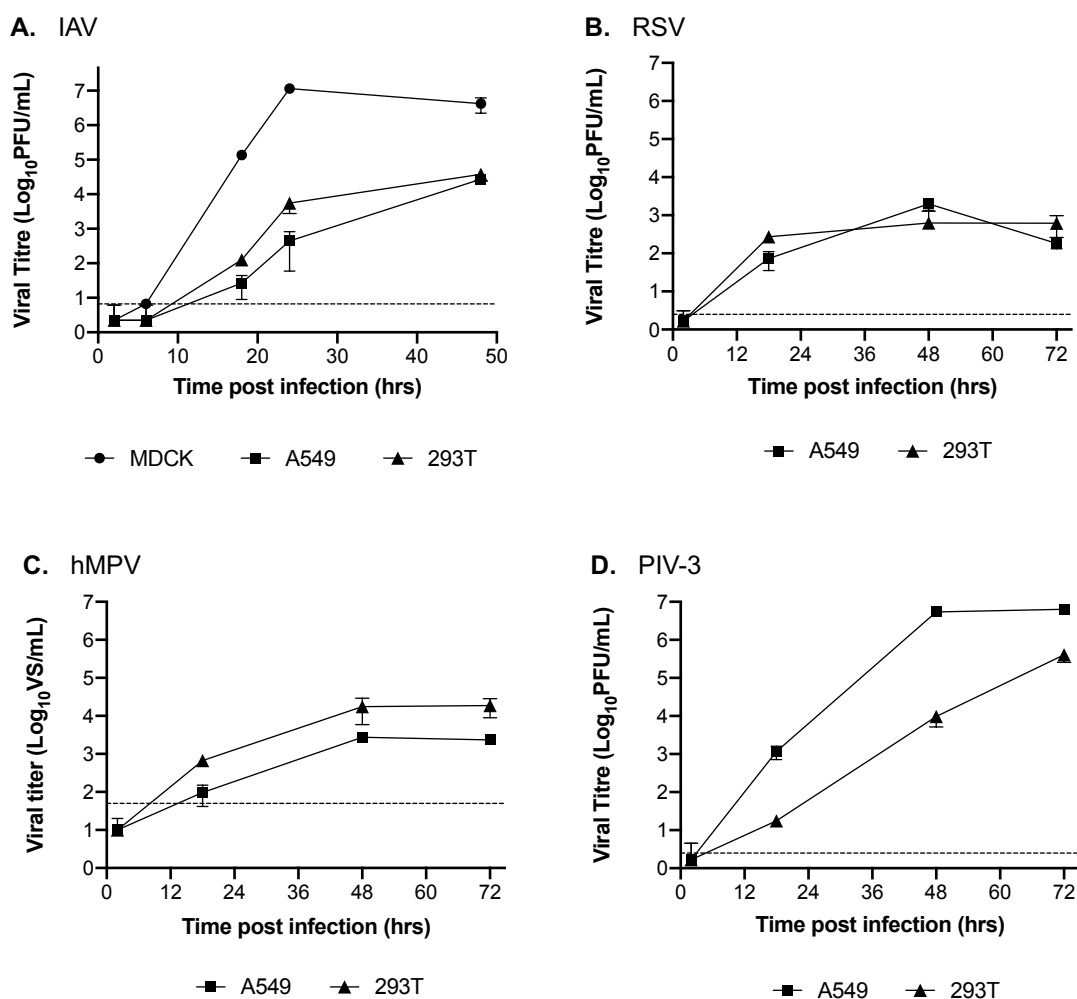
### C. hMPV



### D. PIV-3



**Figure 3.1(ii). Susceptibility of MDCK, A549 and 293T cells to infection by IAV, RSV, hMPV and PIV-3.** MDCK, A549 and 293T cells were seeded into 12-well tissue culture plates as described in Materials and Methods. After overnight culture, cells were infected for 1 hr at 37°C with IAV Beij/89 (A), RSV-A2 (B), hMPV CAN-1993-87 (C) or PIV-3 strain 243 (D) at MOI = 0.1, 1 and 10 PFU/cell, washed and cultured an additional 7 (IAV) or 17 hr (RSV, hMPV, PIV-3). Cells were then washed, fixed, permeabilized and stained with mAbs to detect IAV NP (A), RSV F (B) hMPV NP (C) or PIV-3 HN (D) proteins. The percentage of virus-infected cells was then determined using flow cytometry. Representative FACs plots are shown. Bar graphs show mean  $\pm$  SD from triplicate samples.

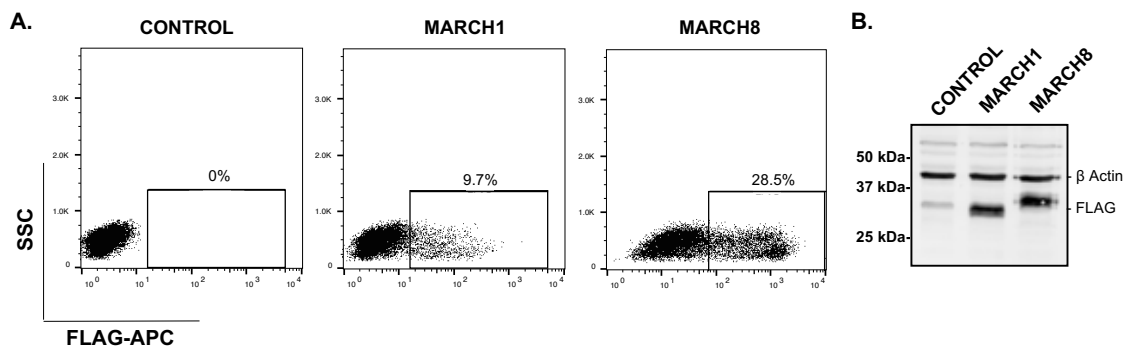


**Figure 3.2. Growth of IAV, RSV, hMPV and PIV-3 in MDCK, A549 and 293T cells.** (A) After seeding and overnight culture, MDCK, A549 and 293T cells were infected for 1 hr at 37°C with IAV Beij/89 at MOI 0.01 PFU/cell washed and cultured in the presence of exogenous trypsin (0.5 µg/mL). Supernatants harvested at 2, 6, 18, 24 and 48 hr p.i. were clarified by centrifugation (3000 rpm, 5 min) and titers from infectious virus determined by standard plaque assay using MDCK cells. (B, C, D) After seeding and overnight culture, A549 and 293T cells were infected for 1 hr at 37°C with RSV-A2, hMPV CAN-1993-87 or PIV-3 strain 243 at MOI 0.01 PFU/cell. After washing, tissue culture plates of infected cells (i.e. with wells containing both cell-associated virus and virus in supernatants) were frozen at 2, 18, 48 and 72 hr p.i., then thawed, vortexed and clarified by centrifugation (3000 rpm, 5 min). Titters of infectious virus were determined by standard plaque assay using Hep2 cells for RSV and PIV-3 or ViroSpot using LLC-MK2 cells for hMPV. Results show mean ± SD from triplicate samples. The dashed lines indicate the limit of detection of the plaque assay and ViroSpot assay.

and clarified before titres of infectious virus were quantified at different time points by standard plaque assay on Hep2 cells (RSV and PIV) or ViroSpot (VS) assay on LLC-MK2 (hMPV). Both RSV and hMPV replicated productively in A549 and 293T cells, reaching peak titres at 48 hr post-infection (**Figure 3.2B/C**). PIV-3 replicated to high titres in both cell lines, albeit at a slower rate in 293T cells (**Figure 3.2D**). Based on susceptibility to infection and ability to support virus replication, A549 and 293T cells appear to be good candidates for subsequent studies examining the capacity of MARCH proteins to modulate respiratory viral infections.

### **3.2.2. Generation of cell lines with ectopic expression of different MARCH-family proteins.**

In preliminary experiments to generate stable cell lines expressing different MARCH proteins, 293T cells were transiently transfected with pcDNA3 plasmids encoding human MARCH1 and MARCH8, each of which contained a N-terminal FLAG tag to enable detection of the expressed protein. As a negative control (CONTROL), pcDNA3 encoding a cytoplasmic version of hen egg ovalbumin (lacking the sequence for cell-surface trafficking)) without a FLAG tag was used [217]. Expression of FLAG-tagged proteins was evaluated by flow cytometry at 48 hr post-transfection. As seen in **Figure 3.3A**, compared to CONTROL-transfected cells, FLAG-positive cells were detected by flow cytometry, suggesting that both MARCH1 and 8 proteins were expressed in 293T cells. To confirm that the FLAG signal detected by flow cytometry corresponded to a protein of the expected size, lysates of transfected cells were subjected to western blot using



**Figure 3.3. Expression of FLAG-tagged MARCH1 and MARCH8 following transfection of 293T cells.** Cultures of 293T cells were transfected with pcDNA3 plasmids (i) encoding human MARCH1 or MARCH8 expressing a N-terminal FLAG tag, or (ii) encoding cytoplasmic hen egg ovalbumin (CONTROL) lacking a FLAG-tag. **(A)** At 48 hr post-transfection cells were washed, fixed, permeabilized and stained with anti-FLAG-APC mAb and the percentage of FLAG-positive cells was determined by flow cytometry. Representative dot plots (gated on viable cells) are shown. **(B)** At 48 hr post-transfection, cell lysates were subjected to SDS PAGE under reducing conditions, transferred to a polyvinylidene difluoride (PVDF) membrane and probed with an anti-FLAG mAb. Membranes were also probed with an anti- $\beta$ -actin mAb to confirm similar amounts of cellular protein were loaded for each sample.

the same anti-FLAG mAb. Using this approach, bands corresponding to the predicted molecular weight of MARCH1 (~32 kDa) and MARCH8 (~34 kDa) were detected in lysates from cells transfected to express the relevant MARCH protein, but not in CONTROL cells expressing untagged ovalbumin (**Figure 3.3B**). The low-intensity bands observed represent non-specific antibody binding, as untransfected cells also showed the same banding pattern (data not shown).

Given that pcDNA3 encodes for resistance to the antibiotic Geneticin (G418), A549 cells transfected with the plasmids described above were cultured in the presence of G418 to select for A549 cell lines with stable expression of MARCH1 and MARCH8. As 293T cells are inherently resistant to G418 they could not be used for this purpose [224]. Flow cytometry confirmed expression of MARCH1 or MARCH8 proteins at 48 hr post-transfection, however expression was subsequently lost following culture in the presence of G418 (data not shown). Despite strategies to improve the transfection efficiency of A549 cells (e.g. different lipid-based transfection reagents, electroporation) we could not generate stable lines of FLAG-positive cells following culture in G418. These data indicate this was not a suitable experimental approach to generate stable human cell lines expressing particular MARCH proteins.

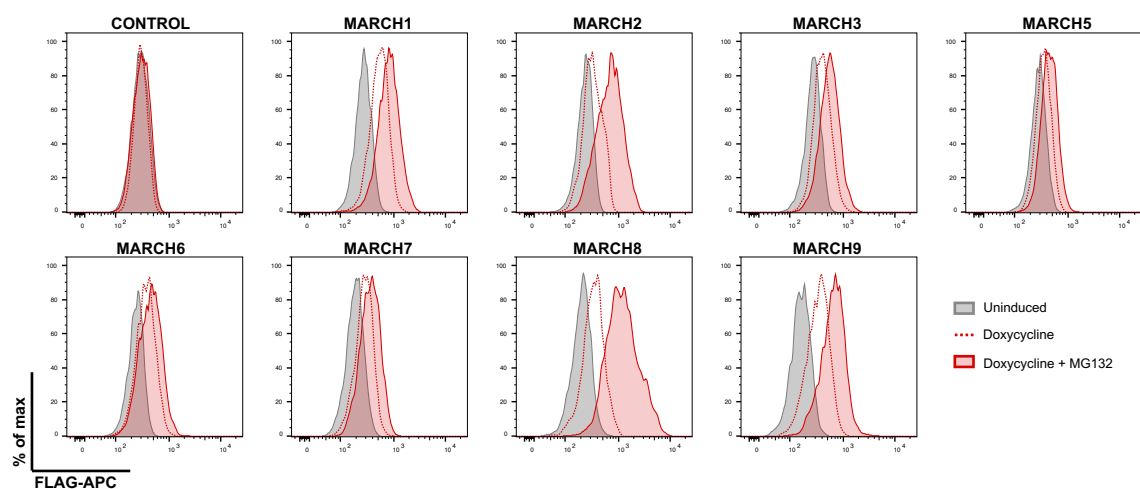
As an alternative, we next used a lentivirus expression system to generate cell lines with stable expression of different MARCH proteins under a doxycycline (DOX)-inducible promoter. This system relies on the use of a transfer plasmid carrying the coding sequence of the protein of interest under the control of the tetracycline responsive TRE promoter (**Figure 2.1**, Chapter 2, Materials and Methods). Additionally, this plasmid also encodes the fluorescent protein mCherry under the constitutive ubiquitin promoter, to



allow selection of transduced cells by cell sorting. To generate the lentivirus particles, 293T cells were transfected with the transfer plasmid, together with 2 packaging plasmids and 1 envelope plasmid carrying the vesicular stomatitis virus envelope glycoprotein (VSV-G). We produced 9 different lentiviral stocks, which were subsequently used to transduce 293T cells to generate cell lines with DOX-inducible stable expression of either human MARCH1, MARCH2, MARCH3, MARCH5, MARCH6, MARCH7, MARCH8 or MARCH9. A schematic representation of the MARCH proteins studied, including the location of RING-CH and transmembrane domains (TM), as well as location of the N-terminal FLAG tag, are shown in **Figure 3.4**. Lentiviral particles carrying untagged cytoplasmic ovalbumin were used to transduce 293T cells to generate a negative control cell line (CONTROL).

To generate cell lines, 293T cells were transduced with the respective lentiviral particles and, 72 hr later, cell sorting was used to enrich mCherry-positive cells (**Figure 2.2**, Chapter 2, Materials and Methods). To evaluate DOX-inducible expression of the different MARCH proteins, cells were treated with DOX for 24 hr and intracellular FLAG expression was analyzed by flow cytometry. Due to the rapid turnover of some MARCH family proteins, the proteasomal inhibitor MG132 was added 20 hr after the addition of DOX to avoid protein degradation prior to analysis. As seen in **Figure 3.5**, the addition of DOX resulted in upregulated expression of FLAG in each cell line expressing different FLAG-tagged MARCH proteins, but not in cells expressing untagged ovalbumin (CONTROL). Interestingly, different levels of FLAG expression were observed depending on the MARCH protein expressed. Despite multiple attempts, the expected sizes of the





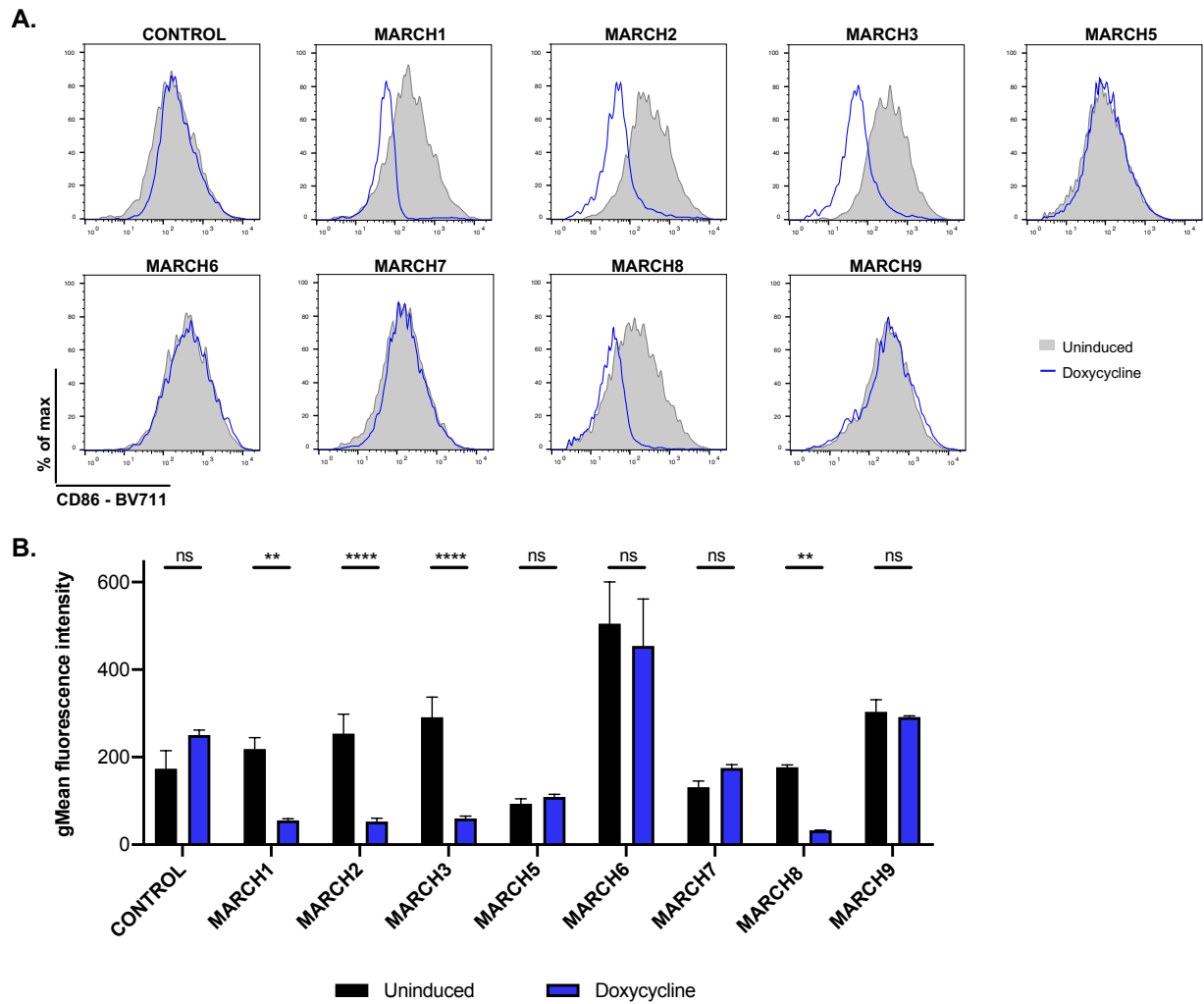
**Figure 3.5. DOX-inducible expression of MARCH proteins in lentivirus-transduced 293T cells.** 293T cells transduced with lentivirus to generate cell lines with DOX-inducible expression of different MARCH proteins were cultured in media alone (grey, filled) or in media supplemented with 1 $\mu$ g/mL DOX (red, open). At 20 hr post-DOX treatment, 10  $\mu$ M of MG132 was added to media to limit protein degradation (red, filled). At 24 hr post DOX treatment, cells were washed, fixed, permeabilized and stained with anti-FLAG-APC mAb. Intracellular FLAG expression was determined by flow cytometry. Representative histograms are shown.

different MARCH family proteins could not be confirmed by western blot due to low levels of protein expression.

### **3.2.3. Functionality of DOX-inducible MARCH proteins.**

MARCH proteins are known to play important roles in the regulation of immune responses, mainly due to their ability to control the turnover of key immunomodulatory molecules, such as MHC I and MHC II, as well as some co-stimulatory molecules, including CD81 and CD86 (reviewed in [181]). However, different MARCH proteins show different specificity regarding the particular host proteins that they target.

To validate the functionality of expressed MARCH proteins after DOX induction, their ability to downregulate cell-surface CD86 was tested. Studies to date have reported that CD86 can be downregulated by at least some MARCH-family proteins, including MARCH1, MARCH2 and MARCH8 (reviewed in [181]). As 293T cells do not express endogenous CD86, a plasmid encoding human CD86 together with the ZsGreen fluorescent protein was transfected into each cell line and levels of cell-surface CD86 were determined by flow cytometry. After DOX induction, cells expressing MARCH1, MARCH2 and MARCH8 showed reduced cell-surface expression of CD86 (**Figure 3.6**). Of interest, our studies demonstrate that MARCH3 also targeted CD86 for degradation. DOX-inducible expression of MARCH 5/6/7/9 did not result in altered expression of cell surface CD86 relative to the respective uninduced control.



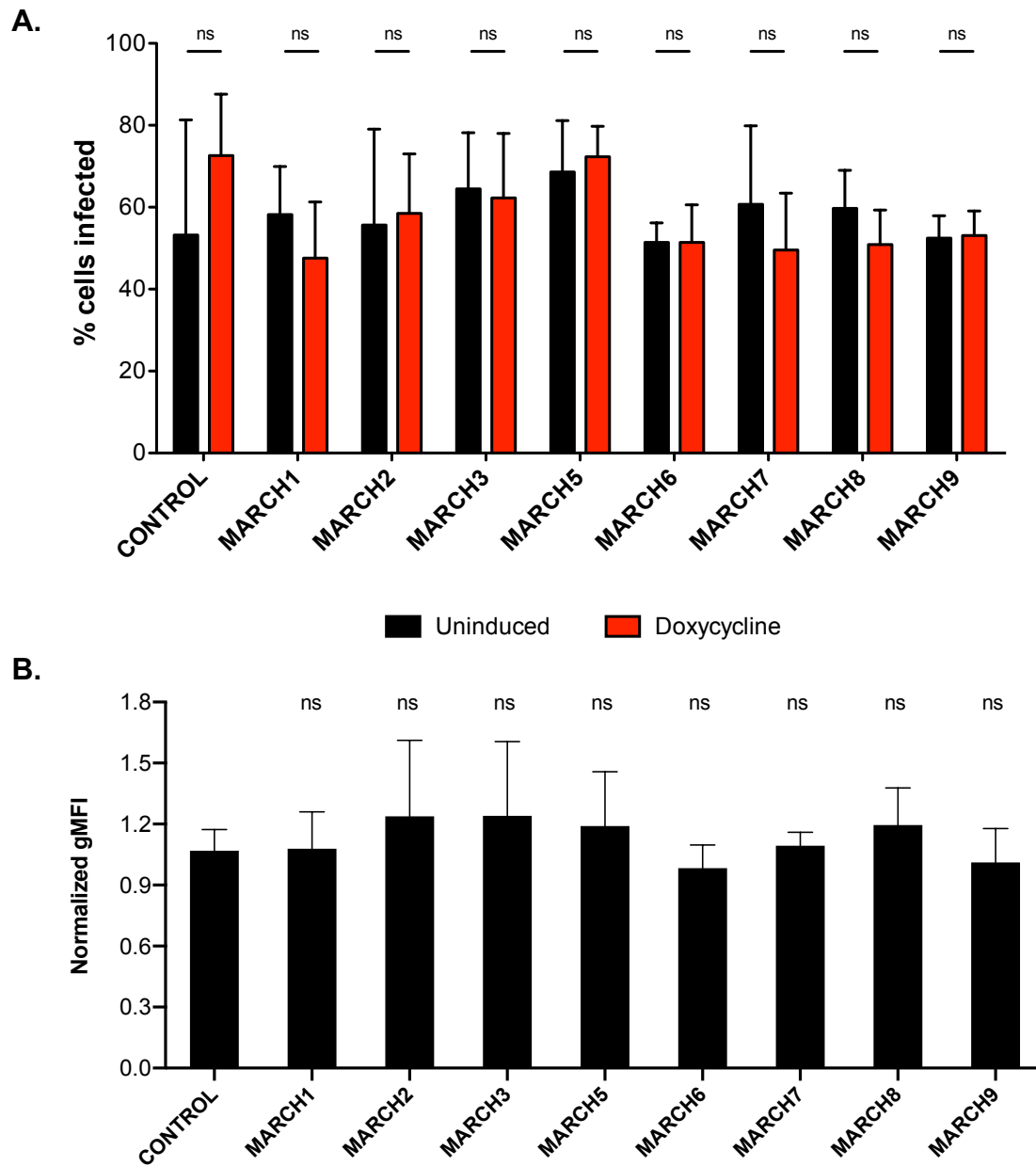
**Figure 3.6. Cell lines with DOX-inducible expression of MARCH1/2/3/8 proteins downregulate cell-surface expression of CD86.** 293T cells with DOX-inducible expression of different MARCH proteins were transfected with a plasmid encoding CD86 and the fluorescence protein ZsGreen. At 12 hr post-transfection, cells were left untreated (grey, filled) or treated with 1 $\mu$ g/mL DOX (blue, empty) to induce MARCH protein expression. At 24 hr post-DOX treatment, cells were washed and stained with anti-CD86-BV711 mAb. Levels of CD86 on the surface of ZsGreen-positive transfected cells were analyzed by flow cytometry. **(A)** Representative histograms of CD86 expression are shown. **(B)** Geometric mean fluorescence intensity ( $\pm$  SD) from triplicate samples are shown. Data are representative of 2 independent experiments. A one-way ANOVA with Tukey's post-test analysis was performed to compare uninduced and DOX-induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

#### **3.2.4. Use of cell lines with DOX-inducible expression of different MARCH proteins to investigate their potential to modulate IAV infection.**

A wide range of host restriction factors have been described that can target different stages in the replicative cycle of a broad range of viruses. As a first screen to assess the antiviral capacity of different MARCH proteins against respiratory viruses, we designed experiments to assess the impact of DOX-inducible MARCH expression on “early” and “late” stages of the virus replicative cycle. To assess impact on early stages of IAV infection, we measured expression of newly-synthesized viral nucleoprotein (NP) using flow cytometry. By assessing expression of viral NP, any major effects of DOX-inducible MARCH protein expression in modulating attachment, entry, fusion, transcription and translation of viral and messenger RNA could be identified. To assess any impact on late stages of IAV infection, we determined the titre of infectious particles released from virus-infected cells to assess for any impact on virus assembly, budding and/or exit from infected cells.

##### *3.2.4a. Effect of MARCH proteins on the early stages of IAV infection.*

To evaluate the ability of different MARCH family proteins to modulate the early stages of IAV infection, 293T cells were cultured in the presence (induced) or absence (uninduced) of DOX, washed and then infected with Beij89 at a MOI of 2.5 PFU/cell. At 8 hr post-infection, the percentage of cells expressing intracellular viral NP was determined by flow cytometry. As seen in **Figure 3.7A**, no significant differences were observed in the percentage of NP-positive IAV-infected cells between CONTROL cells, or for any cell lines with DOX-inducible expression of different MARCH proteins, cultured



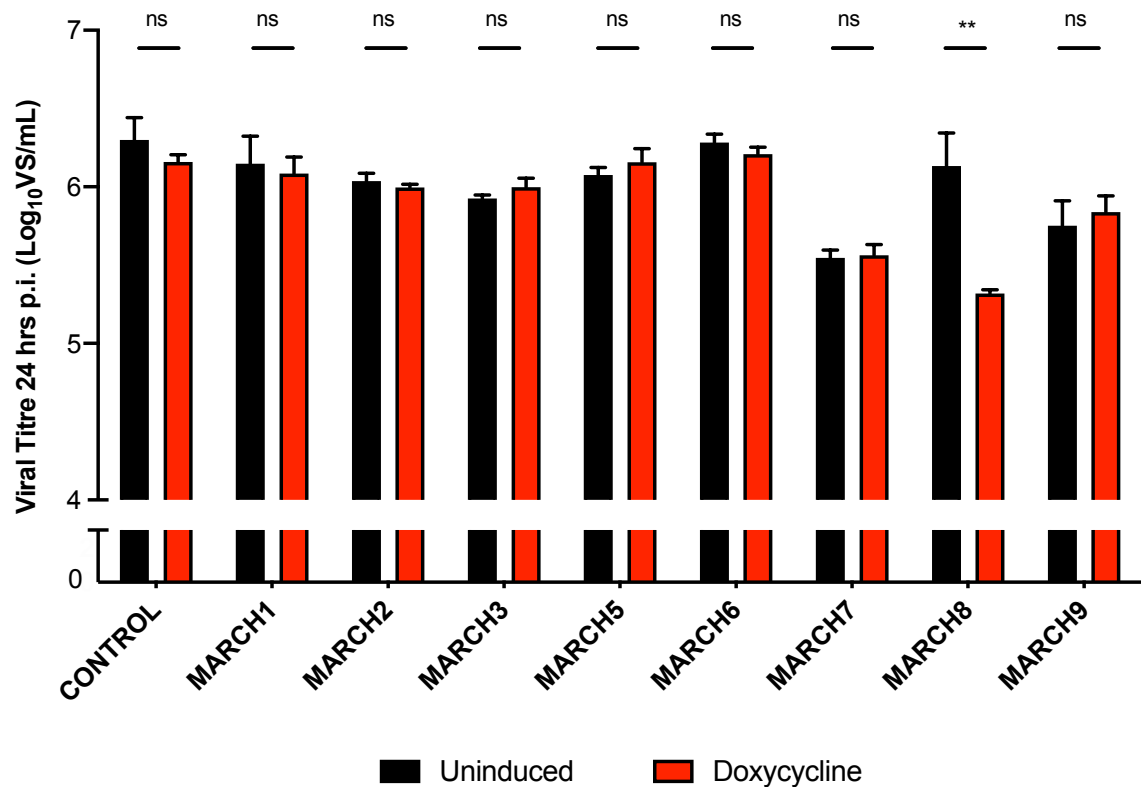
**Figure 3.7. DOX-inducible expression of different MARCH proteins in 293T cells does not result in major differences in the early stages of IAV infection, as assessed by intracellular expression of IAV-NP.** 293T cells cultured for 24 hr in media (black bars) or media supplemented with 1 $\mu$ g/mL DOX (red bars) were washed, then infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI 2.5 PFU/cell. After washing, cells were cultured an additional 7 hr and then washed, fixed, permeabilized and stained with NP-FITC mAb. **(A)** The percentage of infected cells was determined by flow cytometry for intracellular expression of IAV NP. Data shown are the mean from 3 independent experiments ( $\pm$  SD). A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant. **(B)** Geometric mean fluorescence intensity (gMFI) measures were normalized (DOX/uninduced) and plotted (mean  $\pm$  SD). A one-way ANOVA analysis with Dunnett's post-test analysis was performed to compare each normalized gMFI against the CONTROL. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

in the presence or absence of DOX. In addition to the overall percentage of NP-positive cells, a reduction in the geometric mean fluorescence intensity (gMFI) of NP expression would be consistent with a role for MARCH proteins in limiting the degree of virus replication within infected cells. However, no major differences in the normalized gMFIs were observed when the CONTROL condition was compared against each cell line expressing different MARCH proteins (**Figure 3.7B**). Overall, these data suggest that DOX-inducible expression of MARCH1/2/3/5/6/7/8/9 in 293T cells does not result in a major block in the early stages of IAV infection, at least when assessed by the production of newly-synthesized viral NP.

#### *3.2.4b. Effect of MARCH proteins on the late stages of IAV infection.*

To evaluate the ability of different MARCH family proteins to modulate late stages of IAV infection, 293T cells with DOX-inducible expression of different MARCH proteins were cultured in the presence (induced) or absence (uninduced) of DOX, washed and then infected with Beij89 at a MOI of 5 PFU/cell. At 24 hr post-infection, cell supernatants were collected and titrated on MDCK cells using a ViroSpot assay. As seen in **Figure 3.8**, DOX-inducible expression of MARCH8 was associated with a ~90% reduction in the titre of infectious particles released compared to uninduced MARCH8 cells. No significant differences in viral titre were observed between DOX-induced CONTROL cell lines, or between any of the cells expressing different MARCH proteins, when compared with their respective cell line cultured under uninduced conditions.



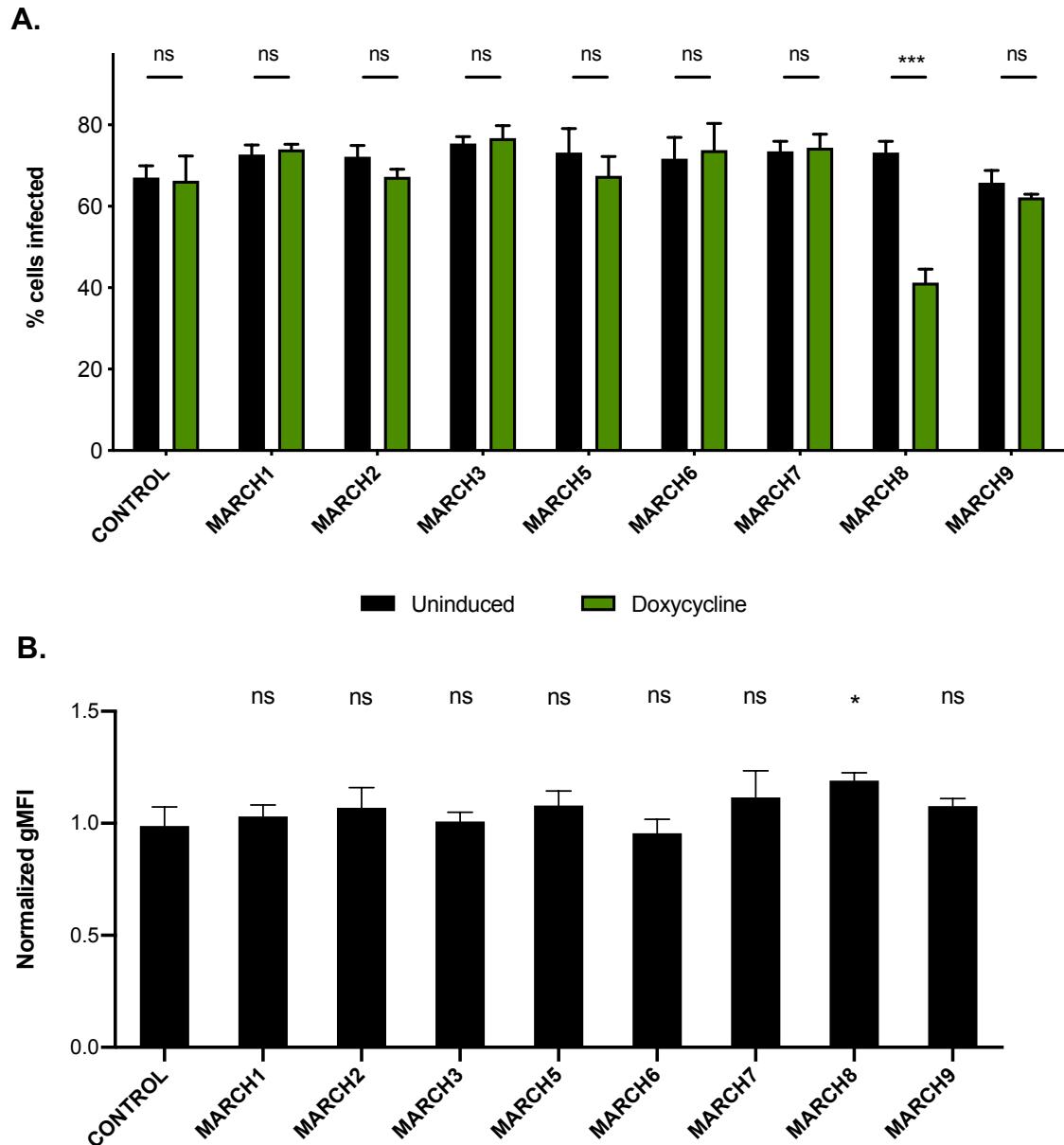


**Figure 3.8. The impact of DOX-inducible expression of different MARCH proteins on the release of infectious IAV from 293T cells.** 293T cells cultured for 24 hr in media (black bars) or media supplemented with 1 $\mu$ g/mL DOX (red bars) were washed, then infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr post-infection, supernatants were harvested and clarified, and titres of infectious virus in supernatants were determined by ViroSpot assay using MDCK cells. Mean virus titres from triplicate samples ( $\pm$  SD) are shown. Limit of detection is 0.85 Log<sub>10</sub> VS/mL. Data are representative of 2 independent experiments. A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

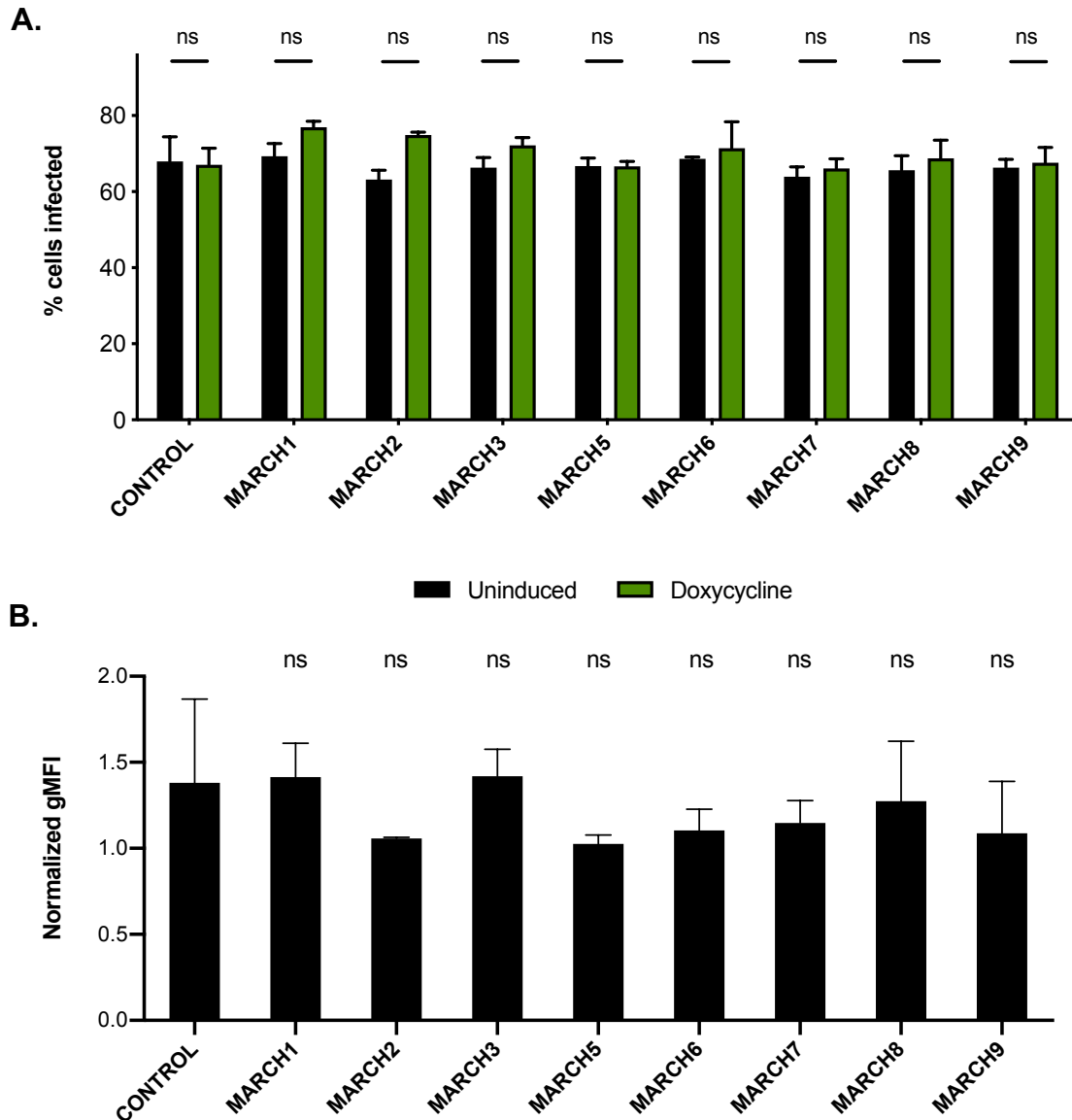
### **3.2.5. Use of cell lines with DOX-inducible expression of different MARCH proteins to investigate their potential to modulate infection by other respiratory viruses.**

#### *3.2.5a. Effect of MARCH proteins on the early stages of pneumovirus infection.*

To determine if different MARCH proteins might modulate other respiratory virus infections, we used a similar strategy to that outlined for IAV to assess their impact on “early” or “late” events in the replicative cycle of the pneumoviruses RSV and hMPV. To examine the impact on early events in virus replication, 293T cells cultured in the presence (induced) or absence (uninduced) of DOX, were infected with RSV-A2 (**Figure 3.9**) or hMPV CAN-1993-87 (**Figure 3.10**) at a MOI of 2 PFU/cell. At 18 hr post-infection, the percentage of cells expressing intracellular viral NP was determined by flow cytometry. As seen in **Figure 3.9A**, DOX-induced expression of MARCH8 was associated with a decrease in the percentage of cells infected with RSV-A2, whereas expression of the other MARCH proteins did not reduce the percentage of RSV NP-positive cells relative to their respective uninduced control. When examining the normalized gMFI of RSV NP within infected cells, MARCH8-induced cells showed a significantly higher ratio DOX/uninduced compared to CONTROL cells (**Figure 3.9B**). No significant differences were observed among the other cell lines. Interestingly, DOX-inducible expression of the different MARCH proteins did not result in a reduced percentage of hMPV-infected cells relative to their respective uninduced cell line (**Figure 3.10A**), nor did it alter expression levels of intracellular hMPV NP, as determined by gMFI (**Figure 3.10B**).



**Figure 3.9. DOX-inducible expression of MARCH8 in 293T cells restricts early events in RSV infection, as assessed by intracellular expression of RSV-NP.** 293T cells cultured for 24 hr in media (black bars) or media supplemented with 1 $\mu$ g/mL DOX (green bars) were washed, then infected for 1 hr at 37°C with RSV-A2 at MOI 2 PFU/cell. After washing, cells were cultured an additional 17 hr and then washed, fixed, permeabilized and stained with NP-FITC mAb. **(A)** The percentage of infected cells was determined by flow cytometry for intracellular expression of RSV-NP. The mean of triplicate samples ( $\pm$  SD) are shown. Data are representative of 2 independent experiments. A two-way ANOVA analysis with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant. **(B)** Geometric mean fluorescence intensity (gmFI) measures were normalized (DOX/uninduced) and plotted (mean  $\pm$  SD). A one-way ANOVA analysis with Dunnett's post-test analysis was performed to compare each normalized MFI against the CONTROL. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.



**Figure 3.10. DOX-inducible expression of different MARCH proteins in 293T cells does not result in major differences in early events in hMPV infection, as assessed by intracellular expression of hMPV-NP.** 293T cells cultured for 24 hr in media (black bars) or media supplemented with 1 $\mu$ g/mL DOX (green bars) were washed, then infected for 1 hr at 37°C with hMPV CAN-1993-87 at MOI 2 PFU/cell. After washing, cells were cultured an additional 17 hr and then washed, fixed, permeabilized and stained with NP-FITC mAb. **(A)** The percentage of infected cells was determined by flow cytometry for intracellular expression of hMPV-NP. The mean of triplicate samples ( $\pm$  SD) are shown. Data are representative of 2 independent experiments. A two-way ANOVA analysis with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant. **(B)** Geometric mean fluorescence intensity (gMFI) measures were normalized (DOX/uninduced) and plotted (mean  $\pm$  SD). A one-way ANOVA analysis with Dunnett's post-test analysis was performed to compare each normalized MFI against the CONTROL. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

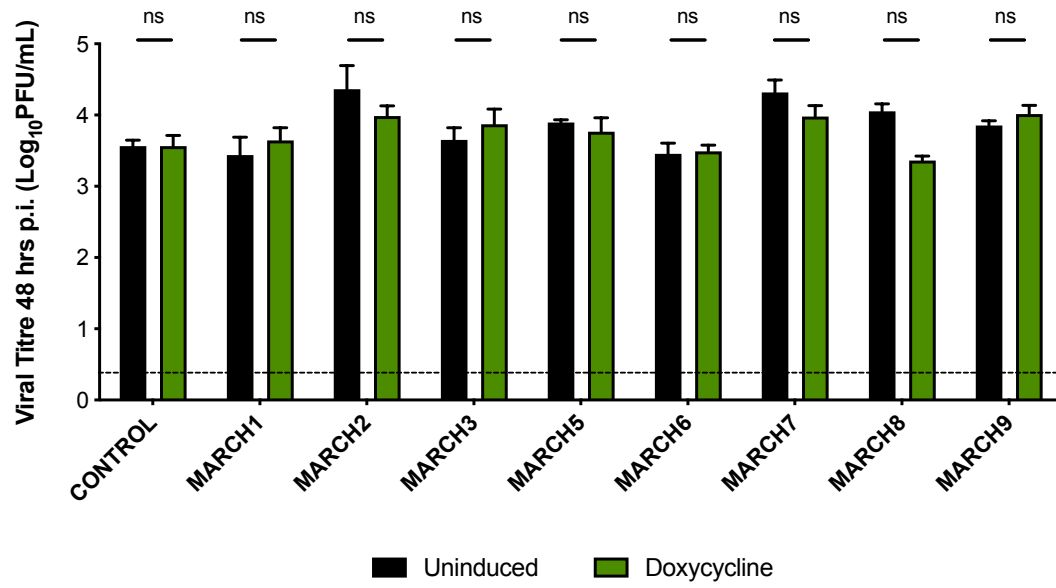
### *3.2.5b. Effect of MARCH proteins on the early stages of paramyxovirus infection.*

Following the same strategy, we assessed the ability of DOX-inducible expression of different MARCH proteins to modulate infection of 293T cells by paramyxovirus PIV-3. Surprisingly, the susceptibility of all transduced 293T cell lines, including the CONTROL cell line, was dramatically reduced (<5%) when compared to non-transduced parental 293T cells (~30%, data not shown). In an attempt to overcome this issue, we used the same lentivirus system to generate A549 cell lines (CONTROL and with DOX-inducible expression of different MARCH proteins), however all transduced A549 cell lines also showed markedly reduced susceptibility to PIV-3 infection compared to untransduced A549 cells (data not shown). Together, these results suggest that the lentivirus system used in our studies may have altered one or more cellular pathways that are critical during the replicative cycle of PIV-3, but not for IAV, RSV or hMPV replication.

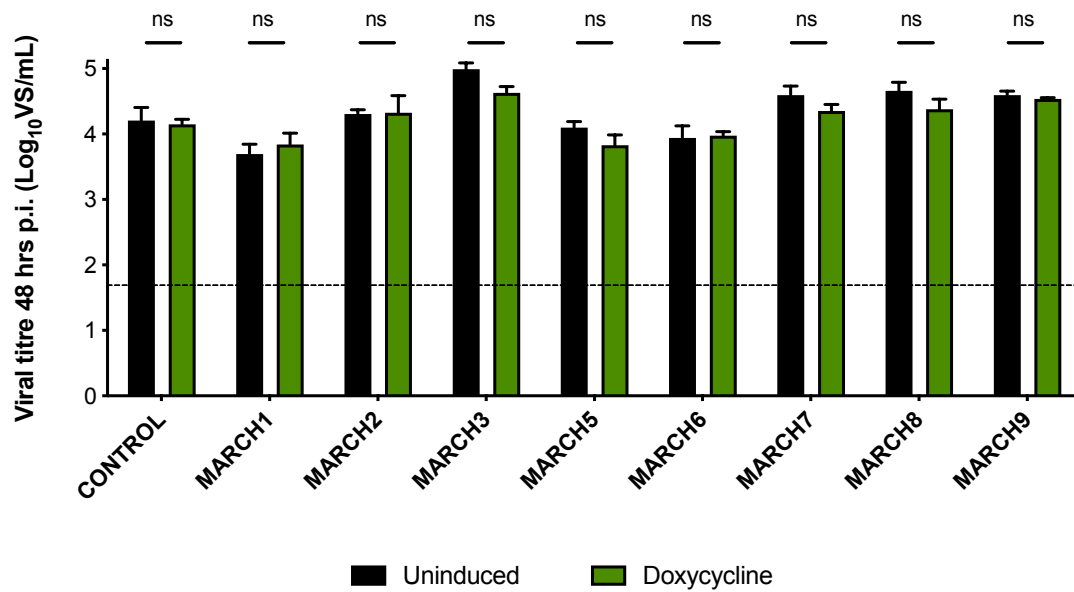
### *3.2.5c. Effect of MARCH proteins on the late stages of pneumovirus infection.*

To determine whether DOX-inducible expression of particular MARCH proteins might modulate the late stages of pneumovirus replication, 293T cells were cultured in the presence (induced) or absence (uninduced) of DOX, washed and then infected with RSV A2 or hMPV CAN-1993-87 at a MOI of 2 PFU/cell. At 48 hr post-infection, the amount of cell-associated plus cell-free virus particles (i.e. “total virus”) was determined. For RSV, DOX-induced expression of MARCH8 resulted in a modest, but not statistically significant, reduction in the titres of total infectious virus particles at 48 hr post-infection (**Figure 3.11A**). For hMPV, DOX-inducible expression of different MARCH proteins did not result in any significant changes in the titres of total virus detected at 48 hr

### A. RSV



### B. hMPV



**Figure 3.11. Impact of DOX-inducible expression of different MARCH proteins on cell-associated and cell-free pneumovirus particles released from 293T cells.** 293T cells cultured for 24 hr in the absence (Uninduced, black bars) or presence of 1 $\mu$ g/mL DOX (Doxycycline, green bars) were washed, then infected for 1 hr at 37°C with RSV-A2 (A) or hMPV CAN-1993-87 (B) at MOI 2 PFU/cell. Cells were then washed and cultured for 47 hr. At 48 hr post-infection, total virus (i.e. cell-associated plus cell-free) was harvested from each well, clarified and titres of infectious virus were determined by plaque assay using HEp2 cells for RSV A2 or by ViroSpot assay on LLC-MK2 cells for hMPV. Mean virus titres from triplicate samples ( $\pm$  SD) are shown. Dashed lines indicate the limit of detection of the plaque assay and ViroSpot assay. Data are representative of 2 independent experiments. A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

post-infection, relative to each respective uninduced cell line (**Figure 3.11B**).

#### *3.2.5d. Effect of MARCH proteins on the late stages of paramyxovirus infection.*

Given the poor susceptibility of transduced 293T and A549 cell lines to PIV-3 infection (as assessed by flow cytometry for the viral HN protein), we did not determine the titres of virus released from transduced 293T cells infected with PIV-3.

### **3.3. Discussion.**

In this chapter, we generated tools to study the ability of different MARCH-family proteins to modulate respiratory virus infections. We first aimed to develop appropriate systems to overexpress different MARCH proteins in human epithelial cells, allowing us to then screen for their ability to modulate early or late events in the replication of different respiratory viruses. We focused on using 293T cells to develop cell lines with stable expression of particular MARCH proteins, due to their capacity to support infection and growth by different respiratory viruses (**Figure 3.1-2**), together with their high susceptibility to transfection [225]. Given the high degree of sequence homology and ability to modulate similar immunological ligands, initial studies to generate overexpressing cell lines focused on MARCH1 and MARCH8. While we could detect expression of MARCH1 and MARCH8 following transfection of 293T cells with appropriate pcDNA3 expression vectors (**Figure 3.3**), we were unable to apply G418 selection to these cultures, as 293T cells are already resistant to this antibiotic. Therefore, we attempted to transfect and select for stable expression of MARCH1 and

MARCH8 in A549 cell lines. However, multiple attempts to generate A549 cells with stable constitutive expression of MARCH1 or MARCH8 following pcDNA3 transfection, G418 selection and single cell cloning were not successful (data not shown). One possible explanation for this result is that maintaining long-term constitutive expression of these MARCH proteins could be detrimental for different cellular processes. Supporting this idea, it is known that expression of most MARCH-family proteins is tightly regulated by several post-translational modifications. For example, antigen presenting cells (APCs) maintain low level of expression of the murine MARCH1 protein which has been estimated to have a half-life of less than 30 minutes due to rapid protein turnover controlled by autoubiquitination, internalization and degradation [185, 226]. Similar autoubiquitination capacity has been also described for other members of the family, including MARCH5-9 (reviewed in [227]), suggesting that controlled expression of MARCH family proteins is important for appropriate cellular functions. Additionally, overexpression of MARCH8 has been associated with increased apoptosis and inhibition in cell proliferation, at least in the human lung adenocarcinoma cell lines A549 and H1299 [228].

As an alternative approach, we generated 293T cell lines with DOX-inducible expression of MARCH1 and MARCH8. Subsequently, we generated additional cell lines with DOX-inducible expression of MARCH2/3/5/6/7/9. These particular MARCH proteins were chosen for further study based on their reported distribution in human tissues, including the airways (**Table 1.1**). It is noteworthy that pcDNA3-driven expression in 293T cells resulted in markedly higher levels of MARCH1/8 proteins (**Figure 3.3B**) compared to the DOX-inducible system as none of the DOX-inducible MARCH proteins could be detected



by western blot using anti-FLAG mAb. However, subsequent immunoprecipitation studies did confirm expression of DOX-inducible FLAG-tagged MARCH1/8 in 293T cells (data not shown). Given our inability to generate stable cell lines with constitutive expression of MARCH1/8, it is possible that the reduced levels of DOX-inducible MARCH proteins might have less impact on normal cellular functions in 293T cells, as well as allowing for more controlled expression of MARCH proteins during specific experimental conditions. However, addition of DOX does complicate our ability to study some important aspects of viral infection, as this antibiotic can have off-target effects that affect cellular metabolism, including modulation of cytokine production [229].

After generating 293T cells with DOX-inducible expression of different MARCH proteins, FLAG staining indicated that after DOX induction, levels of protein expression differed between cell lines (**Figure 3.5**). For example, DOX induced MARCH6 and MARCH7 expression was modest when compared with the uninduced condition, perhaps due to their larger molecular weight (~103 kDa and 78 kDa, respectively) relative to other family members (**Figure 3.4**). However, MARCH5 was also expressed to lower levels compared with other MARCH proteins of similar size. It is possible that intracellular localization of (MARCH5 in the mitochondria, MARCH6 in the ER and MARCH7 in the cytoplasm and nucleus (**Table 1.1**)) also impacts expression levels and/or sensitivity to anti-FLAG mAb binding and detection by flow cytometry. While inclusion of the proteasome inhibitor MG132 stabilized expression of some MARCH, including MARCH1/2/8/9, it is possible that degradation of at least some MARCH proteins also occurs in a different compartment to the proteasome, such as the lysosome. Consistent with this, it has been reported that murine MARCH1 can be stabilized, at least partially, using lysosomal

inhibitors [226]. Additionally, it cannot be ruled out that immortalized cell lines such as 293T cells might not contain all of necessary machinery for optimal expression of certain MARCH proteins. Expression levels of particular MARCH proteins are likely to be one important factor impacting our ability to assess their ability to modulate respiratory virus infection *in vitro*.

Next, we designed experiments to confirm the functionality of at least some MARCH-family proteins. Although particular MARCH proteins can target different substrates, a number show overlapping specificity to ubiquitinate and modulate expression of immune molecules such as Class I (MARCH4/8/9) and Class II (MARCH1/8) MHC, as well as costimulatory molecules such as CD81 (MARCH4/8) and CD86 (MARCH1/2/8). As a first step towards validating functionality, we examined the impact of different MARCH proteins on CD86 expression. As expected, induced expression of MARCH1, MARCH2 and MARCH8 downregulated CD86 from the surface of transfected cells (**Figure 3.6**). Interestingly, MARCH3 also downregulated cell-surface CD86 expression. Considering that MARCH2 and MARCH3 share 76% protein sequence identity (reviewed in [181]), it is perhaps not surprising that these proteins target similar ligands in this inducible overexpression system. Given its endosomal localization (**Table 1.1**), it would be of interest to silence or delete endogenous MARCH3 to confirm its ability to modulate CD86. It was particularly encouraging to confirm that MARCH1, 2 and 8 downregulated CD86 expression, given these 3 MARCH are the only ones so far reported to display antiviral activity against any virus. As the goal of our studies was to generate cell lines to allow for screening of antiviral activity (to identify candidate MARCH to subsequently study in more detail) we did not perform exhaustive studies to confirm functionality of

each individual MARCH protein. Rather, we focused on regulation of CD86 to confirm generation of 293T cell lines with inducible expression of a number of functional MARCH proteins. In future studies it will be important to confirm functionality of DOX-induced expression of the other MARCH protein against reported cellular targets, including MARCH5 (MAVS), MARCH6 (squalene monooxygenase, SM) and MARCH9 (CD4) (reviewed in [181]), although validation of MARCH7 may be more challenging as no definitive cellular targets have been reported to date.

We used 293T cells with DOX-inducible expression of FLAG-tagged MARCH proteins to screen for the ability of different MARCH to inhibit early or late events in the replication cycle of IAV, RSV and hMPV. Our rationale for screening “early” events during virus infection was that any reduction in production of newly-synthesized viral proteins would indicate a block in one or more preceding steps in the virus replicative-cycle (i.e. attachment, entry, genomic replication, translation of mRNA, protein synthesis). The particular step could then be explored in more detail in subsequent studies. When screening IAV infection, none of the MARCH proteins tested were able to reduce the percentage of NP-positive IAV-infected cells at 8 hr post-infection (**Figure 3.7**). This was an interesting result, as MARCH2 and MARCH3 are known to localize to endosomal compartments (**Table 1.1**) and IAV entry occurs via HA-mediated fusion with endosomal membranes (reviewed in [230]). Moreover, our studies indicate that MARCH2 and MARCH3 are functional in our system, at least in terms of downregulating cell-surface CD86 expression.

In the case of RSV infection, we observed that MARCH8 expression was associated with a reduction in the percentage of RSV NP-positive cells at 18 hr post-infection (**Figure**

**3.9).** Although the route of RSV entry can differ depending on the virus strain and the particular cell type used, it is widely accepted that RSV can fuse directly with the plasma membrane of many cells to initiate infection (reviewed in [40]). Considering that MARCH8 can also localize to the plasma membrane (**Table 1.1**), it is possible that MARCH8 somehow interferes with viral fusion, preventing release of the RSV genome to the cytosol of the target cell and this, in turn, could result in the reduced percentage of NP-positive cells. Of interest, MARCH8 expression did not alter the percentage of NP-positive hMPV-infected cells (**Figure 3.10**). Like RSV, hMPV entry pathways can also vary with cell type, however it is well established that this virus can also fuse directly at the plasma membrane of some cell types (reviewed in [231]). In future studies, it would be of interest to determine the specific entry pathways used by RSV and hMPV to enter 293T cells to see if this might help to explain the differential impact of MARCH8 expression on infection by these two viruses.

The mechanisms by which MARCH8 expression limits early events in RSV infection remain to be determined. Whilst MARCH8-mediated inhibition of RSV infection has not been studied further in this thesis, it would be of interest to determine if MARCH8 mutants lacking a functional RING-CH domain [199, 218] could also inhibit the early stages of RSV infection, as this would provide insight regarding steric hinderance of RSV entry by inducible expression of MARCH8 lacking E3 ligase activity. Indeed, the ability of MARCH8 to modulate HIV-1 [210] or flavivirus infections [212] has been reported to be dependent on a functional RING-CH domain. Previous studies in our laboratory have used experimental approaches to define steps during early infection by IAV in macrophages, including attachment, entry, genomic replication and mRNA transcription

[232], and similar approaches could be developed to understand MARCH8-mediated inhibition of RSV. Moreover, results obtained with RSV-A2 and hMPV CAN-1993-87 should be confirmed with additional virus strains, as well as using other human cells with DOX-inducible expression of MARCH8. In particular, the use of human airway epithelial cell lines such as A549 or BEAS-2B would be particularly informative, given these cells represent more appropriate target cells for respiratory virus replication.

Surprisingly, we were unable to determine the impact of the MARCH family proteins as potential antiviral factors against the paramyxovirus PIV-3, as transduction of 293T cells with lentivirus, followed by cell sorting to select for mCherry-positive transduced cells, resulted in cell lines which were no longer susceptible to infection by this virus. Similar results were obtained when using lentivirus for cells with DOX-inducible expression of cytoplasmic ovalbumin (CONTROL) or for expression of any MARCH protein studied. It is possible that lentivirus transduction and subsequent cell sorting selected for cells lacking or with reduced expression of cell-surface attachment factors or receptors that are required for PIV-3 infection. It is well known that IAV and PIV-3 can use  $\alpha$ 2,3- and  $\alpha$ 2,6-linked sialic acids as attachment factors [233, 234], however PIV-3 preferentially uses  $\alpha$ 2,3-linked sialic acids for attachment to the cell-surface. Although preliminary evidence indicated that 293T cells showed reduced expression of cell-surface  $\alpha$ 2,3-linked sialic acids after lentiviral transduction and cell sorting (data not shown), this was associated with reduced susceptibility to PIV-3, but not IAV, infection. It is, however, possible that the specificity of the IAV HA and the PIV-3 HN of the virus strains used may contribute to differences observed between these viruses. Whatever the case, PIV-3 has not been used further in the studies described in this thesis.

Finally, to assess the impact of different MARCH proteins on the later stages of respiratory virus infection, we measured titres of infectious virus released from and/or associated with virus-infected cells after allowing sufficient time for virus replication to be completed. For IAV, only MARCH8 expression was observed to impact titres of infectious virus released from infected cells (**Figure 3.8**). Given that MARCH8 did not block the early steps of IAV infection (as measured by NP expression at 8 hr post-infection), this finding indicates that MARCH8 is interfering with later stages in the virus replicative cycle, perhaps virus assembly, budding or release from infected cells. Alternatively, MARCH8 expression could interfere with production of viral proteins other than the viral NP. Of interest, while MARCH1 and MARCH8 exhibit a high degree of sequence homology and share many immunological ligands (reviewed in [181]), we did not observe any reduction in IAV titres released from 293T cells expressing MARCH1. Furthermore, while MARCH1/2/8 were reported to reduce the infectivity of nascent HIV-1 particles released from virus-infected cells [191, 210, 211], and MARCH1/2/8 all downregulated CD86 expression from 293T cells (**Figure 3.6**), only MARCH8 inhibited the late stages of IAV infection. The ability of MARCH8 to mediate antiviral activity against IAV is the major focus of subsequent chapters presented in this thesis.

For pneumoviruses, MARCH8 showed a tendency to reduce the viral titre of infectious particles associated and released from RSV-infected cells during the late stages of infection. Although this was not significant across two independent experiments (**Figure 3.11A**). Contrary to what was observed against IAV, MARCH8 expression was also associated with a reduction in the percentage of cells expressing RSV-NP 18 hr post infection, suggesting that this block observed during early stages of the virus-replicative

cycle could be responsible of the reduction in the viral titres observed during late stages of infection. If that is the case, it would be of interest to take advantage of our DOX-inducible system to turn on the expression of MARCH8 immediately after virus entry, to examine the impact of MARCH8 expression only during the late stages of virus replication, post-entry. Future studies could also investigate the impact of a lower MOI to infect cells, determining titres of “total” (cell-associated plus supernatant) virus in samples harvested at different timepoints after infection and comparing titres of “total” virus (as described herein) and “cell-free” virus released into the supernatant of RSV-infected cells. Considering that pneumoviruses are known to bud from the plasma membrane, it is possible that MARCH8 could mediate “canonical” downregulation of viral glycoproteins from the surface of the RSV-infected cells, thereby affecting the infectivity of the particles released. If so, it would be interesting to measure the levels of expression of RSV proteins known to localize at the plasma membrane during infection (F, G and SH). Analysis of protein sequences from Uniprot indicated that each of these viral proteins contain acceptor sites for ubiquitin in their cytoplasmic tails, suggesting that any could be targeted by MARCH8. Our data show a clear effect of MARCH8 expression on infectious entry by RSV, but the role of MARCH8 in modulating RSV replication and release requires further investigation.

Finally, while MARCH8 appears to modulate RSV infection, our data indicate that hMPV infection and replication in 293T cells was not impacted by DOX-inducible expression of MARCH8 (**Figure 3.11B**). Analysis of protein sequences from Uniprot indicated that the cytoplasmic tails of the F, G and SH envelope glycoproteins of hMPV also express acceptor sites for ubiquitination, however these do not seem to be targeted by

MARCH8, at least under the conditions used in our experiments. It is also interesting to note that MARCH1 expression was not associated with restriction against either pneumovirus. As mentioned before, MARCH1 and MARCH8 are expressed in similar compartments within the cell and, when expressed in the same cell, can target identical substrates [177]. An important first step to understand the differential effect of MARCH1/8 against pneumoviruses would be to confirm their intracellular localization after DOX-induction. If localized to similar compartments, it is possible that ubiquitination capacity may differ between MARCH1 and MARCH8. Considering that the replicative cycles of RSV and hMPV remain poorly understood, it is possible that each pneumovirus requires different host proteins to complete replication, opening the possibility that particular host proteins relevant to RSV infection could be targeted exclusively by MARCH8 and not by MARCH1. Conversely, if the localization of both proteins is different, it is possible that the presence or absence of MARCH8 is interfering with a specific step of the replicative cycle of RSV and not hMPV.





## Chapter 4

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# **Investigating the antiviral activity of MARCH8 against influenza viruses.**

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#### **4.1. Introduction.**

MARCH8 was the first mammalian homologue of the viral E3 ligases to be characterized. It is expressed in a variety of tissues, but its highest expression is in the lungs [177]. Even though it has been difficult to determine its endogenous subcellular localization, immunofluorescence studies using ectopically overexpressed tagged versions of MARCH8 have shown that it is localized at the cell surface and in early and late endosomes [177, 181].

As for the majority of MARCH-family proteins, MARCH8 has a RING-CH domain in the N-terminal end of its sequence which is responsible for interactions with the E2 conjugating enzyme that has been charged with ubiquitin. Different studies have shown that MARCH8 can interact directly with target proteins, allowing the transfer of ubiquitin to lysine residues located in the cytoplasmic tail of these targets. To date, different domains of MARCH8 have been implicated in direct interactions with specific target proteins, including its transmembrane domains, the connecting loop between these transmembrane domains and the C-terminal cytoplasmic tail [199, 218, 235].

Among its functions, MARCH8 has been implicated in the control of human viral infections. In this context, MARCH8 has been reported to reduce the infectivity of HIV-1 particles released from infected cells, by reducing incorporation of the envelope glycoprotein on the surface of nascent virions during the late stages of replication [210]. Specifically, this study showed that MARCH8 interacts with and downregulates the envelope (Env) glycoprotein from the surface of HIV-1 infected cells in a RING-dependent manner. However, it remains to be determined if the HIV-1 Env protein is

directly ubiquitinated by MARCH8. Similarly, MARCH8 can also downregulate VSV-G from the surface of transfected cells, reducing its incorporation into the surface of pseudotyped viruses. Although there is no experimental evidence of direct ubiquitination, it has been shown that MARCH8 can interact and target VSV-G to lysosomes for degradation [210]. Interestingly, a more recent report has shown that in addition to restricting virus replication, MARCH8 can also act as a proviral protein and is essential during the replicative cycle of some members of the *Flaviviridae* family. Here, MARCH8 was identified as the E3 ligase responsible of the K63 polyubiquitination of the non-structural 2 (NS2) protein of hepatitis C virus (HCV) [212]. It has been proposed that this polyubiquitination allows the binding of NS2 with the ESCRT-0 component hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), an essential step during HCV envelopment [213].

The previous chapter identified MARCH8 as a potent restriction factor against IAV strain Beij89, acting during the late stages of infection. In this chapter we aimed to further characterize the anti-IAV activity of MARCH8 and gain insights regarding the mechanisms by which MARCH8 inhibits the late stages of IAV infection. Our findings confirm that MARCH8 mediates restriction of a broad range of influenza viruses relevant to human health and that its antiviral activity is dependent on a functional RING-CH domain. Moreover, this does not appear to be mediated by the same mechanism as MARCH8-mediated restriction of HIV-1, since viral glycoproteins were not downregulated from the surface of infected cells. Our studies suggest that induction of MARCH8 expression altered the protein composition and morphology of nascent virions released from infected cells and this, in turn, is associated with reduced IAV infectivity.

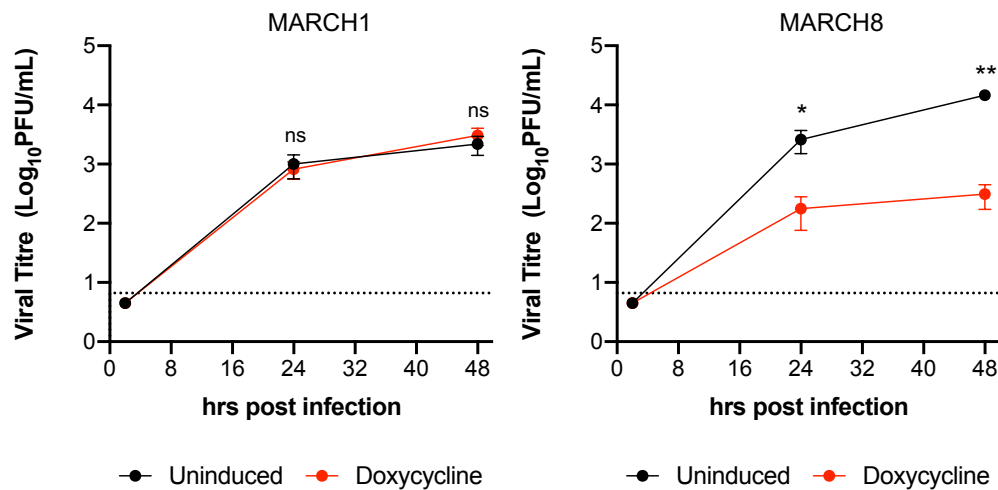
When site-directed mutagenesis was used to replace lysine by arginine residues in the cytoplasmic tails of the viral HA, NA and M2 proteins, or in the M1 protein, we found that all of the recombinant viruses still remained sensitive to MARCH8-mediated restriction. Finally, we report that endogenous MARCH8 can mediate modest, but significant, antiviral activity against IAV *in vitro*.

## **4.2. Results.**

### **4.2.1. Further characterization of the antiviral activity of MARCH8 against influenza viruses.**

#### *4.2.1.1. MARCH8, but not the closely related MARCH1, mediates antiviral activity during multicycle IAV infection.*

To confirm MARCH8-mediated restriction of IAV infection, 293T cells with DOX-inducible expression of MARCH8 or the closely related MARCH1 protein (89% amino acid sequence conservation within RING-TM1/2) were cultured in the presence or absence of DOX, washed and then infected with IAV strain Beij89 at a MOI of 0.005 PFU/cell in the presence of exogenous trypsin to promote multicycle growth. This approach was designed to highlight more subtle differences in the replication efficiency of Beij89 in the presence of inducible MARCH1 or MARCH8 proteins. Supernatants collected at 2, 24 and 48 hr post-infection were then titrated on MDCK monolayers to determine titres of infectious virus. As seen in **Figure 4.1**, virus titres in all supernatants markedly increased

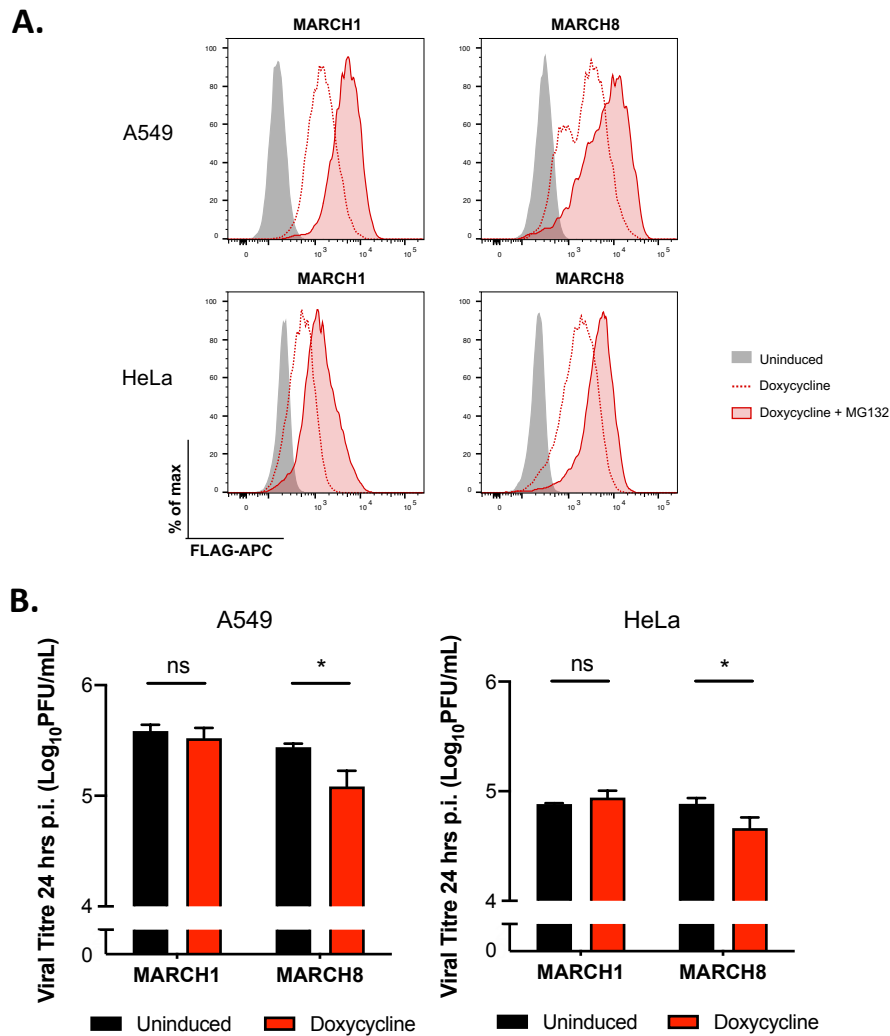


**Figure 4.1. The impact of DOX-inducible expression of MARCH1 or MARCH8 on multicycle growth of IAV in 293T cells.** 293T cells cultured for 24 hr in the absence (Uninduced, black circles) or presence of 1 $\mu$ g/mL DOX (Doxycycline, red circles) were washed, then infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI 0.005 PFU/cell. Cells were then washed and cultured in the presence of exogenous trypsin (0.5  $\mu$ g/mL) to facilitate multi-cycle virus replication. Supernatants harvested at 2, 24 and 48 hr p.i. were clarified by centrifugation (3000 rpm, 5 min) and titres of infectious virus determined by plaque assay using MDCK cells. Mean virus titres from triplicate samples ( $\pm$  SD) are shown. The dashed lines indicate the limit of detection. Data are representative of 2 independent experiments. A two-tailed unpaired Student's t-test was performed to compare uninduced and DOX-induced conditions at each timepoint. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

between 2 and 24 hr, with a more modest increase by 48 hr. Inducible expression of MARCH1 did not alter viral titres at any timepoint, whereas viral titres were markedly reduced at 24 hr (~95%) and at 48 hr (~98%) after MARCH8 induction compared to uninduced cells. These data highlight that MARCH8, but not the closely related MARCH1 protein, is a potent restriction factor of IAV strain Bejj89.

#### *4.2.1.2. Induction of MARCH8 expression in different cell lines mediates antiviral activity against IAV.*

Cells and cell lines of different origins are likely to exhibit different properties which may relate to distinct physiological functions in the body. Thus, epithelial cells from the kidneys (e.g. 293T cells) may exhibit different properties to those derived from other organs. Therefore, adenocarcinoma epithelial cells from the cervix (HeLa) or lung (A549) were also engineered to express DOX-inducible MARCH1 and MARCH8 proteins. After lentivirus transduction and cell sorting (as described in Chapter 2: Materials and Methods, **Figure 2.2**), flow cytometry confirmed inducible expression of MARCH1/8 in these cell lines (**Figure 4.2A**). Next, cells cultured in the presence (induced) or absence (uninduced) of DOX were infected with IAV Bejj89 and supernatants harvested at 24 hr p.i. were titrated on MDCK cells. Infection of HeLa and A549 cells with DOX-inducible expression of MARCH8, but not MARCH1, resulted in reduced titres of infectious virus released from infected cells (**Figure 4.2B**). While significant, this inhibition did not appear to be as potent as that seen in 293T cells with DOX-inducible expression of MARCH8. Therefore, subsequent studies to investigate MARCH8-mediated restriction of IAV have mainly utilized 293T cells expressing MARCH8.



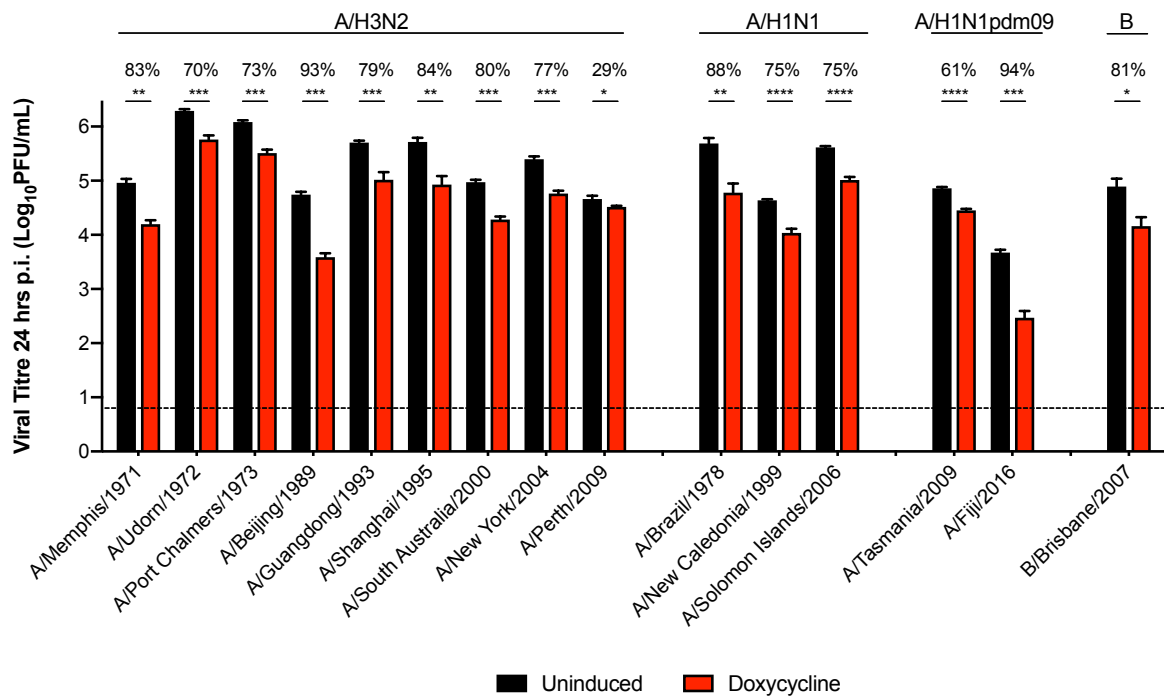
**Figure 4.2. DOX-inducible expression of MARCH8 mediates antiviral activity against IAV in A549 and HeLa cells.** (A) A549 and HeLa cells transduced with lentivirus to generate cell lines with DOX-inducible expression of MARCH1 or MARCH8 were cultured in media alone (grey, filled) or in media supplemented with 1  $\mu$ g/mL DOX (red, open). At 20 hr post DOX treatment, 10  $\mu$ M of MG132 was added to media to limit protein degradation (red, filled). At 24 hr post-DOX treatment, cells were washed, fixed, permeabilized and stained with anti-FLAG-APC mAb. Intracellular FLAG expression was determined by flow cytometry. Representative histograms are shown. (B) A549 and HeLa cells cultured for 24 hr in the absence (Uninduced, black bars) or presence of 1  $\mu$ g/mL DOX (Doxycycline, red bars) were washed, then infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr post-infection, supernatants were harvested and clarified, and titres of infectious virus were determined by plaque assay using MDCK cells. Mean virus titres from triplicate samples ( $\pm$  SD) are shown. The limit of detection of the plaque assay is 0.85 Log<sub>10</sub> PFU/mL. Data are representative of 2 independent experiments. A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.



#### *4.2.1.3. MARCH8 mediates antiviral activity against different seasonal influenza viruses.*

Currently, influenza viruses circulating in the human population include influenza A (A/H1N1pdm/09, A/H3N2) and influenza B viruses. Given that all studies in Chapter 3 focussed on IAV strain Beij89, we next assessed the ability of inducible MARCH8 to mediate antiviral activity against a range of seasonal A/H3N2 (1971-2009), A/H1N1 (1978-2006) and A/H1N1pdm09 (2009-2016) viruses. As seen in **Figure 4.3**, DOX-inducible expression of MARCH8 resulted in a significant reduction in the titres of infectious particles released from cells infected with all strains of IAV tested. Of note, titres of virus released from infected cells did differ following infection with different virus strains and the potency of the antiviral activity also differed depending on the strain of virus used. Moreover, cells infected with IBV strain B/Brisbane/2007 in the presence of inducible MARCH8 also showed significant reduction in titres released from infected cells. Together, these data confirm that MARCH8 mediates restriction against a range of seasonal IAV relevant to human health, as well as against the IBV strain tested in our study.

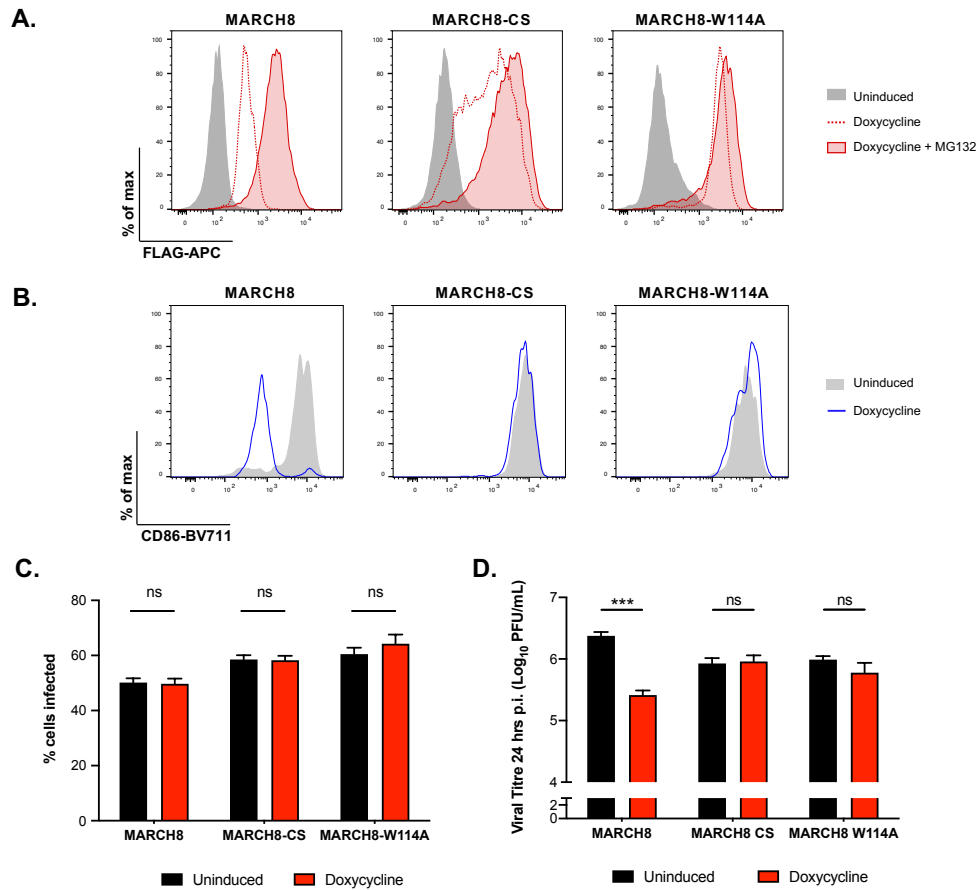
While Beij89 has been used as the representative IAV H3N2 strain in all studies described to date, we identified Braz78 (H1N1) as an IAV strain that grew to 10-fold higher titres than Beij89 and remained sensitive to restriction by MARCH8. The ability to produce high titre IAV was particularly important for subsequent experiments that required concentrated or purified virus (e.g. transmission electron microscopy). Therefore, both Beij89 and/or Braz78 have been used in subsequent studies in this thesis to characterize aspects of MARCH8-mediated restriction of IAV.



**Figure 4.3. DOX-inducible expression of MARCH8 mediates antiviral activity against a broad range of influenza virus strains.** 293T cells with inducible expression of MARCH8 were cultured in the absence (Uninduced, black bars) or presence of 1 µg/mL DOX (Doxycycline, red bars). At 24 hr after DOX induction, cells were washed and infected for 1 hr at 37°C with different strains of influenza virus at MOI 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr post-infection, supernatants were harvested, clarified by centrifugation and titres of infectious virus were determined by plaque assay using MDCK cells. Mean virus titres from triplicate samples (± SD) are shown. The dashed line indicates the limit of detection of the plaque assay. Data are representative of 2 independent experiments. A two-tailed unpaired Student's t-test was performed to compare uninduced and induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant. The % reduction in virus titres from uninduced versus DOX-induced conditions is shown for each cell line.

#### **4.2.2. E3 ligase activity of MARCH8 is essential for its antiviral activity against IAV.**

The ubiquitination capacity of E3 ubiquitin ligases relies on the RING domain. This Zn-binding domain interacts with the E2 ubiquitin-conjugating enzyme allowing the transfer of ubiquitin to the specific substrate. The introduction of mutations into the RING domain known to inactivate E3 ligase activity have been shown to abrogate both the antiviral and the proviral effects of MARCH8 against different viruses [210, 212]. To determine if a functional RING-CH domain was required to mediate antiviral activity against IAV, site-directed mutagenesis was used to incorporate two different mutations known to disrupt interactions between MARCH8 and the E2 ubiquitin-conjugating enzyme: a mutant where all cysteine residues (C) of the RING domain are replaced by serine residues (S, MARCH8-CS) [218] and a mutant where a conserved tryptophan (W) at position 114 is replaced by alanine (A, MARCH8-W114A) [199]. Stable cell lines expressing DOX-inducible MARCH8-CS or MARCH8-W114A, were generated, screened for expression of FLAG-tagged MARCH8 and compared against wild-type MARCH8. As seen in **Figure 4.4A**, expression levels of each RING-CH domain mutant were similar to that of wild-type MARCH8 in 293T cells. To validate each of the mutations, we assessed to ability of the mutant proteins to downregulate cell-surface CD86, as described in Chapter 3. In these experiments, uninduced and DOX-induced cell lines were transfected with a plasmid encoding CD86 and cell surface expression of CD86 was assessed 24 hr after addition of DOX. While DOX-induced wild-type MARCH8 downregulated CD86, cell lines with inducible expression of either MARCH8-CS or MARCH8-W114A did not (**Figure 4.4B**).



**Figure 4.4. DOX-inducible MARCH8 RING-CH mutants do not mediate antiviral activity against IAV in 293T cells.** (A) 293T cells transduced with lentivirus to generate cell lines with DOX-inducible expression of two MARCH8 RING-CH mutant proteins (MARCH8-CS and MARCH8-W114A) were cultured in media alone (grey, filled) or in media supplemented with 1  $\mu$ g/mL DOX (red, open). At 20 hr post-DOX treatment, 10  $\mu$ M of MG132 was added to media to limit protein degradation (red, filled). At 24 hr post-DOX treatment, cells were washed, fixed, permeabilized and stained with anti-FLAG-APC mAb. Intracellular FLAG expression was determined by flow cytometry. Representative histograms are shown. 293T cells with DOX-inducible expression of MARCH8 wild-type were used as a control. (B) 293T cells with DOX-inducible expression of MARCH8, MARCH8-CS and MARCH8-W114A proteins were transfected with a plasmid encoding CD86 and the fluorescence protein ZsGreen. At 12 hr post-transfection, cells were left untreated (grey, filled) or treated with 1  $\mu$ g/mL DOX (blue, empty) to induce expression of the different MARCH8 proteins. At 24 hr post-DOX treatment, cells were washed and stained with anti-CD86-BV711 mAb. Levels of CD86 on the surface of ZsGreen-positive transfected cells were analyzed by flow cytometry. Representative histograms of CD86 expression are shown. (C) 293T cells with DOX-inducible expression of MARCH8, MARCH8-CS and MARCH8-W114A proteins cultured for 24 hr in media (black bars) or media supplemented with 1  $\mu$ g/mL DOX (red bars) were washed, then infected for 1 hr at 37°C with IAV strain A/Braz78 at MOI 2.5 PFU/cell. After washing, cells were cultured an additional 7 hr and then washed, fixed, permeabilized and stained with NP-FITC mAb. The percentage of IAV-infected NP-positive cells was determined by flow cytometry. The mean of triplicate samples ( $\pm$  SD) are shown. Data are representative of 2 independent experiments. (D) 293T cells with DOX-inducible expression of MARCH8, MARCH8-CS and MARCH8-W114A proteins cultured for 24 hr in the absence (Uninduced, black bars) or presence of 1  $\mu$ g/mL DOX (Doxycycline, red bars) were washed, then infected for 1 hr at 37°C with IAV strain A/Braz78 at MOI 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr post-infection, supernatants were harvested, clarified and titres of infectious virus were determined by plaque assay using MDCK cells. Mean virus titres from triplicate samples ( $\pm$  SD) are shown. Limit of detection is 0.85 Log<sub>10</sub> PFU/mL. Data are representative of 2 independent experiments. A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions in (C) and (D). \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

Next, we examined the impact of DOX-inducible expression of MARCH8-CS and MARCH8-W114A on IAV infection. Consistent with previous results, inducible expression of wild-type or mutant forms of MARCH8 did not affect “early” events in the virus replicative-cycle, as determined by assessing the percent of NP-positive IAV-infected cells at 8 hr p.i. (**Figure 4.4C**). However, while MARCH8 wild-type expression reduced titres of infectious virus in cell supernatants harvested at 24 hr p.i., supernatants from cell lines expressing MARCH8-CS and MARCH8-W114A showed similar virus titres in the presence or absence of DOX induction (**Figure 4.4D**). Together, these data confirm that a functional RING-CH domain is essential for the anti-IAV activity of MARCH8, as no restriction was observed by MARCH8-CS or MARCH8-W114A.

#### **4.2.3. Insights into the mechanism/s by which MARCH8 mediates antiviral activity against IAV.**

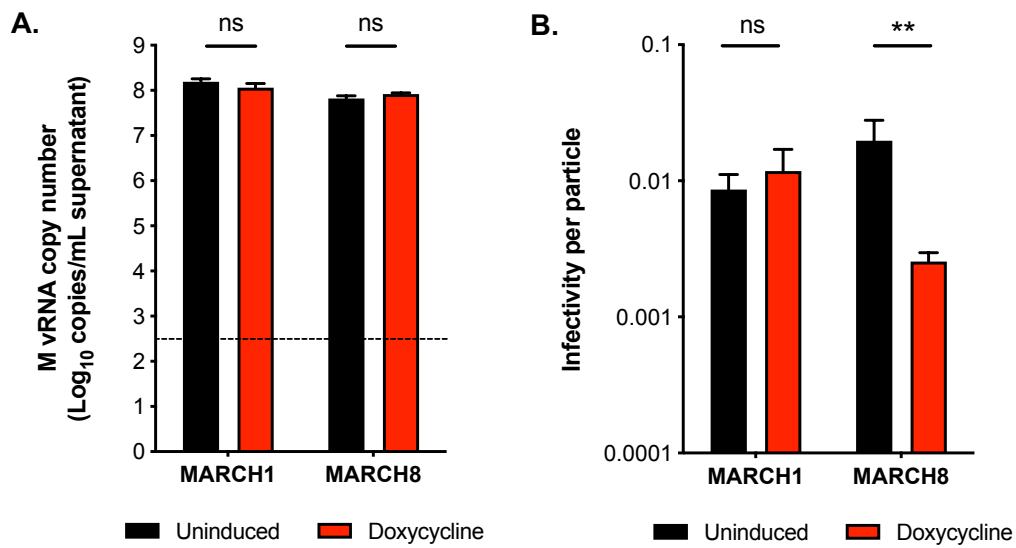
##### *4.2.3.1. Virus particles released from IAV-infected cells in the presence of DOX-inducible MARCH8 show reduced infectivity per particle.*

Induction of MARCH8 expression resulted in reduced titres of infectious particles released from IAV-infected cells. To determine if lower titres represent an overall reduced infectivity per particle or a decrease in the overall number of viral particles released, qPCR was used to quantify copies of viral (v)RNA in the cell-free supernatant of infected cells. Therefore, cells with DOX-inducible expression of MARCH1 or MARCH8 were infected with IAV and, at 24 hr post-infection, vRNA was extracted, converted into cDNA and the number of copies of the viral gene segment encoding the matrix (M)

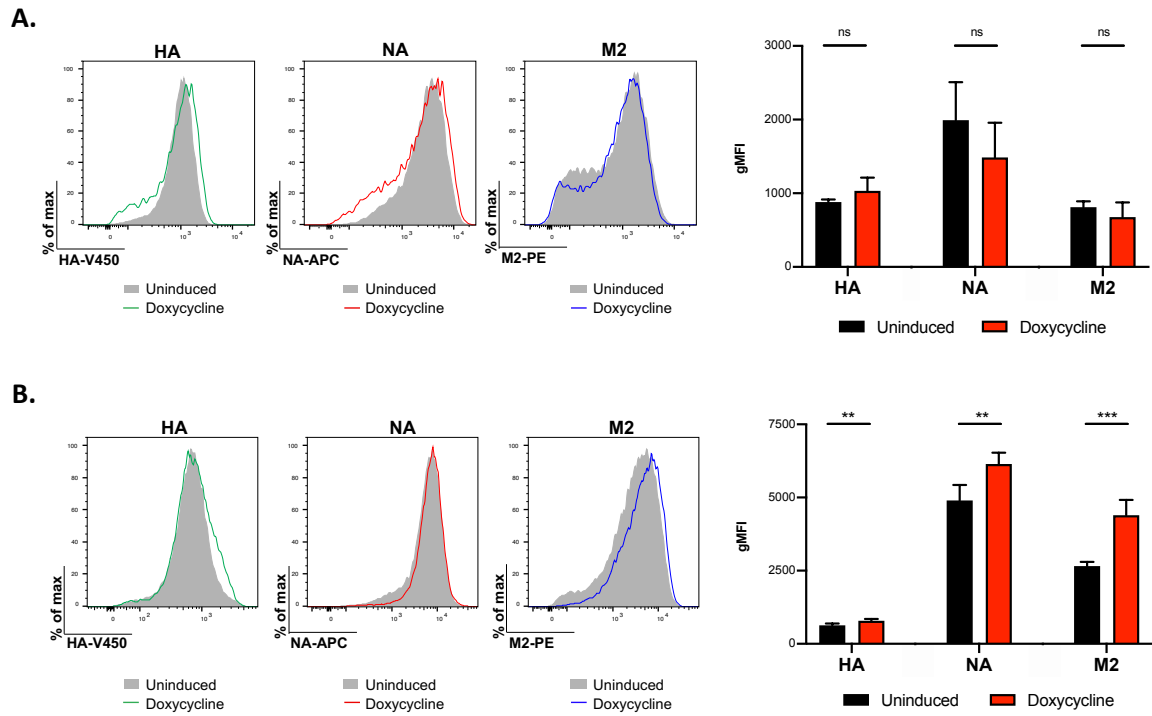
protein were determined. As seen in **Figure 4.5A**, the number of copies of M vRNA segments in the supernatant of infected cells was similar, regardless of DOX-induced expression of MARCH8, suggesting that the number of particles released was the same. To determine infectivity per particle, the ratio between the viral titre (measured by plaque assay) and the total number of particles released (obtained by qPCR) was calculated [219]. Based on this, we conclude that DOX-inducible expression of MARCH8, but not MARCH1, reduced the infectivity per particle of nascent virions released from IAV-infected cells (**Figure 4.5B**).

#### *4.2.3.2. DOX-inducible MARCH8 expression does not result in downregulation of viral glycoproteins from the surface of IAV-infected cells.*

Previous studies have shown that MARCH8 can downregulate expression of viral glycoproteins of HIV-1 (Env) and VSV (G) from the surface of infected cells, reducing their incorporation and therefore infectivity of nascent virus particles released [210]. Given that MARCH8 expression in 293T cells appears to reduce the infectivity of IAV particles released from infected cells, we used flow cytometry to examine expression levels of the three IAV glycoproteins (HA, NA and M2) expressed on the surface of infected cells prior to virus budding. Therefore, DOX-induced and uninduced cells were infected with IAV and cell-surface expression of the viral proteins HA, NA and M2 were determined at 8 hr and 24 hr post-infection. No significant differences were observed in expression levels of each glycoprotein on the surface of uninduced or DOX-induced cells at 8 hr p.i. (**Figure 4.6A**). However, at 24 hr p.i., HA, NA and M2 all showed a modest but significant increase in expression levels on the surface of MARCH8-expressing cells (**Figure 4.6B**)



**Figure 4.5. Effect of DOX-inducible expression of MARCH1 or MARCH8 in the copy number of vRNA matrix (M) gene segment and in the infectivity per particle of IAV released from infected cells.** 293T cells with inducible expression of MARCH1 or MARCH8 were cultured in the absence (Uninduced, black bars) or presence of 1  $\mu$ g/mL DOX (Doxycycline, red bars). At 24 hr after DOX induction, cells were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr p.i., vRNA was extracted from clarified supernatants and the number of copies of M gene vRNA was determined by qPCR. (A) Mean copy numbers ( $\pm$  SD) from triplicate samples are shown. Data are representative of 2 independent experiments. Limit of detection is 2.5 Log<sub>10</sub> copies/mL (Dashed line). (B) Infectivity per particle was calculated as the ratio between the viral titre and M vRNA copy number in cell supernatants. Mean ( $\pm$  SD) values from triplicate samples are shown. Data are representative of 2 independent experiments. A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions in (A) and (B). \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.



**Figure 4.6. Effect of DOX-inducible MARCH8 on expression of the influenza virus HA, NA and M2 proteins on the surface of IAV-infected cells.** 293T cells with inducible expression of MARCH8 were cultured in the absence or presence of 1  $\mu$ g/mL DOX. At 24 hr after DOX induction, cells were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI 1 PFU/cell. After washing, cells were cultured an additional 7 hr (**A**) or 24 hr (**B**). At 8 hr or 24 hr p.i., cells were stained with specific antibodies for cell-surface expression of HA, NA or M2. After surface staining, cells were fixed, permeabilized and stained with NP-FITC mAb to allow for identification of infected cells. The surface expression of HA, NA or M2 on live cells was then determined by flow cytometry. Representative histograms from NP-positive infected cells are shown. Geometric mean fluorescent intensities (gMFI) ( $\pm$  SD) from triplicate samples are shown. Data are representative of 2 independent experiments. A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.



As expected, DOX-inducible expression of MARCH1 did not alter cell-surface expression of viral HA, NA or M2 at 8 hr or 24 hr post-infection (data not shown). These results indicate that MARCH8 does not interfere with synthesis and transport of the viral HA, NA or M2 to the surface of infected cells. Moreover, while MARCH8 mediates antiviral activity against IAV at a late stage of the replication cycle, this appears to be distinct to its antiviral activity against HIV-1 and VSV as MARCH8 expression was not associated with downregulation of viral glycoproteins from the surface of infected cells.

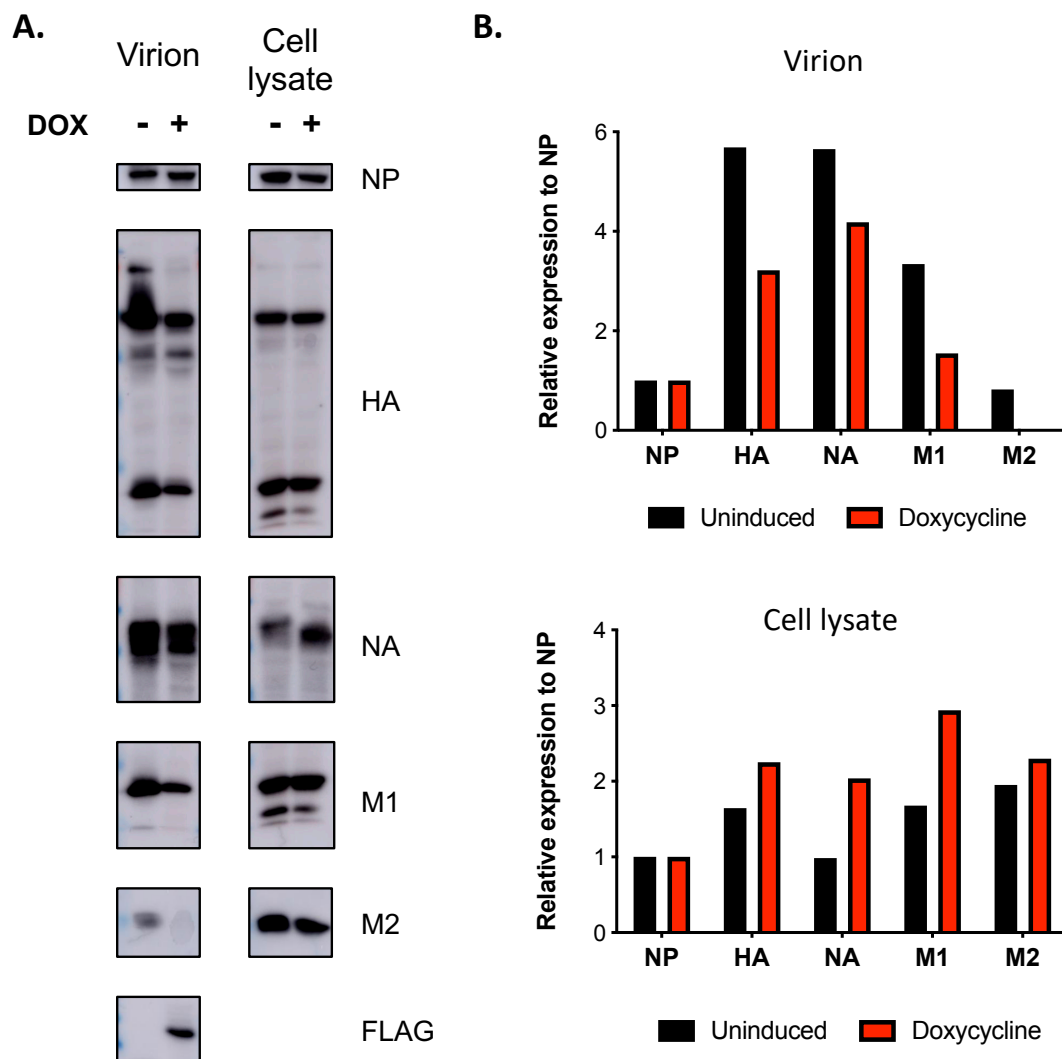
#### *4.2.3.3. Investigating the protein composition of virus particles released from IAV-infected cells with induced MARCH8 expression.*

##### *(a) Western blot analyses of viral proteins in infected cell lysates and newly synthesised virions.*

It is possible that differences in proportion or amount of specific viral proteins in a packaged virion could contribute to the reduced infectivity per particle of IAV released from infected cells in the presence of MARCH8. As a first step towards understanding the protein composition of virions, cells induced to express MARCH8 were infected with IAV and, at 24 hr p.i., cell supernatants containing IAV particles were harvested, concentrated and subjected to western blot analysis using specific antibodies against different structural IAV proteins. Additionally, cell lysates were also prepared to analyse the viral proteins associated with the IAV-infected cells. To facilitate comparison of viral proteins in the presence or absence of MARCH8 expression, the densitometry of the bands obtained by western blot was determined using ImageJ. Densitometry values for specific viral proteins were normalized against the value obtained for the viral NP, since

NP associates with vRNA to form vRNP complexes and we did not detect major differences in levels of vRNA (at least for the M gene segment).

As seen in **Figure 4.7A**, various viral proteins (NP, HA, NA, M1 and M2) were detected in cell lysates from uninduced or DOX-induced 293T cells expressing MARCH8. When normalized to NP expression, densitometry indicated that each of these viral proteins showed increased expression in DOX-induced, compared to uninduced cells (**Figure 4.7B**). When examining the protein composition of virions released from IAV-infected cells in the presence of MARCH8, we observed reduced amounts of HA, NA, M1 and M2 relative to NP when compared to virions released from uninduced cells that did not express MARCH8 (**Figure 4.7A/B**). **Figure 4.7** shows data from a single experiment where all viral proteins were detected in the same virus preparation, however these findings were observed in multiple virus preparations when western blot was performed to detect some, but not all, of these structural IAV proteins (data not shown). Note that while the M2 viral protein was readily detectable in IAV-infected lysates from both uninduced and DOX-induced cells expressing MARCH8, it was only detected at low levels in virions from uninduced cells, consistent with previous studies [236], and not from DOX-induced cells. Importantly, MARCH8 was incorporated into the particles released from DOX-induced cells (as detected using an anti-FLAG mAb) (**Figure 4.7A**). As expected, FLAG-tagged MARCH8 was not detected in cell lysates from DOX-induced cells.



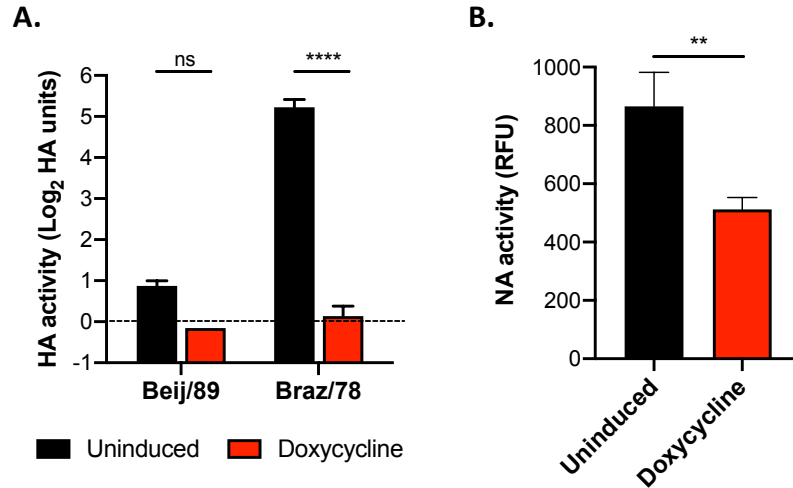
**Figure 4.7. DOX-inducible expression of MARCH8 reduces incorporation of viral structural proteins into newly-synthesized IAV particles released from IAV-infected cells.** 293T cells cultured for 24 hr in the absence or presence of 1 $\mu$ g/mL DOX were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI 5 PFU/cell. Cells were then washed and cultured overnight. **(A)** At 24 hr post-infection, supernatants were harvested, concentrated by ultracentrifugation (as described in Materials and Methods) and subjected to SDS PAGE. At the same time, IAV-infected cells were lysed, and cell lysates were also subjected to SDS PAGE. Reduced samples were transferred to a polyvinylidene difluoride (PVDF) membrane and probed with anti-NP mAb, anti-HA pAb, anti- NA pAb, anti-M1 mAb, anti-M2 mAb or anti-FLAG mAb. **(B)** Band densitometry was measured using ImageJ software and the expression of each viral protein normalized against NP expression was plotted. Black bars represent uninduced conditions and red bars represent DOX-induced conditions. Data shown are from a single experiment.

*(b) HA and NA activity in cell-free supernatant of IAV-infected cells.*

Given that both HA and NA viral proteins were expressed at lower levels in virions released from cells expressing MARCH8 compared to uninduced cells, we next assessed the activity of these proteins in the supernatants of uninduced and DOX-induced cells infected with IAV. Specifically, we assessed the ability of released virions to agglutinate erythrocytes (a function of the viral HA) or cleave MUNANA substrate (a function of the viral NA). As seen in **Figure 4.8A**, DOX-inducible MARCH8 expression resulted in reduced hemagglutination activity in supernatants from cells infected with Braz78, consistent with newly synthesised virions containing less HA per particle in the presence of MARCH8. A similar trend was noted using Beij89, but HA titres were very low in virus released from uninduced cells, and the differences were not significant. Similarly, supernatants from cells infected with Beij89 in the presence of inducible MARCH8 showed reduced levels of cleaved MUNANA, consistent with a reduced NA enzymatic activity (**Figure 4.8B**). Thus, consistent with western blot data (**Figure 4.7**), functional assays confirmed reduced activity associated with the HA and NA glycoproteins in IAV released in the presence of inducible MARCH8. This, in turn, is consistent with reduced infectivity of nascent virions released in the presence of inducible MARCH8.

*4.2.3.4. Alterations in virion morphology following release of virions from IAV-infected cells with induced MARCH8 expression.*

Nascent virions released from IAV-infected cells in the presence of MARCH8 show differences in infectivity, HA and NA activity and in the proportions of a number of

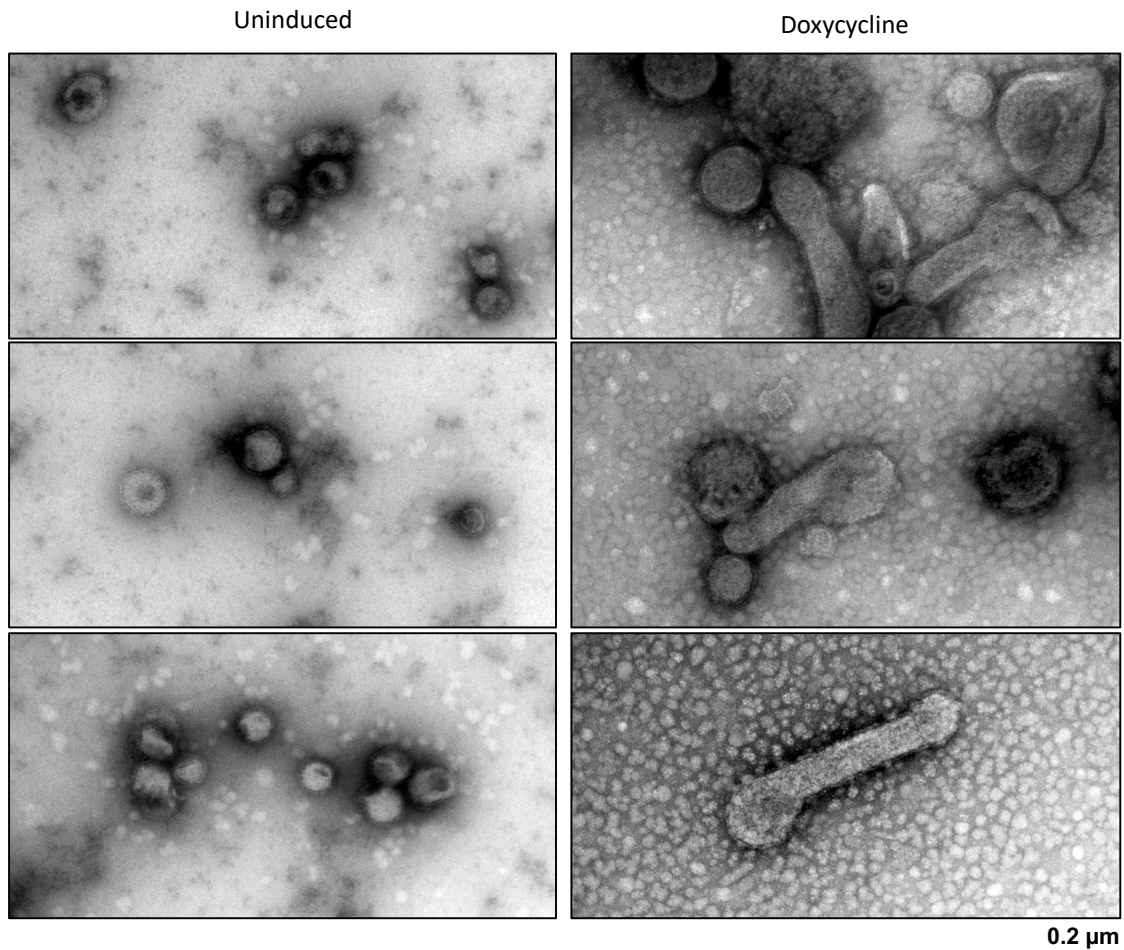


**Figure 4.8. Effect of DOX-inducible expression of MARCH8 on HA and NA activity in supernatants from IAV-infected cells.** 293T cells cultured for 24 hr in the absence (Uninduced, black bars) or presence of 1  $\mu$ g/mL DOX (Doxycycline, red bars) were washed and infected for 1 hr at 37°C with IAV strains A/Beij89 or A/Braz78 at MOI 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr post-infection, supernatants were harvested, clarified and the HA activity was measure by standard hemagglutination assay using 1% vol/vol turkey red blood cells. Mean HA units ( $\pm$  SD) from triplicate samples are shown. Data are representative of 2 independent experiments. The dashed line indicates the limit of detection of the hemagglutination assay. A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant. **(B)** 293T cells cultured for 24 hr in the absence (Uninduced, black bar) or presence of 1  $\mu$ g/mL DOX (Doxycycline, red bar) were washed and infected for 1 hr at 37°C with IAV Beij89 at MOI 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr post-infection, supernatants were harvested, clarified and the NA activity was measured using a MUNANA-based fluorometric assay. Mean relative fluorescence units (RFU) ( $\pm$  SD) from triplicate samples are shown. Data are representative of 2 independent experiments. A two-tailed unpaired Student's t-test was performed to compare uninduced and DOX-induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

structural viral proteins. Therefore, we next used transmission electron microscopy (TEM) to examine the morphology of virions released from infected cells in the presence or absence of DOX-inducible MARCH8. Virus purified from supernatants of MARCH8-induced and uninduced IAV-infected cells were fixed, mounted on grids and negatively stained using uranyl acetate (as described in Materials and Methods). Virus particles released from IAV-infected uninduced cells were generally spherical in shape and quite homogenous in terms of virion size, however particles released from cells expressing MARCH8 were much more heterogeneous, including larger spherical particles (>100 nm) and elongated particles with a more filamentous morphology (**Figure 4.9**).

#### **4.2.4. Investigating if specific lysine residues in the viral HA, NA, M1 and M2 serve as targets for MARCH8-mediated ubiquitination.**

The addition of ubiquitin to specific substrates generally requires lysine (K) residues as acceptor sites for ubiquitination (reviewed in [157]). Interestingly, viral E3 ligases can also use other residues, including serine, threonine and cysteine. However, to date there is no evidence to suggest that MARCH8 can use residues other than K as sites for ubiquitination [212, 218, 235]. Considering that (i) the anti-IAV capacity of MARCH8 is dependent on its E3 ligase activity, (ii) we did not detect any major changes in expression of viral HA, NA and M2 at the surface of IAV-infected cells in the presence or absence of MARCH8, but (iii) multiple structural viral proteins appear to be incorporated into nascent virions at reduced levels in the presence of MARCH8, we investigated if K residues in particular IAV proteins, especially those involved in modulating virus budding

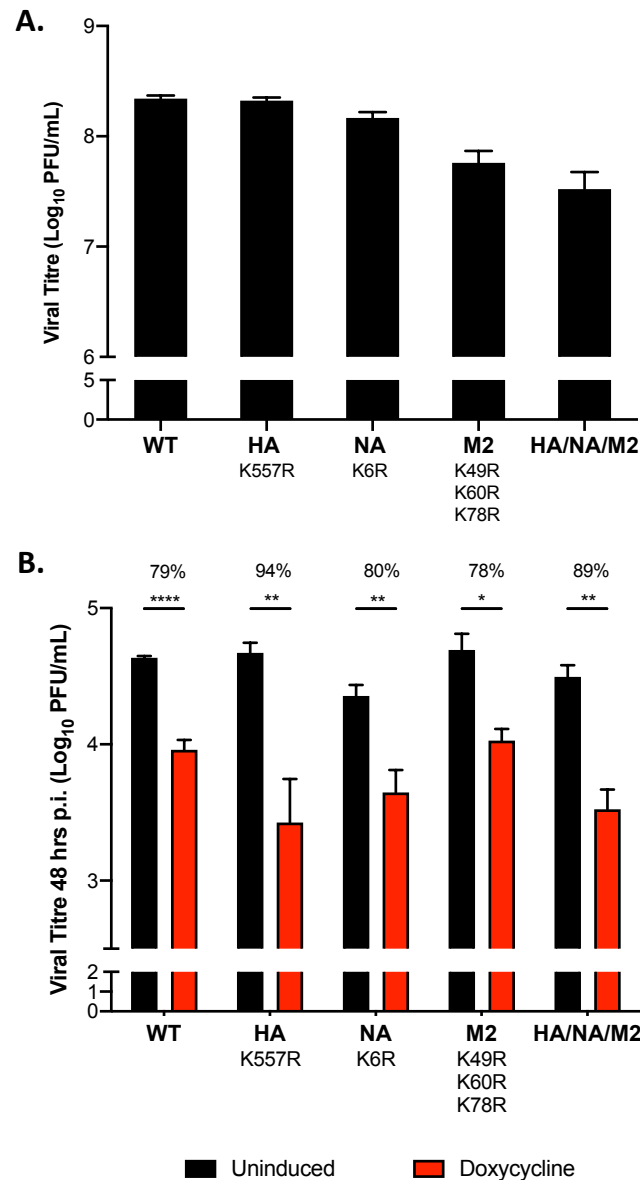


**Figure 4.9. DOX-inducible expression of MARCH8 alters the morphology of newly-synthesized virions released from IAV-infected cells.** 293T cells cultured for 24 hr in the absence (Uninduced) or presence of 1  $\mu\text{g}/\text{mL}$  DOX (Doxycycline) were washed and infected for 1 hr at 37°C with IAV strain A/Braz78 at MOI 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr post-infection, supernatants were harvested, concentrated and purified by ultracentrifugation (as described in Materials and Methods). Purified samples were fixed with 2% (v/v) PFA, then mounted and negatively stained using 1% uranyl acetate before they were examined using a transmission electron microscope. Representative images are shown.

from the surface of IAV-infected cells, might serve as targets for MARCH8-mediated ubiquitination. There are four viral proteins (HA, NA, M1 and M2) known to interact with the plasma membrane during the late stages of IAV infection, with HA, NA and M2 expressed on the surface of the infected cell, while M1 is recruited to budding sites interacting with the inner leaflet of the plasma membrane and the cytoplasmic tails (CT) of HA and NA [30, 237, 238]. Our sequence analysis indicated that all four proteins are predicted to have K residues that could potentially be used as ubiquitin acceptor sites: 1 in the CT of HA, 1 in the CT of NA, 3 in the CT of M2 and 12 distributed throughout M1 (8 in the N-terminal domain, thought to interact with the plasma membrane, and 4 in the C-terminal domain).

Site-directed mutagenesis was used to introduce mutations into the CT of each of the viral proteins, resulting in substitution of K to arginine (R). Using reverse genetic techniques, recombinant viruses carrying these mutations were then generated to determine if specific K residues in any of these viral proteins might be targeted directly by MARCH8. Note that due to availability of appropriate plasmids, all viruses generated by reverse genetics were on a A/Udorn/307/1972 (H3N2) background and that this virus strain is also sensitive to MARCH8-mediated inhibition (**Figure 4.3**). Viruses carrying mutations in HA (K557R), NA (K6R) and M2 (K49R, K60R, K78R), separately or in combination (HA/NA/M2), were generated and mutations confirmed by sequencing of relevant virus genes. Despite some variation in virus titres following growth in eggs (**Figure 4.10A**), all viruses grew to high titres ( $3 \times 10^7$  –  $2 \times 10^8$  PFU/mL), suggesting that none of the mutations altered a site that was critical for productive virus replication in



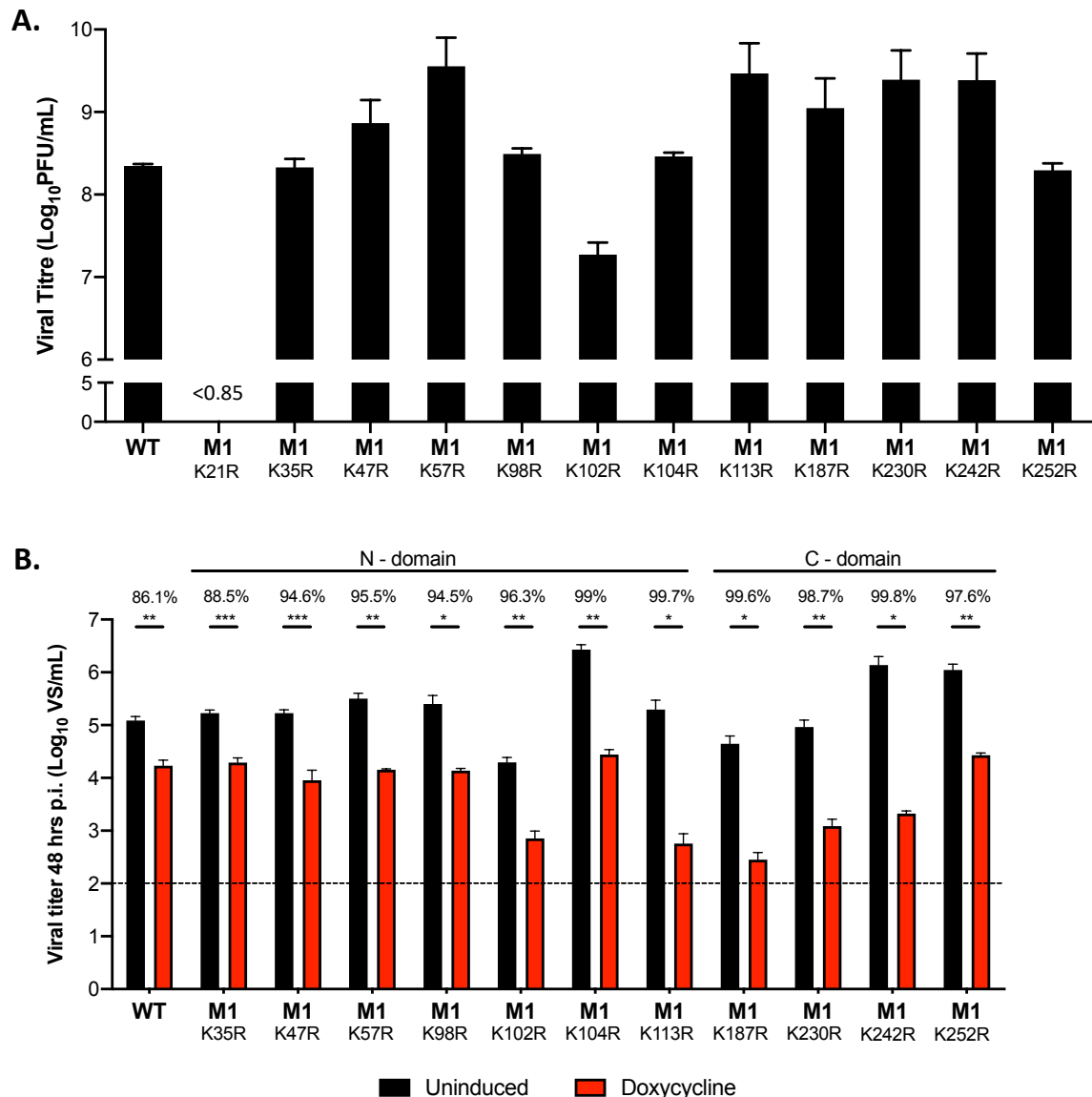


**Figure 4.10. Recombinant IAV with K→R substitutions in the cytoplasmic tails HA, NA and M2 proteins remain susceptible to MARCH8-mediated antiviral activity.** (A) Site-directed mutagenesis was used to introduce mutations in the cytoplasmic tails (CT) of HA, NA and M2. Recombinant viruses carrying wild-type or mutant versions of individual lysine residues (HA K557R; NA K6R), all lysine residues in the CT of one protein (M2 K49R, K60R and K78R), or all lysine residues in the CT of 3 viral proteins (HA/NA/M2) were generated by reverse genetics, as described in Materials and Methods. Mean viral titres from three independent titrations ( $\pm$  SD) are shown. (B) 293T cells with inducible expression of MARCH8 cultured for 24 hr in the absence (Uninduced, black bars) or presence of 1  $\mu$ g/mL DOX (Doxycycline, red bars) were washed, then infected for 1 hr at 37°C with WT or mutant recombinant viruses (as shown on the x-axis) at MOI 0.01 PFU/cell. Cells were then washed and cultured in the presence of exogenous trypsin (0.5 $\mu$ g/mL) to facilitate multi-cycle virus replication. At 48 hr post-infection, supernatants were harvested, clarified and titres of infectious virus in supernatants were determined by plaque assay using MDCK cells. Mean virus titres from triplicate samples ( $\pm$  SD) are shown. Data are representative of 2 independent experiments. A two-tailed unpaired Student's t-test was performed to compare uninduced and DOX-induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant. The % reduction in virus titres from uninduced versus DOX-induced conditions is shown for each cell line. In (A) and (B) the limit of detection is 0.85 Log<sub>10</sub> PFU/mL.

eggs. All titres were sufficient such that virus stocks could be adjusted to equivalent MOI to infect 293T cells with induced expression of MARCH8.

Next, uninduced and DOX-induced cells expressing wild-type MARCH8 were infected at a low MOI (0.01 PFU/cell) with each recombinant virus and at 48 hr post-infection, cell supernatants were collected and titrated on MDCK cells to evaluate the antiviral activity of MARCH8. Note that all viruses replicated to similar levels in uninduced cells confirming that none of the mutations had a major impact on virus replication in 293T cells. Mutant viruses carrying mutations in the CT of HA (K557R), NA (K6R) and M2 (K49R, K60R, K78R) were all restricted by MARCH8, although the potency of this inhibition did vary between viruses (**Figure 4.10B**). Similar results were observed when cells were infected with recombinant viruses containing a combination of K substitutions simultaneously in all three viral proteins (HA/NA/M2, **Figure 4.10B**).

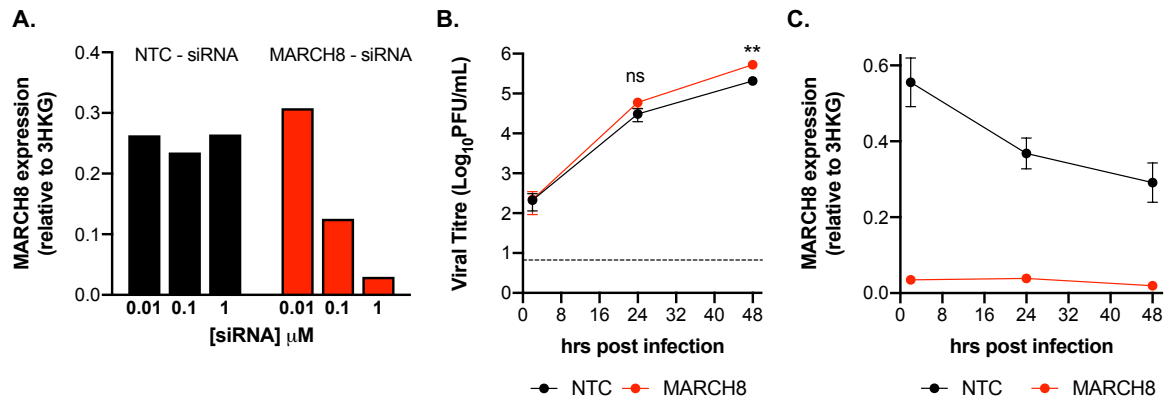
Next, a similar strategy was used to generate viruses with individual mutations (resulting in K to R substitutions) in the viral M1. Despite multiple attempts, mutant viruses carrying a R instead of a K at position 21 (K21R) could not be generated (**Figure 4.11A**). Following infection of uninduced and DOX-induced cells expressing MARCH8 with a low MOI (0.01 PFU/cell) of each virus, cell supernatants collected at 48 hr p.i. were clarified and titrated on MDCK cells. As seen in **Figure 4.11B**, despite some variation in levels of virus titres released from uninduced cells, none of the mutant viruses escaped MARCH8-mediated restriction of virus growth. Interestingly, some of the mutations in M1 actually appeared to result in viruses with an increased susceptibility to restriction by MARCH8.



**Figure 4.11. Recombinant IAV with K→R substitutions in the M1 protein remain susceptible to MARCH8-mediated antiviral activity.** (A) Site-directed mutagenesis was used to introduce individual mutations in the matrix protein 1 (M1). Recombinant viruses carrying wild-type or mutant versions of M1 were generated by reverse genetics. Mean viral titres from three independent titrations ( $\pm$  SD) are shown. Limit of detection is 0.85  $\text{Log}_{10}$  PFU/mL. (B) 293T cells with inducible expression of MARCH8 cultured for 24 hr in the absence (Uninduced, black bars) or presence of 1  $\mu\text{g/mL}$  DOX (Doxycycline, red bars) were washed, then infected for 1 hr at 37°C with WT or mutant recombinant viruses (as shown on the x-axis) at MOI 0.01 PFU/cell. Cells were then washed and cultured in the presence of exogenous trypsin (0.5  $\mu\text{g/mL}$ ) to facilitate multi-cycle virus replication. At 48 hr post-infection, supernatants were harvested, clarified and titres of infectious virus in supernatants were determined by ViroSpot assay using MDCK cells. Mean virus titres from triplicate samples ( $\pm$  SD) are shown. Data are representative of 2 independent experiments. The dashed line indicates the limit of detection. A two-tailed unpaired Student's t-test was performed to compare uninduced and DOX-induced conditions. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant. The % reduction in virus titres from uninduced versus DOX-induced conditions is shown for each cell line.

#### 4.2.5. Investigating the role of endogenous MARCH8 against influenza viruses *in vitro*.

Constitutive and/or inducible expression systems are powerful tools to assess the effect of different proteins in diverse experimental systems and have been widely used to identify and characterize host antiviral restriction factors for many viruses. However, these approaches have limitations, including higher cellular expression levels compared to that of the endogenous protein and/or changes in subcellular protein distribution/localization which may confound interpretation of results. Therefore, assessment of putative antiviral restriction factors by overexpression studies should be complemented by knockdown/knockout studies to validate the role of the endogenous protein in modulating virus infection. Therefore, we used siRNA to knockdown endogenous MARCH8 in host cells to validate an antiviral role in IAV infection. As attempts to detect endogenous MARCH8 protein in 293T, HeLa or A549 cells by western blot were unsuccessful (data not shown) we measured levels of mRNA to validate siRNA-mediated knockdown of MARCH8. First, 293T cells were treated with increasing concentrations of MARCH8-specific siRNA or with a non-targeting control (NTC) and expression levels of MARCH8 determined 72 hr later. As seen in **Figure 4.12A**, specific knockdown of MARCH8 expression was observed with increasing concentrations of MARCH8-specific siRNA. Next, cells pre-treated with 1 mM of siRNA for 72 hr were subsequently infected with a low MOI (0.01 PFU/cell) of IAV strain Beij89 and virus titres determined in clarified cell supernatants collected at various times thereafter. Overall, virus titres increased markedly between 2 and 24 hr p.i., with a further increase by 48 hr p.i. Treatment of cells with MARCH8-specific siRNA resulted in a modest (2.5-fold), but significant, increase in virus titres at 48 hr p.i. (**Figure 4.12B**). Analysis of cell lysates



**Figure 4.12. Effect of MARCH8 siRNA knockdown on the viral titres of infectious IAV released from 293T cells.** (A) Cultures of 293T cells were treated with increasing amounts of siRNAs targeting endogenous MARCH8 (red bars) or non-targeting control (NTC, black bars). At 72 hr post-treatment, total RNA was extracted, converted to cDNA and the expression of MARCH8 was measured by qPCR. To normalize results, three housekeeping genes (HKG) were used: GAPDH (glyceraldehyde 3-phosphate dehydrogenase), RPL13A (ribosomal protein L13a) and TBP (TATA-binding protein). Results from singlicate samples are shown. (B) 293T cells treated for 72 hr with 1 mM siRNA targeting endogenous MARCH8 (red line) or with a non-targeting control (NTC, black line) were washed and infected for 1 hr at 37°C with A/Beij89 at MOI 0.01 PFU/cell. Cells were then washed and cultured in the presence of exogenous trypsin (0.5 μg/mL) to facilitate multi-cycle virus replication. Supernatants harvested at 2, 24 and 48 hr p.i. were clarified by centrifugation (3000 rpm, 5 min) and titres from infectious virus determined by plaque assay using MDCK cells. Mean virus titres from triplicate samples (± SD) are shown. The dashed line indicates the limit of detection of the plaque assay. A two-tailed unpaired Student's t-test was performed to compare NTC and MARCH8 treated cells at each timepoint. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant. (C) Total RNA was extracted from the same infected cells, converted to cDNA and the expression of MARCH8 (relative to GAPDH, RPL13A and TBP) was measured by qPCR. Mean values (± SD) from triplicate samples are shown.

at various times post-infection confirmed efficient knockdown of MARCH8 throughout the experiment, although it should be noted that some reduction of MARCH8 expression was also observed in NTC-treated cells, possibly as a result of virus infection (**Figure 4.12C**).

### **4.3. Discussion.**

MARCH8 was the first member of the mammalian MARCH family proteins to be characterized. As an antiviral protein, MARCH8 is reported to interact with the envelope glycoproteins of HIV-1 and VSV, resulting in their downregulation from the surface of infected cells [210]. This, in turn, was associated with reduced infectivity of virus particles released from infected cells. In these studies, the E3 ligase activity of MARCH8 was essential for antiviral activity, however no evidence was presented to indicate direct MARCH8-mediated ubiquitination of any viral protein. In Chapter 3 we screened different MARCH proteins for their ability to modulate infection by the IAV strain Beij89, reporting that only MARCH8 mediated antiviral activity. MARCH8 did not appear to modulate infectious entry of IAV, but rather reduced titres of infectious particles released from infected cells late in the viral replicative cycle. In this chapter we aimed to investigate the importance of the E3 ligase activity of MARCH8 against IAV, as well as characterize IAV-infected cells and virions released from IAV-infected cells, in the presence or absence of MARCH8. These studies aim to provide mechanistic insights regarding how MARCH8 mediates antiviral activity against IAV.

Initial studies in this chapter used a low MOI and multicycle growth curves to confirm that MARCH8, but not the closely related MARCH1 protein, was a potent restriction factor of IAV strain Beij89 (**Figure 4.1**). This approach was designed to highlight more subtle differences in replication efficiency, given that most protein targets identified to date that are recognized by MARCH8, are also recognized by MARCH1 (reviewed in [181]). Interestingly, MARCH1 is known to be unstable at mRNA and protein level [185, 226], suggesting that while both MARCH1/8 might have the potential to inhibit influenza viruses, the rapid turnover of MARCH1 could result in less protein available to interact with and block influenza viruses release. However, similar levels of DOX-induced expression of MARCH1 and MARCH8 were found in our system prior addition of the proteasomal inhibitor MG132 (Chapter 3, **Figure 3.6**). Next, we generated additional human epithelial cell lines (A549, HeLa) with DOX-inducible expression of MARCH1 or MARCH8 to confirm MARCH8-mediated restriction of IAV strain Beij89 (**Figure 4.2**), although the effect was not as potent as that observed using 293T cells. Differences in potency of MARCH8 restriction of IAV might relate to levels of inducible MARCH8 expressed in each cell line, which could be addressed by staining and measuring intracellular FLAG expression in all cell lines simultaneously. Additionally, confocal microscopy could be used to determine if distribution and/or subcellular localization of induced MARCH8 differs between A549, HeLa and 293T cells. Alternatively, while each cell line can support IAV infection, they do differ in their ability to support growth of Beij89 (**Figure 4.2B** and **Figure 3.2** from Chapter 3) which may alter the spatiotemporal interaction of MARCH8 with its specific(s) targets. Particular cell lines may also differ in expression of other restriction factors which could mask the antiviral effects of MARCH8.

Next, we confirmed that MARCH8 could inhibit a broad range of human seasonal IAV, as well as the one IBV strain tested. While every virus tested was restricted by MARCH8, the potency of inhibition was shown to vary between different strains (**Figure 4.3**). According to available information regarding proteins known to be targeted by MARCH8, only lysine (K) residues in the cytoplasmic tails (CT) of target proteins serve as ubiquitin acceptors. Comparative analysis of the CT of the HA, NA and M2 proteins of all influenza viruses tested showed that each of these viral proteins contained K residues, making it difficult to propose an explanation based on protein sequences alone. When considering the late stages of influenza virus infection, little is known regarding differences in the assembly, budding and egress between A/H1N1, A/H3N2 and IBV, or between different virus strains from the same subtype. Although it is often assumed that all influenza viruses follow the same mechanism to egress from infected cells, strains can differ in their capacity to grow in particular cell types (e.g. **Chapter 3, Figure 3.2**) and in the morphology of virions released from infected cells [239]. This can relate to differences in HA receptor specificity modulating attachment and entry at early stages of influenza virus infection (reviewed in [240]) but could also relate to differences in later steps in the virus replicative cycle, including egress of newly-synthesized infectious particles. Thus, it is possible that some strains could be more or less efficient in trafficking through the secretory pathway of infected cells. If this is the case, an encounter with MARCH8 in a different cellular compartment or at a different time during the viral replicative cycle could explain the differences in potency of inhibition observed between different strains.



As a first step towards defining how MARCH8 mediates anti-IAV activity, we assessed whether the E3 ligase activity of MARCH8 was required for its antiviral activity. Inducible cell lines expressing mutant versions of MARCH8 not able to interact with the E2-conjugating enzyme were generated. Specifically, a mutant with a misfolded RING-CH domain (MARCH8-CS) and a second mutant with a single mutation in the conserved tryptophan at the position 114, which is critical for the interaction of the E2-conjugating enzyme with the RING-CH domain of the E3 ligase (MARCH8-W114A) [199, 218]. While cells expressing wild-type MARCH8 effectively downregulated cell-surface CD86, cells expressing MARCH8-CS or MARCH8-W114A did not, validating the mutations generated (**Figure 4.4B**). Strikingly, cells expressing the MARCH8-CS or MARCH8-W114A variants did not restrict IAV (**Figure 4.4D**). This clearly demonstrates that the E3 ligase activity of MARCH8 is critical for its antiviral activity against IAV, confirming that ubiquitination was required for MARCH8-mediated inhibition of influenza virus replication. The addition of ubiquitin is not only a signal for internalization and degradation, ubiquitination can also serve as a platform for protein interactions as described for MARCH8 in the context of HCV infection. HCV is an enveloped virus requiring ESCRT machinery for its envelopment, however it does not contain the late motifs required for the recruitment of this machinery. Recently, it was shown that MARCH8 polyubiquitinates non-structural protein (NS)2 of HCV, and this ubiquitination is responsible for the recruitment of the ESCRT machinery, via an interaction with the protein HRS [212]. In light of these findings, it is also possible that MARCH8-mediated ubiquitination could be altering important interactions at the site of virus budding from influenza virus-infected cells.

To gain insights regarding the mechanism/s by which MARCH8 blocked the late stages of IAV infection, we have focussed on characterizing IAV-infected cells and the particles released from infected cells in the presence or absence of MARCH8. First, we used qPCR to detect viral genome (M segment) in supernatants from IAV-infected cells as an assay to quantify virus irrespective of particle infectivity. As seen in **Figure 4.5A**, the number of copies of the viral M segment did not change significantly when comparing supernatants from uninduced and DOX-induced cells expressing MARCH8, suggesting that the reduction in infectious titres was the result of a reduced infectivity per particle rather than an overall reduction in the number of particles released. These findings were similar to those reported for MARCH8-mediated inhibition of HIV-1 or pseudotyped particles expressing VSV-G [210], leading us to speculate that MARCH8-mediated restriction of IAV might occur by a similar mechanism.

Therefore, we next investigated if viral envelope glycoproteins were downregulated from the surface of IAV-infected cells in the presence of MARCH8. Cell-surface expression of IAV HA, NA and M2 proteins was determined at 8 hr p.i., an early time point when newly-synthesized viral proteins are transported to the surface of infected cells, but the release of nascent viral particles is negligible. In addition, we examined 24 hr p.i., a later timepoint when virus particles have been and continue to actively bud from the cell surface. To our surprise, neither HA, NA nor M2 showed reduced expression at the surface of virus-infected cells in the presence of MARCH8 (**Figure 4.6**). In fact, we observed a modest increase in the geometric mean fluorescence intensity (gMFI) of HA and NA, and a more pronounced increase in the gMFI of M2, at 24 hr p.i. (**Figure 4.6B**). Increased levels of cell-surface IAV HA, NA and M2 proteins, together with

the higher amounts of viral proteins present in whole-cell lysates of IAV-infected cells expressing MARCH8 (**Figure 4.7**), suggest that a defect in virus assembly and/or budding might result in an accumulation of viral proteins within infected cells as well as reduced incorporation of these viral proteins into nascent particles. Consistent with this, western blot analyses indicated that when normalized to viral NP expression, many structural proteins of the influenza virion (HA, NA, M1, M2) were expressed at reduced levels in particles released from cells in the presence MARCH8 (**Figure 4.7**). In addition to reduced infectivity, virions released in the presence of DOX-inducible MARCH8 show reduced ability to agglutinate erythrocytes (a function of the viral HA) and cleave MUNANA substrate (a function of the viral NA), despite no major deficit in levels of viral genome (qPCR for M segment) (**Figure 4.8**). Together, these data indicate that while MARCH8 expression results in downregulation of HIV-1 and VSV envelope glycoproteins from the surface of infected cells, it does not downregulate HA, NA or M2 from the surface of IAV-infected cells. Therefore, MARCH8 appears to inhibit IAV in a manner distinct from its reported mechanism of action against HIV-1 and VSV.

Western blot analyses proved useful in obtaining preliminary data regarding the protein content of virions released from IAV-infected cells in the presence or absence of DOX-inducible MARCH8. Our studies focussed on detecting viral proteins known to be relevant to the structural integrity of nascent virions. These included (i) the viral HA, which mediates receptor recognition as well as fusion between viral and host membranes, (ii) NA, which cleaves sialic acids from the surface of infected cells to facilitate release of nascent virions, (iii) M2, a proton channel that protects the HA from undergoing premature activation in the secretory pathway and mediates the ESCRT-

independent membrane scission from the infected cell, as well as facilitating dissociation of the viral ribonucleoproteins (vRNPs) from the M1 once the virus has been internalized into the endosomal compartment, and (iv) M1, which facilitates virus assembly and budding by acting like a bridge between HA/NA glycoproteins at the cell surface and the vRNPs, as well as contributing to the membrane deformation that is required for the budding process (reviewed in [18]). To facilitate comparison between virions released from uninduced and DOX-induced cells expressing MARCH8, we normalized our data to the viral NP, an internal protein of the virion. Our rationale for this approach was that despite major differences in virus infectivity, we did not see a significant difference in viral genome (as measured by qPCR for the M segment) in supernatants from infected cells. As NP is the RNA-binding protein responsible for encapsidation of the vRNA, it seemed reasonable to standardize against this protein when examining levels of viral proteins relevant to capsid structure, or the envelope glycoproteins of the virus. Based on this approach, reduced levels of HA, NA, M1 and M2 were detected in virions released from cells expressing MARCH8 which might impact the infectivity of these particles in many ways. For example, particles with reduced levels of HA and/or M2 would be predicted to be less efficient at (i) recognizing sialic acid-containing receptors on target cells to initiate subsequent rounds of viral infection, (ii) mediating dissociation of vRNPs from the matrix protein within the endosomal compartment following virus entry, and (iii) mediating fusion of viral and host membranes, to facilitate release of vRNPs into the cytoplasm for transport to the nucleus to initiate genomic replication.

Results from our western blot analyses can be also used to propose possible target(s) of MARCH8. Reduced amounts of viral HA, NA, M1 and M2 proteins in virions could indicate that these proteins were targeted and downregulated from the surface of infected cells, similar to the mechanism already proposed for MARCH8-mediated inhibition of HIV-1 and pseudotyped particles expressing VSV-G [210], although our data indicate this is not the case for the IAV HA, NA and M2 proteins. Therefore, reduced levels of HA, NA, M1 and M2 in virions released from cells in the presence of MARCH8 likely reflects a more complex scenario. M1 is a key protein during the assembly and budding processes. At the plasma membrane, M1 connects all the components of the virus, interacting with HA, NA and M2, as well as with the inner leaflet of the plasma membrane and with the vRNPs. Thus, a reduction in the amount of these four viral proteins in nascent virions could represent an altered budding process, such that the localization and/or the mechanism of virus budding is altered in the presence of MARCH8. Disruptions in amount and/or localization of M1 itself might also be sufficient to alter incorporation of other viral proteins, given its central role in assembly and budding. Of interest, western blot (using an anti-FLAG mAb) confirmed incorporation of MARCH8 into the newly-formed virions, strengthening the notion that the target of MARCH8 is a viral protein incorporated into the newly-synthesized virions. Our findings that expression of MARCH8-CS and MARCH8-W114A does not mediate restriction of IAV (**Figure 4.4**) also confirm the importance of a functional E3 ligase activity, ruling out the possibility that other features of induced MARCH8 expression (e.g. steric hinderance at the plasma membrane) contribute to restriction of IAV.

Given the alterations in protein composition of virions released from IAV-infected cells in the presence of inducible MARCH8, we next used transmission electron microscopy (TEM) to assess the morphology of virions purified from the supernatants of virus-infected cells. Budding of nascent virions from IAV-infected cells is a highly coordinated process that is dependent on the correct proportion and localization of different viral components, as well as cellular factors. Different IAV can show altered virion morphology following growth in the same cell type depending on viral determinants generally associated with the M segment [239, 241], while a particular IAV strain can also show major differences in morphology when grow in different cell types indicating the importance of cellular determinants, such as the actin microfilament network [242]. Thus, components of the virus and the host cell can impact virus assembly and/or budding, and therefore morphology of the virions released. Given that our western blot data suggested that virions (of the same IAV strain) released from 293T cells in the presence of MARCH8 showed an altered composition of viral proteins, we hypothesised that virus morphology might also be altered. Indeed, when we compared the morphology and size of virions released from uninduced and DOX-induced cells expressing MARCH8, these differences were very clear (**Figure 4.9**). Virions released from uninduced cells were spherical and homogeneous in size (~100-150 nm), whereas those released from MARCH8-induced cells were heterogeneous, including spherical viruses >100 nm and elongated viruses with a filamentous morphology. Of interest, a genetic reassortant IAV virus carrying a HA (from A/Udorn/72) and a NA (from A/WSN/33), both lacking their cytoplasmic tails, also showed a more heterogenous population, including filamentous virions, following growth in embryonated eggs [243]. This suggests that MARCH8-mediated interactions with the cytoplasmic tails of viral

HA/NA proteins could be one possibility underlying the altered virus morphology observed in our studies. Additionally, it is known that both M1 and M2 are important determinants contributing to IAV morphology. For example, replacement of the M segment of an IAV strain that forms spherical particles with the M segment from a filamentous IAV strain is sufficient for the reassortant virus to adopt a filamentous morphology, and vice versa (reviewed in [244]). Thus, it is also possible that differences in M1 and M2 could also contribute to the more filamentous morphology in virions released from cells expressing MARCH8. Whatever the case, the differences observed in virus morphology are consistent with our findings that the composition of viral proteins is altered in virions released from infected cells in the presence of MARCH8. Furthermore, these findings implicate a role for MARCH8 in modulating assembly and/or budding of IAV from infected cells.

MARCH8 transfers ubiquitin to lysine residues in the cytoplasmic tails (CT) of its protein targets. Our data implicated alterations in the expression levels of HA, NA, M1 and M2 proteins in virions released from IAV-infected cells in the presence of MARCH8, as well as suggesting that MARCH8 could potentially modulate IAV assembly and/or budding in infected cells. The CT of the viral transmembrane proteins HA and NA contain a single lysine residue and the CT of the M2 protein contains 3 lysine residues. M1 is not a transmembrane protein but it does contain 8 lysine residues in its N-terminal domain, which is also known to interact with the plasma membrane of infected cells. Thus, we used reverse genetics to generate recombinant viruses where lysine residues were exchanged to arginine residues in the CT of HA, NA and M2 and in M1 alone, and, in some cases, in combination. When cells expressing MARCH8 were then infected with

these recombinant viruses, those viruses carrying mutations in the CT of HA, NA and M2 were restricted to similar levels to that of the recombinant wild-type virus, confirming that these IAV proteins were not the major targets of MARCH8 (**Figure 4.10B**). Although all viruses carrying individual mutations in M1 were inhibited in the presence of MARCH8 (**Figure 4.11B**), we cannot rule out the possibility that M1 is a target of MARCH8, because we were unable to recover recombinant viruses carrying one particular mutation (K21R) (**Figure 4.11A**). Moreover, we did not generate viruses with mutations to exchange multiple lysine residues in the M1, so we cannot not exclude the possibility that there is some redundancy in lysine residues in M1 to serve as targets for MARCH8. Our mutations aimed to generate viruses where lysine residues were exchanged for arginine residues as we reasoned that this conservative change was less like to impact the structure of the mutant proteins, although in future studies there may be some merit in exchanging to alanine residues, as is often done in studies to map amino acid residues of interest.

Ubiquitination of particular residues in the M1 protein has been shown to be important for the replication of IAV, including residues that were targeted in our study. For example, it has been proposed that ubiquitination of M1 at residues K102 and K104 is important for the cellular localization of M1, and therefore for the assembly and budding of IAV [245]. While we recovered a recombinant virus with the K102R substitution, its viral titre in uninduced cells was reduced markedly compared to the wild-type and to other M1 mutants (**Figure 4.11B**). Of note, recombinant viruses carrying mutations and therefore K-to-R amino acid substitutions in the C-terminal region of M1 showed increased sensitivity to MARCH8-mediated restriction. Hence, it is



possible that ubiquitination in the C-terminal region of M1 is also important for the proper assembly of IAV. Consistent with this, it has been shown that SUMOylation, a post-translational modification that binds Small Ubiquitin-like Modifier (SUMO) proteins to lysine residues in target proteins, is important for the morphogenesis of newly-synthesized viruses. More specifically, SUMOylation of M1 at K242, localized at the C-terminal domain, is required for the formation of the M1-vRNP complex [246]. Currently, it is not clear why MARCH8 was more effective at restricting particular mutants with K-to-R substitutions in the C-terminal of M1, however these findings reinforce the idea that the antiviral activity of MARCH8 is linked with modulation of assembly and/or budding during the late stages of IAV replication.



**Generation and characterization of  
MARCH1/MARCH8 chimeric proteins to  
identify domains of MARCH8 required for  
antiviral activity against IAV.**

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## 5.1. Introduction

E3 ubiquitin ligases are critical for the specificity of the ubiquitination process. This specificity is conferred by their ability to interact with target substrates. It is generally accepted that MARCH proteins utilize their transmembrane domains (TMs) to interact with specific targets [182, 235], however some studies have shown the importance of other MARCH protein domains in determining target specificity, such as the cytoplasmic tails (CTs) and the connecting loop between the transmembrane domains [199, 218]. The use of chimeric mutants is one approach that can be used to define important domains essential for specific interactions with a target protein. This can involve switching protein domains between closely related MARCH proteins, where one has been identified as having a functional target specificity and the other has not. For example, MARCH1/MARCH9 chimeric proteins were generated to map the functional domains of MARCH1 responsible for its interaction with major histocompatibility complex II (MHC-II). In this case, portions of MARCH9, which does not downregulate MHC-II, were introduced into MARCH1 and the ability of the chimeric mutants to target MHC-II was evaluated [185].

MARCH proteins can be grouped based on conservation of their protein sequences. For example, MARCH1 and MARCH8 share a common domain organization and their sequence conservation is 65% across the entire protein, and up to 89% if only the RING-TM1/2 segments are compared. While other MARCH proteins can also be grouped based on sequence similarity/identity (e.g. MARCH2/3), the MARCH1/8 pair have the highest sequence conservation amongst this family (reviewed in [181]). In fact, if both MARCH1 and MARCH8 proteins are expressed in the same cell, they have overlapping

substrate recognition, being able to downregulate the same targets (e.g. transferrin receptor and CD86) [177].

MARCH8, but not MARCH1, was observed to be a potent restriction factor against IAV strains, such as Beij89 (Chapter 3 and 4). These findings suggest that MARCH1 and MARCH8 show clear differences in their ability to target at least some strains of IAV. Given that MARCH1/8 share many different substrates (e.g. transferrin receptor, CD86, MHC-II ) and few substrates have been identified that are recognized only by MARCH8 (e.g. CD81), it was of interest to determine if we could identify specific domains and/or residues of MARCH8 that determine its antiviral activity against IAV.

In this chapter we designed several MARCH chimeric mutants by introducing single or multiple domains of MARCH1 into the MARCH8 “backbone”. Then, using the same approach as outlined in chapter 3, we generated lentiviruses to produce stable 293T cell lines with DOX-inducible expression of each chimeric MARCH protein. Given that both MARCH8 and MARCH1 downregulate CD86 from the cell-surface, we evaluated functionality of each chimeric proteins by assessing their ability to modulate CD86 surface expression. We then assessed the susceptibility of cells with inducible expression of chimeric MARCH proteins to early stages of IAV infection (by monitoring expression of intracellular NP-positive cells at 8 hr p.i.), as well as late stages of infection (by measuring titres of infectious virus in supernatants from infected cells). All MARCH1/8 chimeric mutants retained ability to downregulate CD86 expression (confirming proper folding and localization of chimeric proteins), however introduction of single or multiple domains of MARCH1 into MARCH8 did not completely abrogate the IAV-antiviral activity of MARCH8.

## **5.2. Results.**

### **5.2.1. Design of MARCH1/8 chimeric mutants.**

As a first step towards design of the chimeric MARCH proteins, a sequence alignment using Clustal Omega was generated (**Figure 5.1A**). Specific domains of MARCH1/8 proteins were assigned based on information obtained from The Uniprot Consortium [247], and topology and domain organisation are shown in **Figure 5.1B**. Following alignment, it was evident that some domains differed by a single residue (e.g. the transmembrane (TM) domains) while others, such as the cytoplasmic tails (CT), showed a much higher degree of variability. Thus, some chimeric mutants were made using site-directed mutagenesis whereas others were synthesised as a geneblock. Similar to MARCH8 wild-type (WT), a FLAG tag was added at the N-terminal end of each chimeric protein to monitor intracellular expression.

### **5.2.2. Chimeric mutants generated by swapping single domains of MARCH1 into MARCH8.**

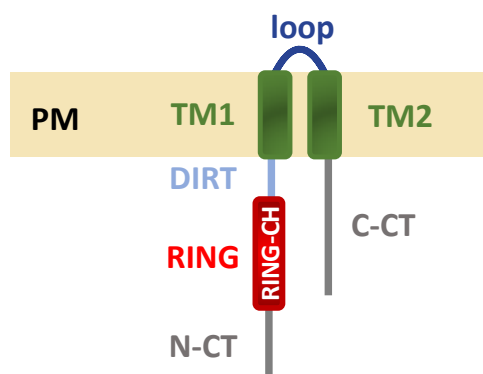
*5.2.2a. Investigating the role of the C-terminal CT in determining the differential ability of MARCH1/8 to mediate anti-IAV activity.*

One of the best characterized proteins targeted by MARCH8 is the transferrin receptor (TfR). Previous studies using deletion mutants have shown that a conserved region in the C-terminal CT of MARCH8 was required for downregulation of cell-surface TfR. Despite correct localization and interaction with TfR, a mutant version of MARCH8

**A.**

[illegible]

**B.**



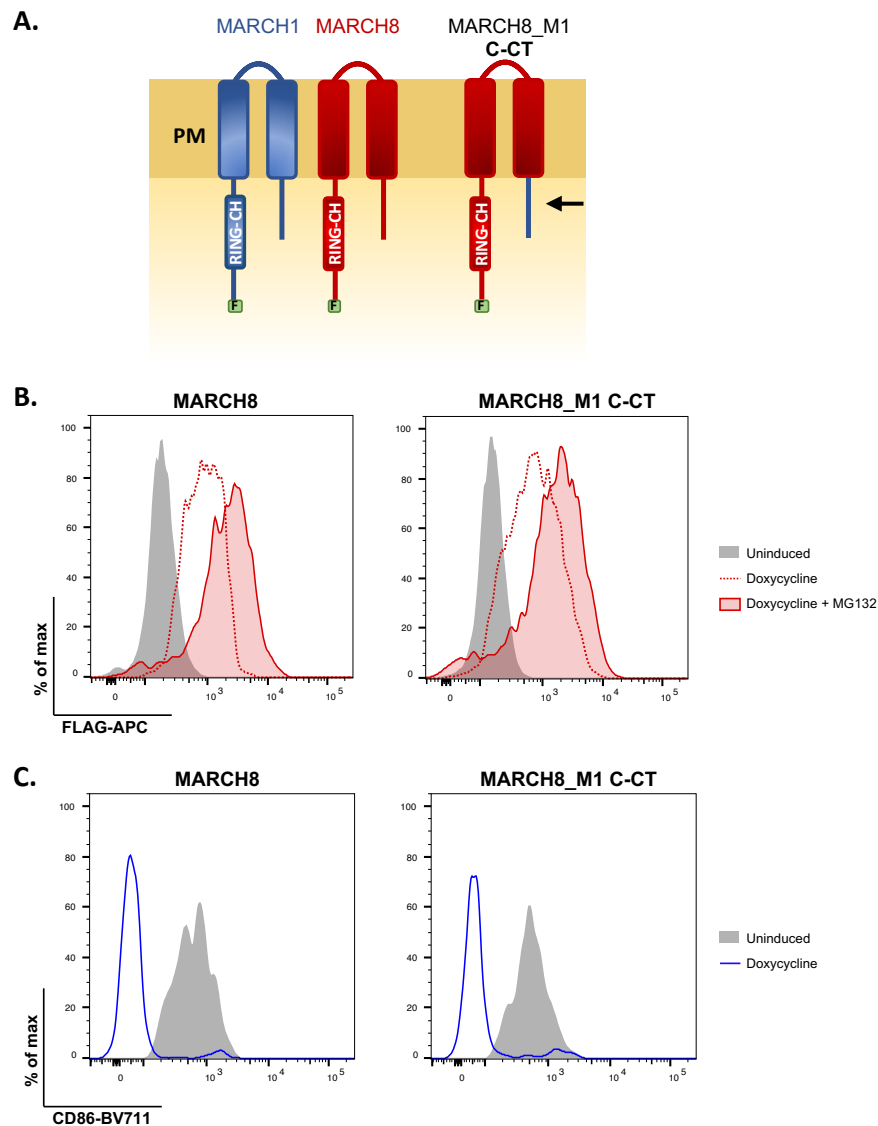
**Figure 5.1. Sequence analysis of MARCH1 and MARCH8 proteins.** (A) Sequence alignment of human MARCH1 and MARCH8 proteins performed using Clustal Omega. N- and C-terminal cytoplasmic tails (N-CT/C-CT) are shaded grey; the RING-CH domain of each protein (RING) is shaded red; the domain in between the RING-CH and the first TM domain (DIRT) are shaded light blue; transmembrane domains (TM1/2) are shaded green and the extraluminal connecting loop between the transmembrane domains of MARCH1/8 (loop) is shaded dark blue. (B) Schematic representation of the predicted topologies of MARCH1/8. Regions are highlighted in different colours corresponding to colours described in (A). Topology is shown relative to the cellular plasma membrane (PM).

lacking 6 conserved amino acids (YVQNCP) in its C-terminal CT failed to ubiquitinate and therefore downregulate TfR [218].

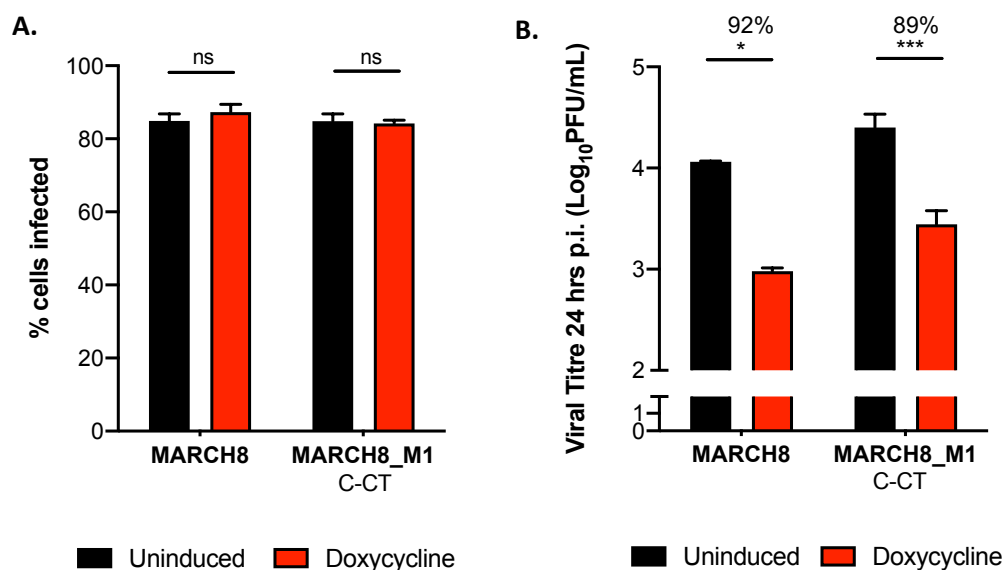
To investigate the importance of the C-terminal CT of MARCH8 during IAV infection, 293T cells were transduced and sorted to generate stable cells lines expressing DOX-inducible MARCH8 with the CT of MARCH1 (MARCH8\_M1 C-CT, **Figure 5.2A**). After induction of MARCH8\_M1 C-CT expression, flow cytometry confirmed that the levels of chimeric protein were similar to those observed for MARCH8 WT (**Figure 5.2B**). As both MARCH1 and MARCH8 can downregulate CD86 expression, we reasoned that any functional chimeric protein generated should also retain this activity. As expected, both MARCH8 WT and MARCH8\_M1 C-CT reduced CD86 expression on the surface of 293T cells (**Figure 5.2C**).

Considering that cell lines expressing MARCH8 WT and MARCH8\_M1 C-CT were generated independently, we next examined susceptibility of both cell lines to IAV infection. In these experiments, uninduced and DOX-induced cell lines were infected with IAV strain Beij89 at MOI 2 PFU/cell and intracellular NP expression was determined at 8 hr p.i. by flow cytometry. As seen in **Figure 5.3A**, both cell lines were equally susceptible to IAV infection. No differences in the geometric mean fluorescent intensity (gMFI) of NP-positive cells were observed between uninduced and DOX-induced conditions (data not shown). To evaluate the role of the C-terminal CT of MARCH8 in its antiviral activity against IAV, cell lines were infected with Beij89 at MOI 5 and, at 24 hr p.i., titres of infectious virus in the supernatant of infected cells were determined. As seen in **Figure 5.3B**, cells expressing MARCH8\_M1 C-CT retained their ability to reduce titres of infectious virus released from infected cells. Thus, introduction of the C-CT from





**Figure 5.2. Generation of a chimeric MARCH8 protein expressing the C-terminal CT of MARCH1.** (A) Schematic representation of the chimeric protein. Domains from MARCH1 and MARCH8 are shown in blue and red, respectively. The FLAG tag is shown in green. Topology is shown relative to the cellular plasma membrane (PM). (B) 293T cells with DOX-inducible expression of a chimeric MARCH8 protein that contains the C-terminal cytoplasmic tail of MARCH1 (MARCH8\_M1 C-CT) were cultured in media alone (grey, filled) or in media supplemented with 1  $\mu$ g/mL DOX (red, dotted line). At 20 hr post-DOX treatment, 10  $\mu$ M of MG132 was added to media to limit protein degradation (red, filled). At 24 hr post-DOX treatment, cells were washed, fixed, permeabilised and stained with anti-FLAG-APC mAb. Intracellular FLAG expression was determined by flow cytometry. Representative histograms are shown. 293T cells with DOX-inducible expression of MARCH8 wild-type (WT) were used as control. (C) 293T cells with DOX-inducible expression of MARCH8 and MARCH8\_M1 C-CT proteins were transfected with a plasmid encoding CD86 and the fluorescent protein ZsGreen. At 12 hr post-transfection, cells were left untreated (grey, filled) or treated with 1  $\mu$ g/mL DOX (blue, empty). At 24 hr post-DOX treatment, cells were washed and stained with anti-CD86-BV711 mAb. Levels of cell-surface CD86 on ZsGreen-positive transfected cells were analyzed by flow cytometry. Representative histograms of CD86 expression are shown.



**Figure 5.3. Impact of the C-terminal CT of MARCH1 on MARCH8-mediated restriction of IAV infection.**

**(A)** 293T cells with DOX-inducible expression of MARCH8 WT and MARCH8\_M1 C-CT proteins cultured for 24 hr in media (black bars) or media supplemented with 1 µg/mL DOX (red bars) were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI = 2 PFU/cell. After washing, cells were cultured an additional 7 hr and then washed, fixed, permeabilized and stained with NP-FITC mAb. The percentage of IAV-infected NP-positive cells was determined by flow cytometry. The mean of triplicate samples (± SD) are shown. Data are representative of 2 independent experiments. **(B)** 293T cells with DOX-inducible expression of MARCH8 WT and MARCH8\_M1 C-CT proteins cultured for 24 hr in the absence (Uninduced, black bars) or presence of 1 µg/mL DOX (Doxycycline, red bars) were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI = 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr p.i., supernatants were harvested and clarified, and titres of infectious virus were determined by plaque assay using MDCK cells. Mean virus titres from triplicate samples (± SD) are shown. Limit of detection is 0.85 Log<sub>10</sub>PFU/mL. Data are representative of 2 independent experiments. The % reduction in virus titres from uninduced versus DOX-induced conditions is shown for each cell line. A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions in **(A)** and **(B)**. \* = p<0.05, \*\* = p<0.01, \*\*\* = p<0.001, \*\*\*\* = p<0.0001, ns = not significant.

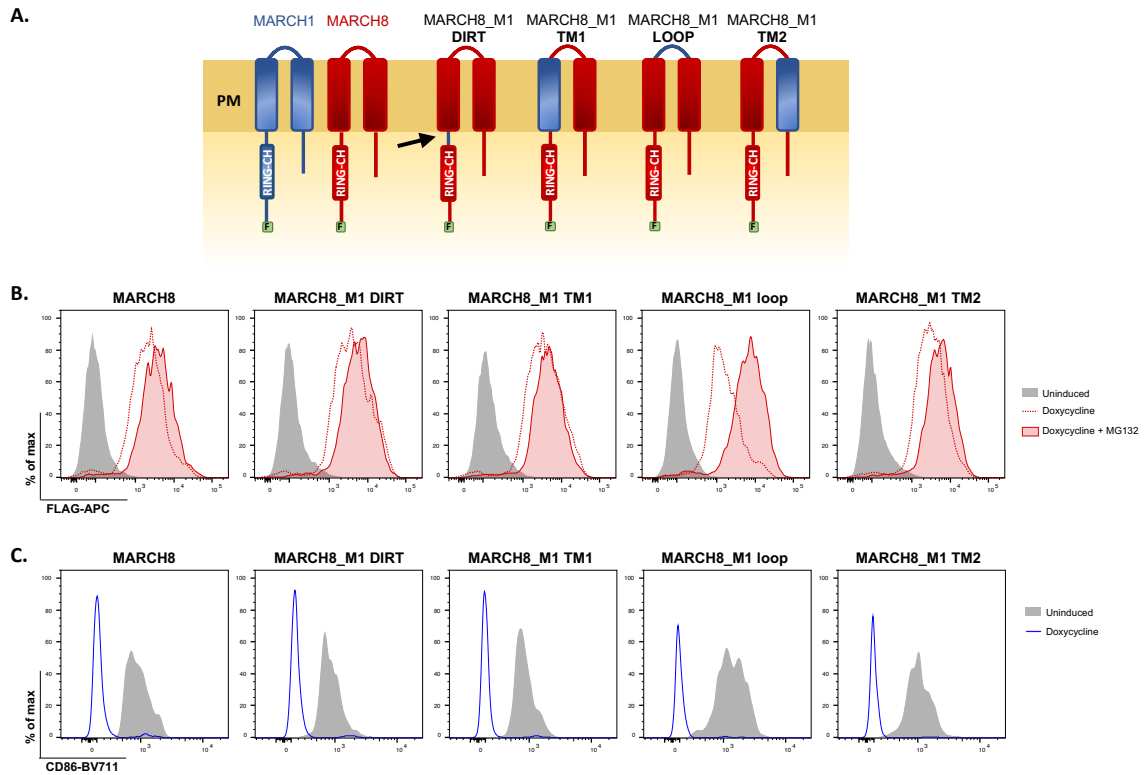
MARCH1 into MARCH8 generated a functional protein that retained ability to downregulate CD86 expression and also inhibited late stages in the IAV replication cycle. This suggests that the MARCH8 C-CT domain is not a key domain responsible for the differential ability of MARCH8, but not MARCH1, to inhibit late stages of IAV replication.

*5.2.2b. Investigating the role of the DIRT, TMs and luminal connecting loop in determining the differential ability of MARCH1/8 to mediate anti-IAV activity.*

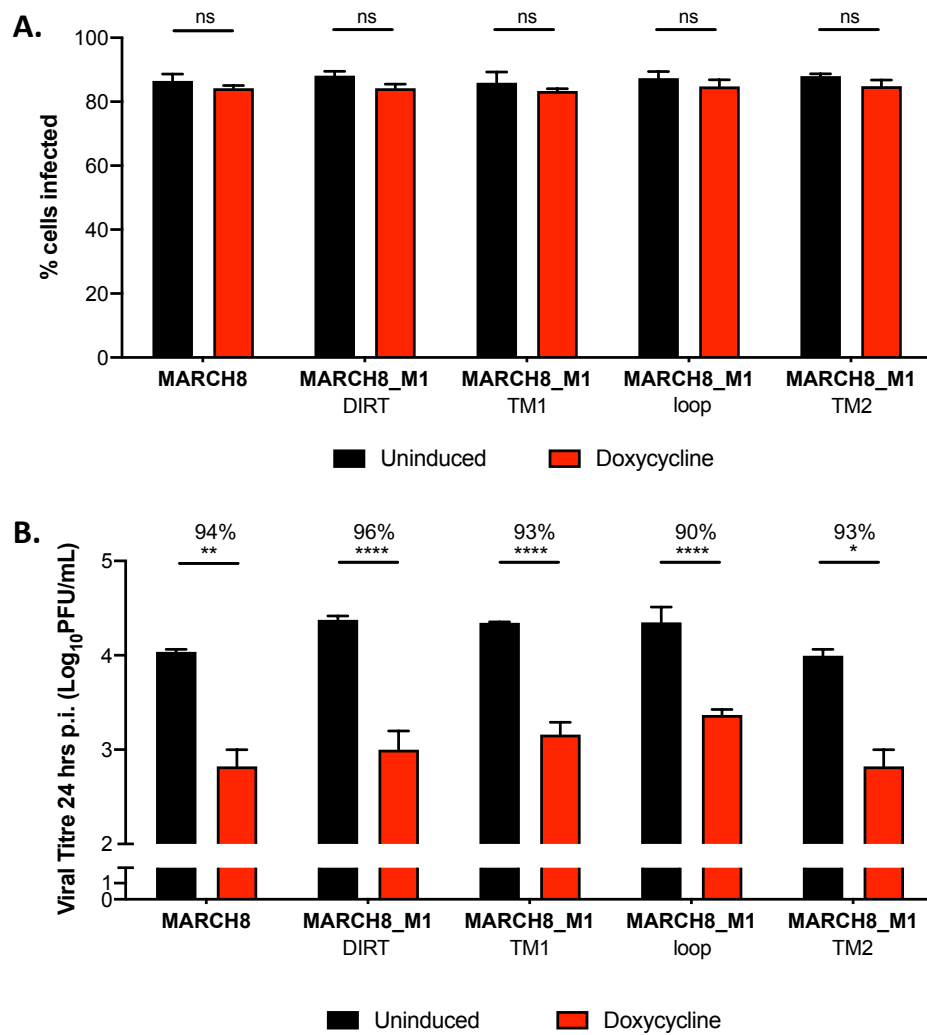
The segment between the RING-CH domain and the second transmembrane (TM2) of MARCH1 and MARCH8 (see **Figure 5.1A/B**) share a high percentage of sequence conservation (reviewed in [181]). Beside the RING-CH and the TMs, other domains among this segment have been involved in the functionality of different MARCH proteins, namely the domain in between the RING-CH and the first transmembrane (DIRT) and the luminal loop that connects the two TMs. Using chimeric mutants (mK3/KSHV-K5), it has been shown that the DIRT of the viral MARCH protein mK3 is critical for its ability to target MHC-I, leading the authors to propose that this region may be involved in recruitment/binding of the E2 conjugating enzyme or in maintaining correct orientation of the RING-CH domain for substrate interaction [248]. Studies using human MARCH proteins have shown that the DIRT of MARCH9 is also important for its ability to downregulate MHC-I [185]. Multiple studies support the role of the TMs in E3 ligase substrate recognition. In murine studies using deletion mutants, interactions between CD86 and MARCH1 were largely dependent on the TM domains of MARCH1 [249]. Similarly, MARCH8 has been shown to recognize specific residues in the TM domains of target proteins, suggesting that TM-TM interactions likely occur [235]. In addition, the first TM and the connecting loop of MARCH8 were implicated as

determinants for functional interactions with the IL-1 receptor accessory protein (IL1RAP) [199]. Given the importance of DIRT, TMs and the luminal connecting loop in interactions between MARCH and target proteins, we generated chimeric mutants with a single domain of MARCH1 swapped into MARCH8 in an attempt to determine the key domain responsible for the differential ability of MARCH8, but not MARCH1, to inhibit late stage IAV replication.

As before, 293T cells with DOX-inducible expression of different chimeric MARCH proteins were generated (**Figure 5.4A**) and expression levels of MARCH8 WT and each chimeric mutant were evaluated by flow cytometry. As seen in **Figure 5.4B**, similar levels of MARCH proteins were observed after DOX induction as indicated by FLAG expression, except in cells expressing MARCH8 with the connecting loop of MARCH1 (MARCH8\_M1 loop). Interestingly, the expression levels of this chimeric protein were recovered when MG132 was added to avoid proteasomal degradation. MARCH8 WT and each of the chimeric proteins reduced cell-surface expression of CD86 to similar levels, confirming functionality of all chimeric proteins (**Figure 5.4C**). Next, we assessed whether the chimeric proteins modulated anti-IAV activity relative to that of MARCH8 WT. As seen in **Figure 5.5A**, cells expressing WT or chimeric MARCH8 proteins showed similar susceptibility of infection by IAV and no differences in the gMFI of NP-positive cells were observed (data not shown). Moreover, supernatants from uninduced and DOX-induced cells infected with IAV demonstrated that induction of MARCH8 WT or any of the chimeric proteins induced a similar reduction in the titres of infectious particles released from IAV-infected cells (**Figure 5.5B**).



**Figure 5.4. Generation of chimeric MARCH8 proteins expressing either the DIRT, TM1, loop or TM2 from MARCH1.** (A) Schematic representation of the chimeric proteins generated. Domains from MARCH1 and MARCH8 are shown in blue and red, respectively. The FLAG tag is shown in green. Topology is shown relative to the cellular plasma membrane (PM). (B) 293T cells transduced with lentivirus to generate cell lines with DOX-inducible expression of different chimeric MARCH8 proteins that contain either the DIRT, TM1, loop or TM2 of MARCH1 (MARCH8\_M1 DIRT, MARCH8\_M1 TM1, MARCH8\_M1 loop and MARCH8\_M1 TM2, respectively) were cultured in media alone (grey, filled) or in media supplemented with 1  $\mu$ g/mL DOX (red, dotted line). At 20 hr post-DOX treatment, 10  $\mu$ M of MG132 was added to media to limit protein degradation (red, filled). At 24 hr post-DOX treatment, cells were washed, fixed, permeabilized and stained with anti-FLAG-APC mAb. Intracellular FLAG expression was determined by flow cytometry. Representative histograms are shown. 293T cells with DOX-inducible expression of MARCH8 WT were used as control. (C) 293T cells with DOX-inducible expression of MARCH8, MARCH8\_M1 DIRT, MARCH8\_M1 TM1, MARCH8\_M1 loop and MARCH8\_M1 TM2 proteins were transfected with a plasmid encoding CD86 and the fluorescent protein ZsGreen. At 12 hr post-transfection, cells were left untreated (grey, filled) or treated with 1  $\mu$ g/mL DOX (blue, empty) to turn on expression of the different MARCH8 proteins. At 24 hr post-DOX treatment, cells were washed and stained with anti-CD86-BV711 mAb. Levels of cell-surface CD86 on ZsGreen-positive transfected cells were analyzed by flow cytometry. Representative histograms of CD86 expression are shown.

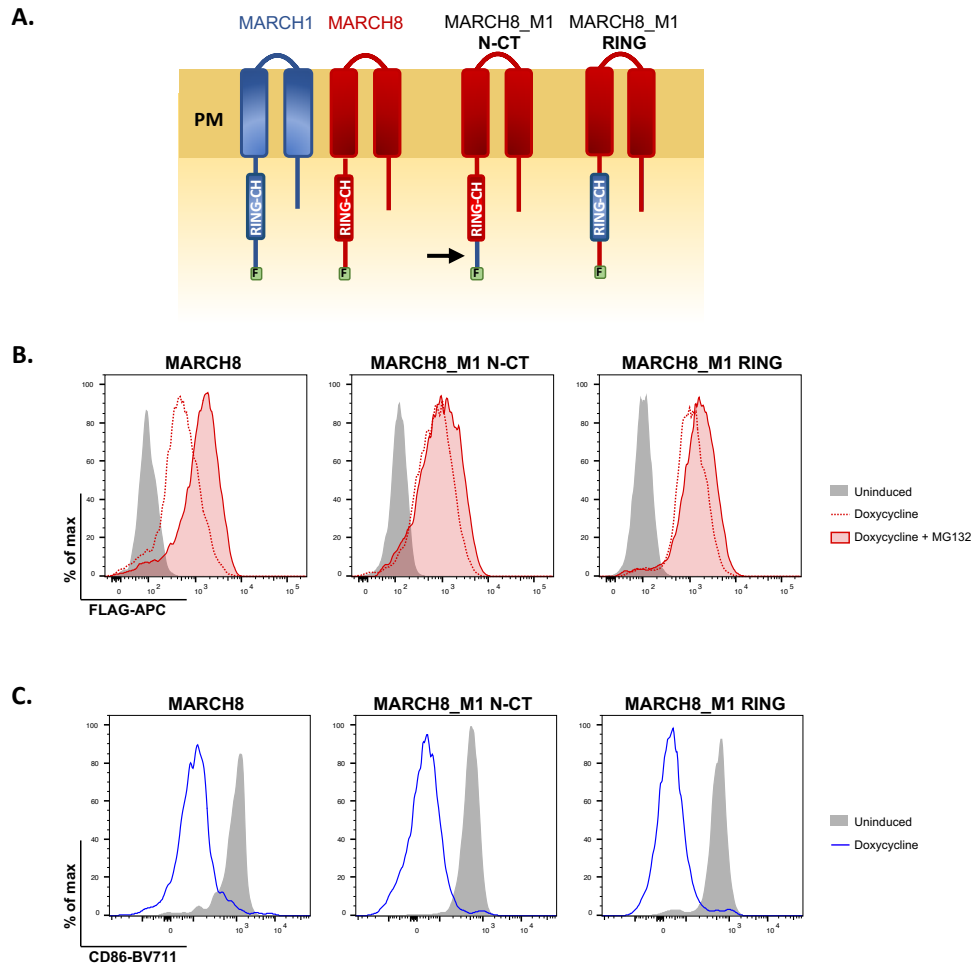


**Figure 5.5. Impact of the DIRT, TM1, loop and TM2 domains of MARCH1 on MARCH8-mediated restriction of IAV infection. (A)** 293T cells with DOX-inducible expression of MARCH8 WT, MARCH8\_M1 DIRT, MARCH8\_M1 TM1, MARCH8\_M1 loop and MARCH8\_M1 TM2 proteins cultured for 24 hr in media (black bars) or media supplemented with 1  $\mu$ g/mL DOX (red bars) were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI = 2 PFU/cell. After washing, cells were cultured an additional 7 hr and then washed, fixed, permeabilized and stained with NP-FITC mAb. The percentage of IAV-infected NP-positive cells was determined by flow cytometry. The mean of triplicate samples ( $\pm$  SD) are shown. Data are representative of 2 independent experiments. **(B)** 293T cells with DOX-inducible expression of MARCH8 WT, MARCH8\_M1 DIRT, MARCH8\_M1 TM1, MARCH8\_M1 loop and MARCH8\_M1 TM2 proteins cultured for 24 hr in the absence (Uninduced, black bars) or presence of 1  $\mu$ g/mL DOX (Doxycycline, red bars) were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI = 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr p.i., supernatants were harvested and clarified, and titres of infectious virus in supernatants were determined by plaque assay using MDCK cells. Mean virus titres from triplicate samples ( $\pm$  SD) are shown. Limit of detection is 0.85 Log<sub>10</sub>PFU/mL. Data are representative of 2 independent experiments. The % reduction in virus titres from uninduced versus DOX-induced conditions is shown for each cell line. A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions in **(A)** and **(B)**. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

*5.2.2c. Investigating the role of the N-terminal CT and RING-CH domain in determining the differential ability of MARCH1/8 to mediate anti-IAV activity.*

Together with the C-terminal CT, the N-terminal CT is the most variable region between the MARCH1 and MARCH8 proteins. Studies using murine MARCH1 determined that the stability of MARCH1 is controlled by sequence elements present in its N-terminal region [226]. The RING-CH domain is the functional domain of the E3 ligases that allows for interactions with the E2 ubiquitin-conjugating enzymes. Interestingly, it has been postulated that differences in the RING domain could influence the recruitment and therefore interactions between E2 conjugating enzymes and E3 ligases (reviewed in [155]). Our studies to date investigating particular domains reported to modulate interactions between MARCH1/8 and their specific substrates (i.e. C-terminal CT, DIRT, TM1/2 and luminal connecting loop) have shown that introduction of single domains from MARCH1 into MARCH8 did not abrogate MARCH8-mediated restriction of IAV. Therefore, we next investigated if introduction of the N-terminal cytoplasmic tail or the RING domain of MARCH1 into MARCH8 could modulate IAV restriction.

Therefore, 293T cells with inducible expression of MARCH8 WT and MARCH8 containing the N-CT of MARCH1 (MARCH8\_M1 N-CT) or the RING-CH of MARCH1 (MARCH8\_M1 RING) were generated. After DOX induction, MARCH expression was evaluated by measuring levels of intracellular FLAG by flow cytometry. As seen in **Figure 5.6B**, both mutants were expressed at higher levels compared to MARCH8 WT although addition of MG132 to limit proteasomal degradation resulted in comparable expression levels of all three proteins. Despite any differences in expression levels, all MARCH proteins

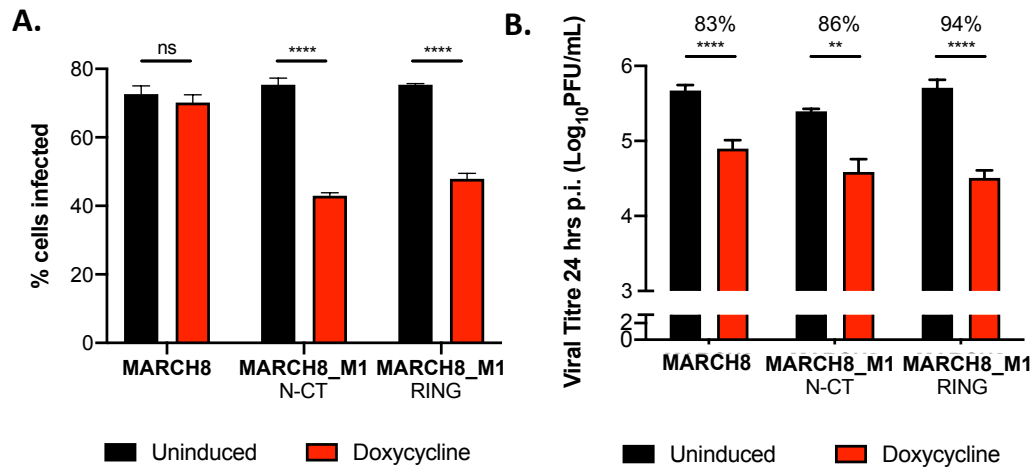


**Figure 5.6. Generation of chimeric MARCH8 proteins expressing either the N-terminal CT or RING-CH domain of MARCH1.** (A) Schematic representation of the chimeric proteins generated. Domains from MARCH1 and MARCH8 are shown in blue and red, respectively. The FLAG tag is shown in green. Topology is shown relative to the cellular plasma membrane (PM). (B) 293T cells transduced with lentivirus to generate cell lines with DOX-inducible expression of different chimeric MARCH8 proteins that contain either the N-terminal CT or RING-CH of MARCH1 (MARCH8\_M1 N-CT and MARCH8\_M1 RING, respectively) were cultured in media alone (grey, filled) or in media supplemented with 1  $\mu$ g/mL DOX (red, dotted line). At 20 hr post-DOX treatment, 10  $\mu$ M of MG132 was added to media to limit protein degradation (red, filled). At 24 hr post-DOX treatment, cells were washed, fixed, permeabilized and stained with anti-FLAG-APC mAb. Intracellular FLAG expression was determined by flow cytometry. Representative histograms are shown. 293T cells with DOX-inducible expression of MARCH8 WT were used as control. (C) 293T cells with DOX-inducible expression of MARCH8, MARCH8\_M1 N-CT and MARCH8\_M1 RING proteins were transfected with a plasmid encoding CD86 and the fluorescent protein ZsGreen. At 12 hr post-transfection, cells were left untreated (grey, filled) or treated with 1  $\mu$ g/mL DOX (blue, empty) to turn on expression of the different MARCH8 proteins. At 24 hr post-DOX treatment, cells were washed and stained with anti-CD86-BV711 mAb. Levels of cell-surface CD86 on ZsGreen-positive transfected cells were analyzed by flow cytometry. Representative histograms of CD86 expression are shown.



downregulated cell-surface CD86, verifying that their functional capacity was maintained (**Figure 5.6C**).

To assess susceptibility of the different cell lines to infection by IAV Bej89, we measured intracellular NP expression at 8 hr p.i. using flow cytometry. To our surprise, while DOX-inducible expression of MARCH8 WT did not alter the percentage of NP-positive virus-infected cells, expression of either MARCH8\_M1 N-CT or MARCH8\_M1 RING reduced the percentage of infected cells from ~75% (uninduced) to ~45% (DOX-induced) (**Figure 5.7A**). Interestingly, the gMFI of NP-positive cells was higher for both chimeric proteins following DOX induction, when compared with the uninduced condition (data not shown). To investigate if the expression of these chimeric proteins modulated the late stages of IAV replication, cell supernatants collected from uninduced and DOX-induced cells 24 hr after infection were titrated by plaque assay on MDCK cells. Note that the MOI used in the experiments performed to evaluate the late stages of infection is 5 PFU/cell to ensure high levels of infection. This is particularly important in this experiment, given the differences in the percentage of infected cells observed in **Figure 5.7A** using a MOI of 2 PFU/cell. As seen in **figure 5.7B**, expression of either chimeric protein resulted in a significant reduction in the viral titres of infectious particles released from infected cells. Of interest, MARCH8 WT and MARCH8\_M1 RING showed a similar potency of restriction, whereas restriction exerted by MARCH8\_M1 N-CT was less potent, although still significant.

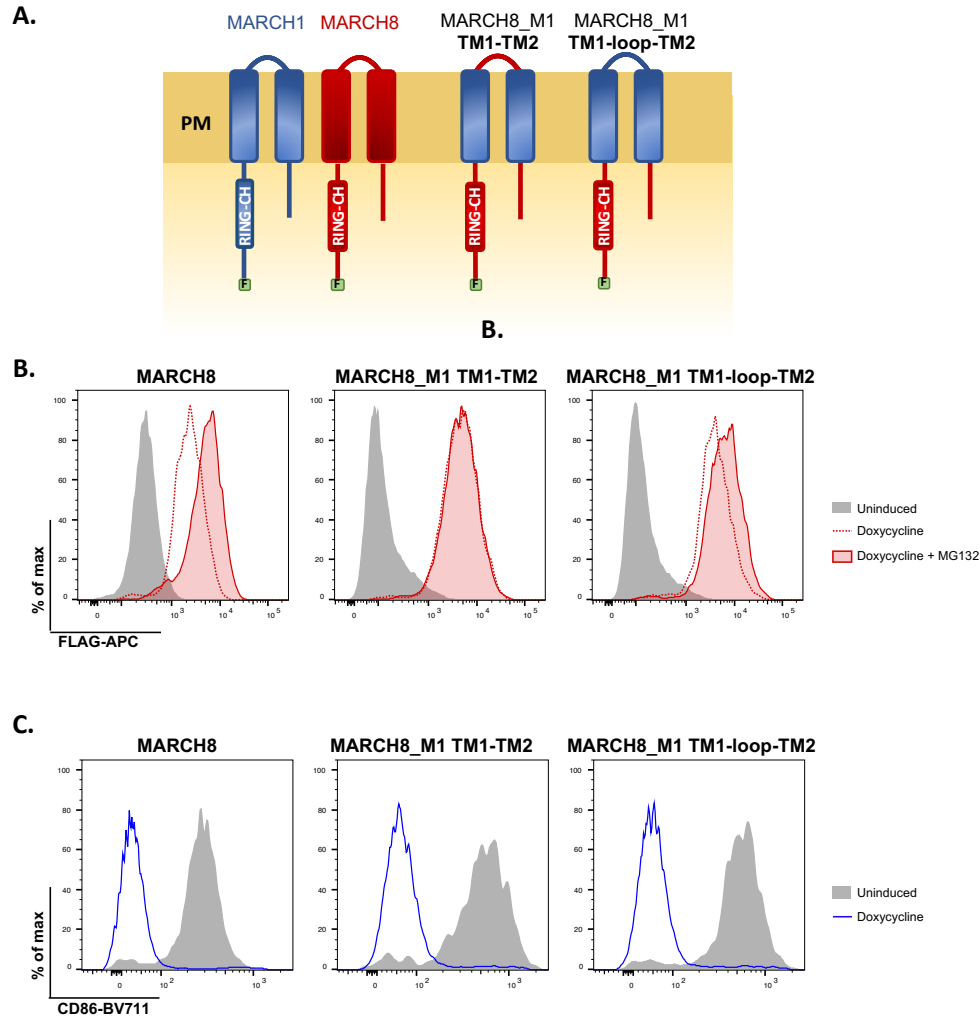


**Figure 5.7. Impact of the N-terminal CT and RING-CH domains of MARCH1 on MARCH8-mediated restriction of IAV infection.** (A) 293T cells with DOX-inducible expression of MARCH8 WT, MARCH8\_M1 N-CT and MARCH8\_M1 RING proteins cultured for 24 hr in media (black bars) or media supplemented with 1 µg/mL DOX (red bars) were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI = 2 PFU/cell. After washing, cells were cultured an additional 7 hr and then washed, fixed, permeabilized and stained with NP-FITC mAb. The percentage of IAV-infected NP-positive cells was determined by flow cytometry. The mean of triplicate samples (± SD) are shown. Data are representative of 2 independent experiments. (B) 293T cells with DOX-inducible expression of MARCH8 WT, MARCH8\_M1 N-CT and MARCH8\_M1 RING proteins cultured for 24 hr in the absence (Uninduced, black bars) or presence of 1 µg/mL DOX (Doxycycline, red bars) were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI = 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr p.i., supernatants were harvested and clarified, and titres of infectious virus in supernatants were determined by plaque assay using MDCK cells. Mean virus titres from triplicate samples (± SD) are shown. Limit of detection is 0.85 Log<sub>10</sub> PFU/mL. Data are representative of 2 independent experiments. The % reduction in virus titres from uninduced versus DOX-induced conditions is shown for each cell line. A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions in (A) and (B). \* = p<0.05, \*\* = p<0.01, \*\*\* = p<0.001, \*\*\*\* = p<0.0001, ns = not significant.

### 5.2.3. Chimeric mutants generated by swapping multiple domains of MARCH1 into MARCH8.

Swapping single domains of MARCH1 into a MARCH8 “backbone” did not identify a specific domain of MARCH1 that could abrogate the antiviral activity of MARCH8 against IAV, suggesting that a more complex interplay between MARCH8 and IAV is responsible for its antiviral activity. Several studies have shown or proposed that E3 ligases not only recognise specific targets directly, but may also interact with adaptor proteins which, in turn, interact with target proteins. For example, the “proximity model” has been proposed to explain how the viral E3 ligase mK3 mediates downregulation of MHC-I, whereby mK3 instead of interacting directly with the substrate, forms a complex with the host proteins TAP and Tpn through its C-terminal domain, promoting the correct orientation of the MHC-I molecule. Thus, multiple regions of mK3 are required for the formation of the complex between mK3, the E2 ubiquitin-conjugating enzyme and TAP/Tpn/MHC-I to facilitate the polyubiquitination and downregulation of MHC-I from the cell-surface [248].

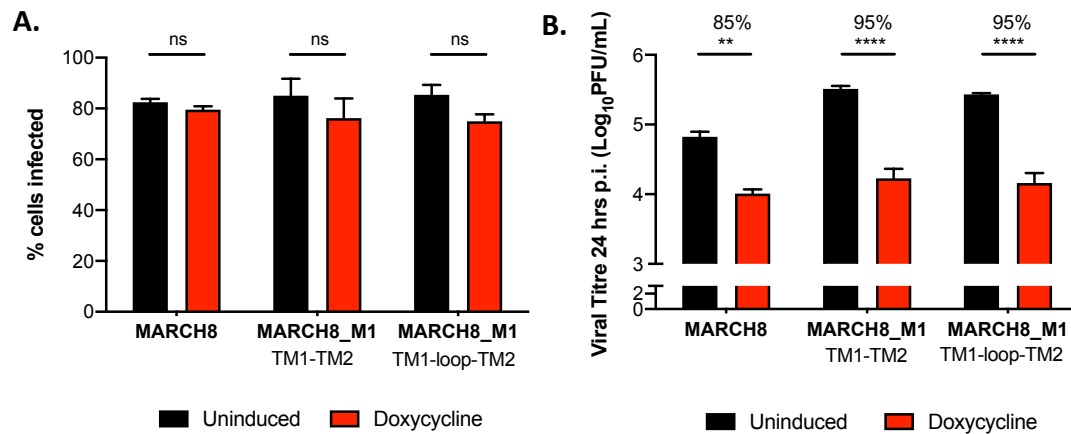
To explore the possibility that multiple domains of MARCH8 could be required for its antiviral activity, two additional chimeric proteins were generated; (i) MARCH8\_M1 TM1-TM2, where both TMs from MARCH1 replaced those from MARCH8, and (ii) MARCH8\_M1 TM1-loop-TM2, where, both TMs and the connecting loop from MARCH1 replaced those from MARCH8. As before, expression levels and functionality of each chimeric protein were determined relative to MARCH8 WT. As seen in **Figure 5.8B**, higher levels of FLAG expression were observed after DOX-induction of each chimeric protein compared to MARCH8 WT. Of note, only WT and MARCH8\_M1 TM1-loop-TM2



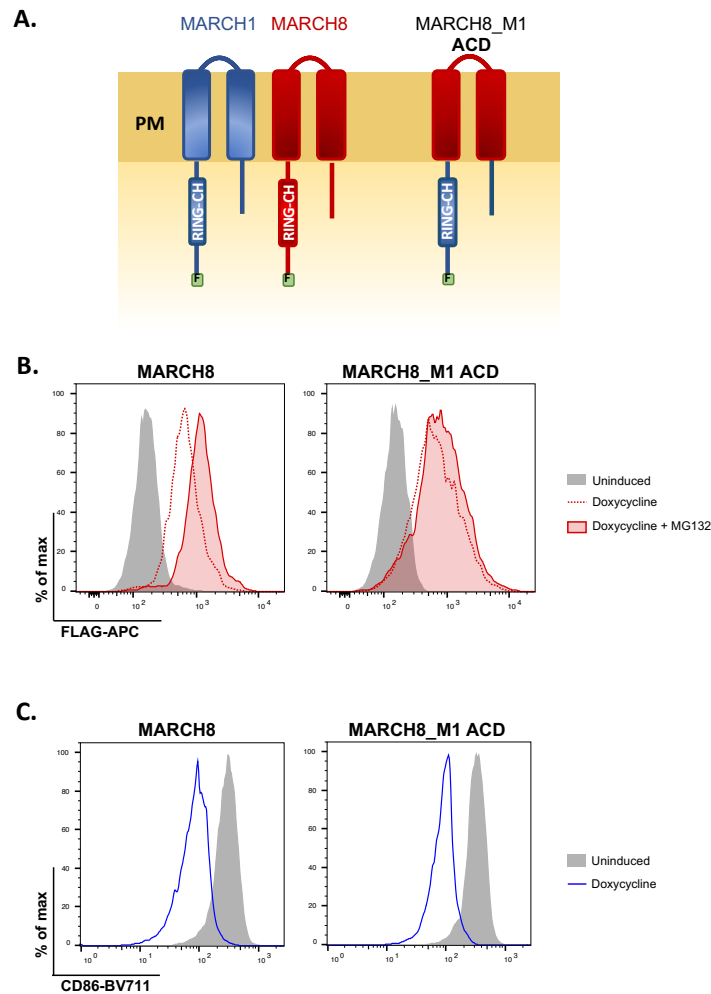
**Figure 5.8. Generation of chimeric MARCH8 proteins expressing either the TM1-TM2 or the TM1-loop-TM2 domains of MARCH1.** (A) Schematic representation of the chimeric proteins generated. Domains from MARCH1 and MARCH8 are shown in blue and red, respectively. The FLAG tag is shown in green. Topology is shown relative to the cellular plasma membrane (PM). (B) 293T cells transduced with lentivirus to generate cell lines with DOX-inducible expression of different chimeric MARCH8 proteins that contain either the TM1-TM2 or the TM1-loop-TM2 of MARCH1 (MARCH8\_M1 TM1-TM2 and MARCH8\_M1 TM1-loop-TM2, respectively) were cultured in media alone (grey, filled) or in media supplemented with 1  $\mu$ g/mL DOX (red, dotted line). At 20 hr post-DOX treatment, 10  $\mu$ M of MG132 was added to media to limit protein degradation (red, filled). At 24 hr post-DOX treatment, cells were washed, fixed, permeabilized and stained with anti-FLAG-APC mAb. Intracellular FLAG expression was determined by flow cytometry. Representative histograms are shown. 293T cells with DOX-inducible expression of MARCH8 WT were used as control. (C) 293T cells with DOX-inducible expression of MARCH8, MARCH8\_M1 TM1-TM2 and MARCH8\_M1 TM1-loop-TM2 proteins were transfected with a plasmid encoding CD86 and the fluorescent protein ZsGreen. At 12 hr post-transfection, cells were left untreated (grey, filled) or treated with 1  $\mu$ g/mL DOX (blue, empty) to turn on expression of the different MARCH8 proteins. At 24 hr post-DOX treatment, cells were washed and stained with anti-CD86-BV711 mAb. Levels of cell-surface CD86 on ZsGreen-positive transfected cells were analyzed by flow cytometry. Representative histograms of CD86 expression are shown.

showed increased expression following addition of MG132. When functionality of the DOX-inducible proteins was evaluated, both chimeric proteins downregulated CD86 from the cell surface to a similar extent as MARCH8 WT (**Figure 5.8C**). Next, we assessed the ability of the chimeric proteins to modulate IAV infection. As for MARCH8 WT, inducible expression of MARCH8\_M1 TM1-TM2 or MARCH8\_M1 TM1-loop-TM2 did not alter the percentage of NP-positive IAV-infected cells detected at 8 hr post-infection (**Figure 5.9A**) nor did it alter expression levels of intracellular NP, as determined by gMFI (data not shown). To determine if the chimeric proteins had lost the capacity to restrict late stages of IAV infection, uninduced and DOX-induced cells were infected with IAV Beij89 at MOI 5 and, 24 hr after infection, viral titres were determined in cell supernatants from virus-infected cells. As seen in **Figure 5.9B**, both MARCH8\_M1 TM1-TM2 and MARCH8\_M1 TM1-loop-TM2 restricted IAV replication efficiently, as seen by a reduction in viral titres compared to their respective uninduced control. Of interest, the restriction of IAV by each of the chimeric proteins was more potent than that exhibited by MARCH8 WT.

As a final attempt to identify critical domains of MARCH8 that distinguish it from MARCH1 in its ability to mediate antiviral activity against IAV, a chimeric mutant expressing all cytoplasmic domains of MARCH1 (i.e. N-CT, RING-CH, DIRT and C-CT, known as MARCH8\_M1 ACD) was generated. After DOX induction, expression levels of the chimeric protein were slightly higher than those of MARCH8 WT and a similar trend was observed when MG132 was added (**Figure 5.10B**). Induction of both WT and MARCH8\_M1 ACD resulted in downregulation of CD86, confirming functionality of the chimeric protein (**Figure 5.10C**). In line with what was observed when single domains



**Figure 5.9. Impact of the TM1-TM2 and the TM1-loop-TM2 domains of MARCH1 on MARCH8-mediated restriction of IAV infection.** **(A)** 293T cells with DOX-inducible expression of MARCH8 WT, MARCH8\_M1 TM1-TM2 and MARCH8\_M1 TM1-loop-TM2 proteins cultured for 24 hr in media (black bars) or media supplemented with 1  $\mu$ g/mL DOX (red bars) were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI = 2 PFU/cell. After washing, cells were cultured an additional 7 hr and then washed, fixed, permeabilized and stained with NP-FITC mAb. The percentage of IAV-infected NP-positive cells was determined by flow cytometry. The mean of triplicate samples ( $\pm$  SD) are shown. Data are representative of 2 independent experiments. **(B)** 293T cells with DOX-inducible expression of MARCH8 WT, MARCH8\_M1 TM1-TM2 and MARCH8\_M1 TM1-loop-TM2 proteins cultured for 24 hr in the absence (Uninduced, black bars) or presence of 1  $\mu$ g/mL DOX (Doxycycline, red bars) were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI = 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr p.i., supernatants were harvested and clarified, and titres of infectious virus in supernatants were determined by plaque assay using MDCK cells. Mean virus titres from triplicate samples ( $\pm$  SD) are shown. Limit of detection is 0.85 Log<sub>10</sub> PFU/mL. Data are representative of 2 independent experiments. The % reduction in virus titres from uninduced versus DOX-induced conditions is shown for each cell line. A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions in **(A)** and **(B)**. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.



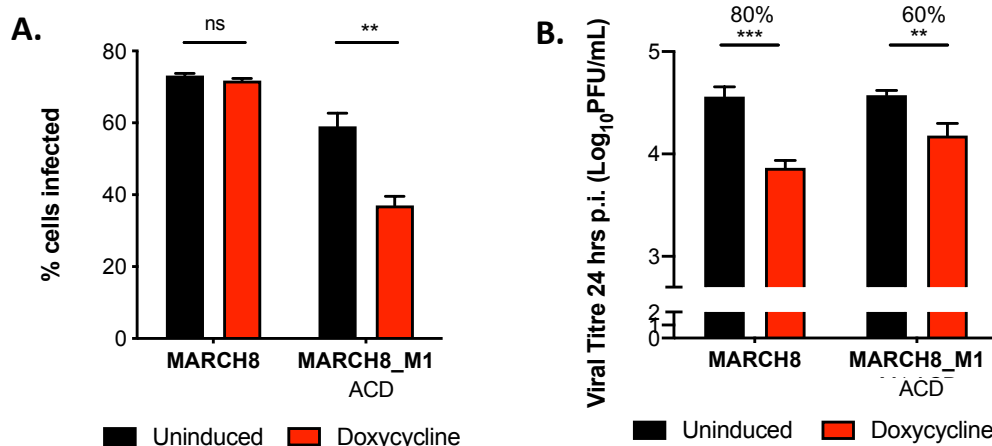
**Figure 5.10. Generation of chimeric MARCH8 proteins expressing All Cytoplasmic Domains of MARCH1.** (A) Schematic representation of the chimeric protein generated. Domains from MARCH1 and MARCH8 are shown in blue and red, respectively. The FLAG tag is shown in green. Topology is shown relative to the cellular plasma membrane (PM). (B) 293T cells transduced with lentivirus to generate cell lines with DOX-inducible expression of a chimeric MARCH8 protein containing all the cytoplasmic domains of MARCH1 (MARCH8\_M1 ACD) were cultured in media alone (grey, filled) or in media supplemented with 1  $\mu\text{g}/\text{mL}$  DOX (red, dotted line). At 20 hr post-DOX treatment, 10  $\mu\text{M}$  of MG132 was added to media to limit protein degradation (red, filled). At 24 hr post-DOX treatment, cells were washed, fixed, permeabilized and stained with anti-FLAG-APC mAb. Intracellular FLAG expression was determined by flow cytometry. Representative histograms are shown. 293T cells with DOX-inducible expression of MARCH8 WT were used as control. (C) 293T cells with DOX-inducible expression of MARCH8 and MARCH8\_M1 ACD proteins were transfected with a plasmid encoding CD86 and the fluorescent protein ZsGreen. At 12 hr post-transfection, cells were left untreated (grey, filled) or treated with 1  $\mu\text{g}/\text{mL}$  DOX (blue, empty) to turn on expression of the different MARCH8 proteins. At 24 hr post-DOX treatment, cells were washed and stained with anti-CD86-BV711 mAb. Levels of cell-surface CD86 on ZsGreen-positive transfected cells were analyzed by flow cytometry. Representative histograms of CD86 expression are shown.

were swapped (MARCH8\_M1 N-CT and MARCH8\_M1 RING, **Figure 5.7A**), cells with DOX-inducible expression of MARCH8\_M1 ACD were less susceptible to IAV infection, corroborating that expression of the N-CT and/or RING-CH domain of MARCH1 in MARCH8 appears to impact early events in IAV infection (**Figure 5.11A**). Moreover, the gMFI of NP-positive IAV-infected cells was higher after DOX-induction of MARCH8\_M1 ACD expression compared to uninduced conditions (data not shown). Finally, to evaluate the ability of MARCH8\_M1 ACD to restrict IAV infection, uninduced and DOX-induced cells were infected with IAV at MOI 5, and cell supernatants harvested 24 hr later were titrated on MDCK cells (**Figure 5.11B**). Although the reduction in viral titres of infectious particles released was less pronounced in supernatants from DOX-induced MARCH8\_M1 ACD compared to MARCH8 WT, it was still a significant reduction. Thus, these data indicate that introduction of the cytoplasmic domains of MARCH1 was not sufficient to abolish the antiviral effect of MARCH8 against IAV.

### 5.3. Discussion

E3 ubiquitin ligases confer specificity to the ubiquitination process by interacting with specific targets. Considering the diverse array of cellular processes regulated by ubiquitination, there is obvious potential to develop novel therapeutics that target the E3 ligase/substrate interaction. However, this is not an easy task. Although it has been found in many cases that E3 ligases can interact with specific motifs of the target protein directly, this is not necessarily the general mechanism of interaction. This is highlighted by the fact that the majority of the proteins targeted by the same E3 ligase do not share





**Figure 5.11. Impact of all cytoplasmic domains of MARCH1 on MARCH8-mediated restriction of IAV infection.** **(A)** 293T cells with DOX-inducible expression of MARCH8 WT, and MARCH8\_M1 ACD proteins cultured for 24 hr in media (black bars) or media supplemented with 1  $\mu$ g/mL DOX (red bars) were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI = 2 PFU/cell. After washing, cells were cultured an additional 7 hr and then washed, fixed, permeabilized and stained with NP-FITC mAb. The percentage of IAV-infected NP-positive cells was determined by flow cytometry. The mean of triplicate samples ( $\pm$  SD) are shown. Data are representative of 2 independent experiments. **(B)** 293T cells with DOX-inducible expression of MARCH8 WT and MARCH8\_M1 ACD proteins cultured for 24 hr in the absence (Uninduced, black bars) or presence of 1  $\mu$ g/mL DOX (Doxycycline, red bars) were washed and infected for 1 hr at 37°C with IAV strain A/Beij89 at MOI = 5 PFU/cell. Cells were then washed and cultured overnight. At 24 hr p.i., supernatants were harvested and clarified, and titres of infectious virus in supernatants were determined by plaque assay using MDCK cells. Mean virus titres from triplicate samples ( $\pm$  SD) are shown. Limit of detection is 0.85 Log<sub>10</sub>PFU/mL. Data are representative of 2 independent experiments. The % reduction in virus titres from uninduced versus DOX-induced conditions is shown for each cell line. A two-way ANOVA with Bonferroni's post-test analysis was performed to compare uninduced and DOX-induced conditions in **(A)** and **(B)**. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

a common sequence or motif that is recognised. Additionally, E3 ligases use different regions to interact with their specific substrates, including transmembrane regions and cytoplasmic tails, adding further complexity to the E3 ligase/substrate recognition process.

In previous chapters we found that MARCH8, but not MARCH1, was able to restrict IAV. That was of particular interest, especially because it is known that when both proteins are ectopically expressed in the same cell, they generally target the same substrates. Of note, exceptions have been reported as only MARCH8 recognized certain substrates such CD81 and CD44 [182].

Based on these findings, in this chapter we aimed to utilise the differential antiviral activity of MARCH1 compared to MARCH8 to identify the critical domain/s of MARCH8 required for its activity against influenza viruses. To do this, we generated a panel of MARCH1-MARCH8 chimeric mutants to screen for proteins that retained functional activity against CD86 (a property shared by MARCH1 and MARCH8) but had lost the ability to block late stages of IAV infection. Our initial approach was to swap individual domains of MARCH1 into the MARCH8 “backbone”. First, we generated a mutant version of MARCH8 expressing the C-terminal CT of MARCH1. Interestingly, if protein sequences of these regions are compared, many residues are conserved, especially closer to the TM, including the residues identified previously as responsible for the downregulation of TfR [218]. To generate a mutant version of MARCH8 expressing the second TM of MARCH1, all that was required was the introduction of a single conservative substitution (L213V, both non-polar residues) into MARCH8. To generate a MARCH8 mutant expressing the connecting loop from MARCH1, mutations were

introduced which gave rise to 4 amino acid substitutions (Q188N, A189D, T190N and I192V). Most of these substitutions were between residues with similar properties, except for the exchange of alanine (A), a small non-polar residue, to aspartic acid (D), a negatively charged amino acid. To generate the chimeric protein carrying the first TM from MARCH1, another conservative substitution (M156F, both highly hydrophobic), was introduced into the MARCH8 backbone. To swap the DIRT domain of MARCH8 to MARCH1, a conservative substitution (S149T, both polar residues containing a hydroxyl group) was introduced into MARCH8. To swap the RING domain of MARCH8 to MARCH1, 10 amino acid substitutions were required, most of which were conservative, except for T-to-C (polar to non-polar change), A-to-S (non-polar to polar change) and Q-to-H (polar uncharged to polar positively charge). The final single domain mutant that we generated contained the N-terminal CT of MARCH1 in the MARCH8 backbone. Of note, the N-terminal CTs of MARCH1/8 not only vary in terms of protein sequence, but also in their length.

Although we did observe some variation in the expression levels of the different chimeric proteins, each “single domain swap” mutant generated was functional (at least based on their ability to downregulate CD86 from the cell surface of ZsGreen/CD86-transfected cells). As cell lines expressing particular chimeric proteins were generated at different times, the cell line with DOX-inducible MARCH8 WT was included in each assay for comparison. Based on this, the first chimeric protein to display differences in expression level was MARCH8\_M1 loop (**Figure 5.4B**). This chimeric protein appeared to be expressed at lower levels in steady state, but at similar levels to MARCH8 WT in the presence of proteasomal inhibitor MG132. These results suggest that MARCH8\_M1 loop

may be turned over more rapidly than MARCH8. This result was unexpected, especially because the stability of MARCH1 has been associated with its N-terminal domain, at least in studies using murine MARCH1 [226]. Our data suggest that the loop region could possibly contribute to the relative instability of MARCH1 compared to MARCH8. Unexpected results were also obtained when chimeric proteins expressing the N-terminal CT or the RING-CH domain of MARCH1 were generated. In this case, both chimeric proteins showed higher levels of expression compared to MARCH8 WT (**Figure 5.6B**). This finding corroborates the association of the N-terminal region with the stability of the MARCH proteins, however the results obtained were surprising, as deletions in the N-terminal region of murine MARCH1 resulted in superior protein stability of the mutants proteins, suggesting that this region was involved in more rapid turnover of murine MARCH1 [226]. In our studies, substituting the N-terminal region of MARCH8 with the equivalent region of MARCH1 actually increased protein stability. Considering the importance of intracellular regulation of MARCH proteins, we were concerned that increased levels of chimeric MARCH protein expression could impact cell viability after DOX induction. However, we did not observe any significant changes in cell viability in flow cytometry analyses (data not shown), although we did observe a reduction in the percentage of infected cells expressing intracellular NP 8 hr after IAV infection. Of all chimeric proteins generated by swapping a single domain of MARCH1 into the MARCH8 backbone, only these two (N-CT and RING) were associated with a reduced susceptibility to IAV infection, indicating an effect on early stages of infection (**Figure 5.7A**). These findings suggest that DOX-induced expression might alter intracellular localization/activity of MARCH8\_M1 N-CT or MARCH8\_M1 RING and/or target cellular proteins which modulate critical pathways required for early events in

IAV infection, such as receptor recognition and endocytosis. Interestingly, even when the total percentage of cells expressing viral NP was reduced, the amount of NP generated within each infected cell was markedly higher, suggesting either increased cellular susceptibility to infection, and/or a higher capacity to synthesize viral proteins.

The impact of MARCH8\_M1 N-CT and MARCH8\_M1 RING on early events in IAV infection complicates our ability to definitively assess their impact on late stage IAV infection. Although we always use a higher MOI (5 PFU/cell) to evaluate the late stages of IAV infection (to ensure that all cells were infected and therefore capable of producing viral progeny), we cannot disregard that, even when all cells were infected, the differences observed during early stages on infection will have an impact later during infection. Nevertheless, after titrating supernatants, each chimeric protein expressing single domains of MARCH1 in the MARCH8 backbone was found to block late stages of IAV infection to significant levels relative to the appropriate uninduced control, however we did observe variation in the potency of inhibition when compared to MARCH8 WT (**Figures 5.3B/5B/7B**). One possible explanation for this could be differences in expression levels, stability and/or intracellular localization of each chimeric protein. While we have included MARCH8 WT for reference in each experiment described, a single experiment should be performed which includes all chimeric proteins, as well as MARCH1 and MARCH8, to obtain more complete data. Importantly, by swapping the RING domains, we were able to discard the possibility that the differential antiviral effect observed between MARCH1 and MARCH8 was due to the use of a different E2-conjugating enzyme. E2s can be classified according to the ubiquitination that they produce: E2s that transfer ubiquitin (Ub) to a substrate residue (monoubiquitination),

E2s that transfer Ub to another Ub (polyubiquitination) or promiscuous E2s that can do both [155]. Thus, depending on the E2 that is recruited, different Ub chains can be added to a substrate and this, in turn, can determine its fate within the cell [250]. Altogether, our results suggested that the differential antiviral activity observed between MARCH1 and MARCH8 does not depend on a unique functional domain expressed by MARCH8. In future studies, we will exploit the DOX-inducible system to infect cells expressing MARCH8\_M1 N-CT and MARCH8\_M1 RING prior to addition of DOX and then determine virus titres released 24/48 hr later. This should allow us to uncouple the impact of these chimeric proteins on early IAV infection and to focus on their effects during late stages of IAV replication.

Considering that a more complex interaction involving multiple domains of MARCH8 could be responsible for its antiviral activity against IAV, we then decided to swap multiple domains of MARCH1 into MARCH8. We generated a chimeric protein expressing both TMs from MARCH1 and another expressing both TMs and the connecting loop of MARCH1 in a MARCH8 backbone. Although sequence conservation of TM1-loop-TM2 between MARCH1 and MARCH8 is very high (only 6 out of 61 amino acids are different), we generated these chimeric proteins given the important roles reported for the TMs and the loop in interactions between E3s and specific substrates. Expression levels of these chimeric proteins were comparable to those of MARCH8 WT, however the addition of MG132 did not increase expression of the protein containing only the TMs from MARCH1 (MARCH8\_M1 TM1-TM2) (**Figure 5.8B**). This result suggests that incorporation of the 2 TMs from MARCH1 reduced protein turnover, which is a surprising result considering that we did not observe a similar phenomenon when both

TMs were replaced individually (MARCH8\_M1 TM1 and MARCH8\_M1 TM2). Interestingly, the chimeric protein that also contained the loop of MARCH1 (MARCH8\_M1 TM1-loop-TM2) showed a slight increase in protein expression in the presence of MG132, indicative of an intermediate phenotype between MARCH8 WT and MARCH8\_M1 TM1-TM2. Previously, we found that incorporation of the connecting loop of MARCH1 appeared to reduce the stability of MARCH8 (**Figure 5.4B**). Therefore this “intermediate” phenotype could result from the stability provided by the TMs of MARCH1, countered by the instability associated with MARCH1 loop. When the capacity to restrict late stages of IAV infection was assessed, both MARCH8\_M1 TM1-loop-TM2 and MARCH8\_M1 TM1-TM2 were more potent inhibitors than MARCH8 WT (**Figure 5.9B**). Most likely, the potent antiviral activity of these chimeric proteins reflects their enhanced intracellular expression when compared to MARCH8 WT.

Finally, we generated a chimeric protein expressing all cytoplasmic domains of MARCH1 with the TM1-loop-TM2 domains of MARCH8. Consistent with results obtained by swapping individual domains, this chimeric protein was expressed at higher levels than MARCH8 WT (**Figure 5.10B**), and also reduced the susceptibility of DOX-induced cells to IAV infection as measured by expression of viral NP at 8 hr post-infection (**Figures 5.11A**). Interestingly, this chimeric protein induced a relatively modest, but significant reduction (60%) in infectivity of virus particles released from infected cells compared to >85% observed for all other chimeric proteins (**Figure 5.11B**). Although this result suggests that the domains facing the cell-cytoplasm are important for the antiviral capacity of MARCH8, other regions also appear to be required for its anti-IAV activity, suggesting a complex interplay between E2(s), MARCH8 and the target protein/s.

Moreover, we have not determined the percentage of NP-positive cells at 8 hr post-infection with the higher MOI used to study virus release, nor do we know if the impact of the MARCH8\_M1 ACD protein on early IAV replication has subsequent effects on the late stages of IAV infection. Again, it will be of particular interest to infect uninduced MARCH8\_M1 ACD cells to promote equivalent virus entry and infection, before addition of DOX to induce MARCH expression and perform assessment of infectious viral titres released from infected cells. This appears to be the most accurate way to uncouple any effects on particular chimeric proteins on early (NP-positive cells at 8 hr post-infection) and late (infectivity titres in supernatants from cells at 24 hr post-infection) stages of IAV replication. Although we were unable to do these experiments before thesis submission, they will be performed upon resumption of research activities in our institute, once the SARS-CoV2 lockdown has been lifted.



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## Chapter 6

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# **Evaluating the role of endogenous MARCH8 during IAV infection *in vivo*.**

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## 6.1. Introduction.

Cell culture approaches, including the DOX-inducible expression system used in previous chapters, provide many advantages to study specific host-encoded antiviral restriction factors. However, these results should be interpreted with caution and complemented by additional studies to confirm the importance of such restriction factors both *in vitro* and *in vivo*, when endogenous levels of expression are observed. Animal models are particularly important to assess the role of antiviral restriction factors in the context of a whole animal, providing a more wholistic insight as to whether a particular host protein can modulate the severity of infection or disease. Genetically defined “knockout mice” which lack production of specific host proteins are widely used to evaluate the role of endogenous proteins and their contribution during viral and other pathogenic infections.

A recurrent problem in the identification and characterization of host antiviral restriction factors is the discrepancy between results obtained *in vitro* and *in vivo* (e.g. BST-2 [97, 136, 137]). The use of constitutive or inducible overexpression systems can identify host proteins with potent antiviral capacity *in vitro* but some of these do not appear to modulate infection *in vivo*. This could be perhaps due to physiological expression levels *in vivo* are different to high levels *in vitro*, limited expression of the protein of interest in relevant cells/tissues *in vivo* or redundancy in antiviral mechanisms between different host factors.

Considering the potent antiviral capacity exhibited by MARCH8 *in vitro*, in this chapter we aimed to evaluate the role of endogenous MARCH8 during IAV infection *in vivo*. qPCR

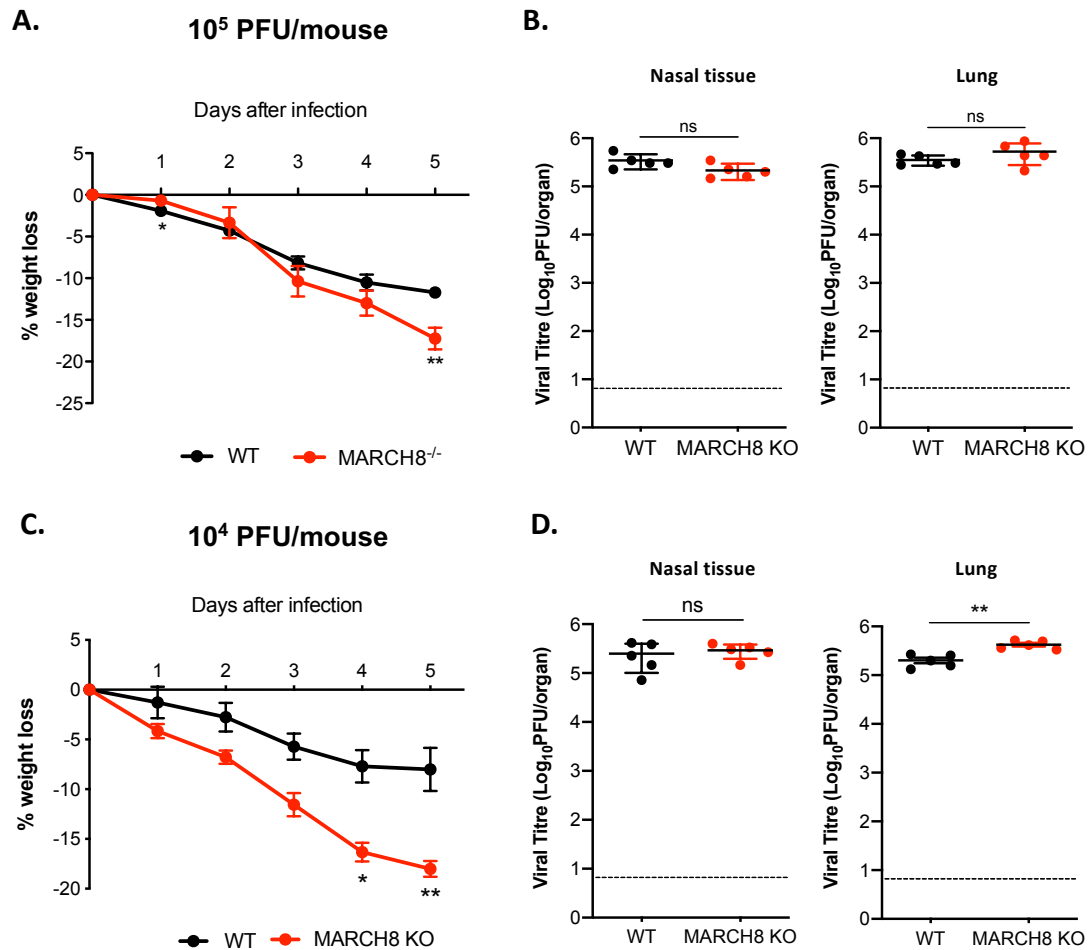
studies regarding expression levels of different MARCH proteins in human tissue samples indicated broad expression of MARCH8 in different tissues, however its highest expression was found in samples derived from lung [177], supporting a possible role for endogenous MARCH8 at controlling IAV infection.

In this chapter, wild-type (WT, C57BL/6) and MARCH8 knockout mice (KO) were infected with IAV and various parameters of infection were determined at different times thereafter. In addition, primary macrophages recovered from animals were infected with IAV *in vitro* to assess the impact of endogenous MARCH8 expression on the “early” and “late” stages of IAV infection. Overall, our studies showed that mice deficient in MARCH8 expression displayed enhanced weight loss and viral replication in the airways compared to WT controls. This is consistent with an important role for endogenous MARCH8 as an antiviral restriction factor, limiting IAV replication and disease severity *in vivo*. Interestingly, no major defects were observed in the ability of MARCH8 KO mice to clear IAV infection, suggesting that these mice were able to mount an effective antiviral response despite not expressing MARCH8. Finally, primary murine macrophages from MARCH8 KO mice did not show enhanced viral replication after IAV infection, when compared to primary macrophages recovered from WT mice. Of note, genetic analysis showed that the MARCH8 KO mice used in this study have a disruption in the NK locus, therefore a new MARCH8 KO mice strain has been generated using CRISPR/Cas9 technology to confirm the role of MARCH8 during IAV infection *in vivo* in future studies.

## 6.2. Results.

### 6.2.1. Optimizing infectious dose of IAV for inoculation of wild-type and MARCH8 KO mice.

Mice are the most widely used *in vivo* model to study IAV infection. Generally, mice are inoculated intranasally with mouse-adapted IAV strains and different parameters are measured to evaluate pathogenesis, disease and/or immunity to infection. For our studies, we have used HKx31, a reassortant virus bearing the internal components of the mouse-adapted A/PR/8/34 (PR8, H1N1) strain and the HA and NA from A/Aichi/2/68 (H3N2). First, C57BL/6 (hereafter referred to as WT) and MARCH8 KO mice were inoculated via the intranasal route with  $10^4$  or  $10^5$  PFU of IAV in a total volume of 50  $\mu$ L, representing a total respiratory tract (TRT) infection. Weight loss was monitored daily, and mice were killed and the viral load in the nose and lungs was determined at day 5 post-infection (p.i.). As seen in **Figure 6.1A/C**, MARCH8 KO mice lost significantly more body weight than the WT mice at both inoculum doses tested, and this was significant at days 1/5 and days 4/5 p.i. for  $10^5$  and  $10^4$  PFU, respectively. To compare viral loads, nasal tissues and lungs were homogenized and infectious IAV titres in clarified supernatants determined by standard plaque assay. As seen in **Figure 6.1B/D**, IAV replicated efficiently in nose and lung after inoculation with either dose of virus. No significant differences were observed in viral titres in the nasal tissue from WT or MARCH8 KO mice (left panels). However, while virus titres in lungs were not significantly different following inoculation with  $10^5$  PFU, titres were significantly higher in the lungs of MARCH8 KO mice at day 5 p.i. following inoculation with  $10^4$  PFU. These data provide

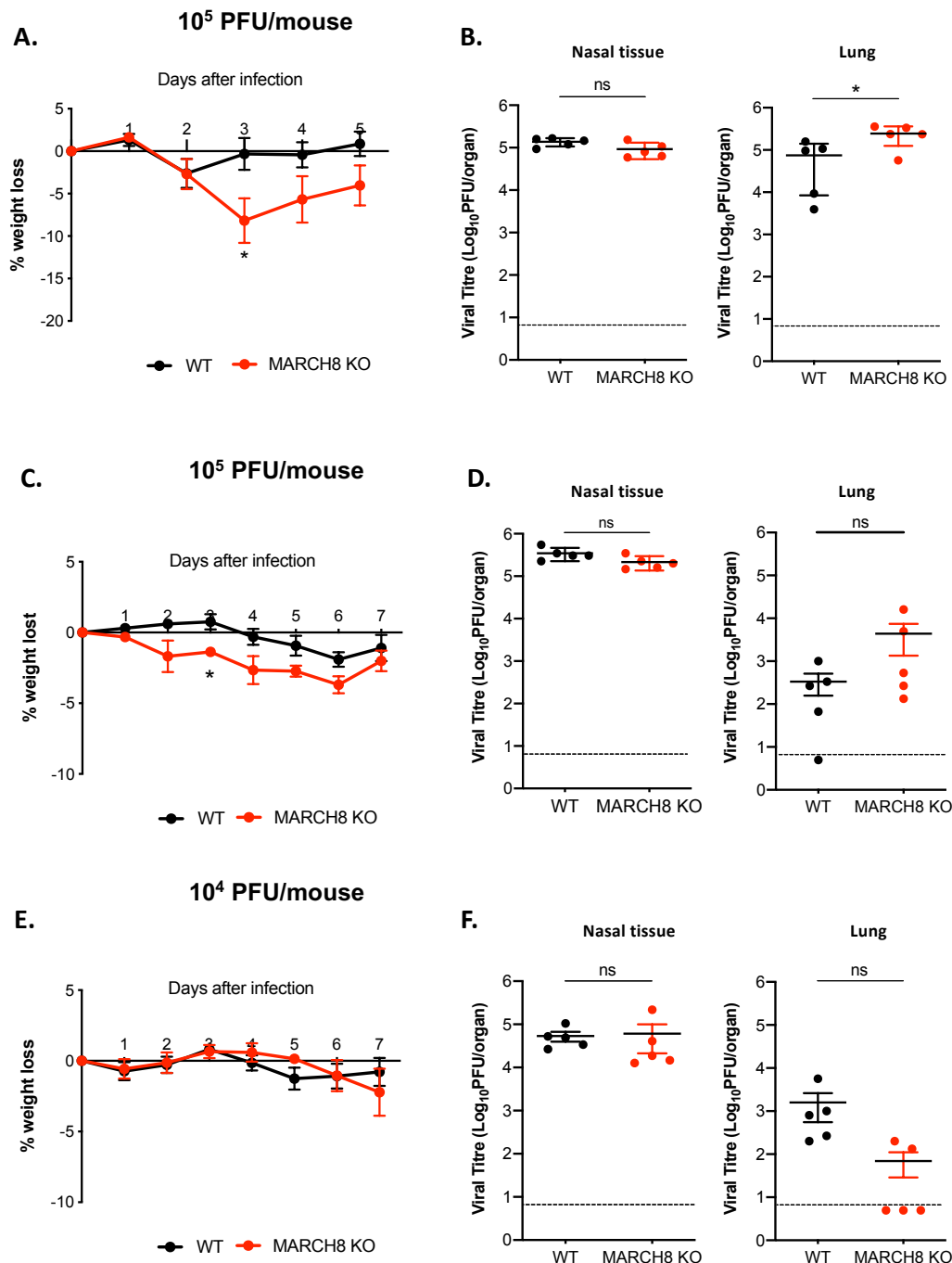


**Figure 6.1. Weight loss and virus replication in the airways following a total respiratory tract (TRT) infection of wild-type and MARCH8 KO mice with IAV.** C57BL6 wild-type (WT) or MARCH8 KO mice were inoculated intranasally with 50  $\mu$ L of PBS containing 10<sup>4</sup> or 10<sup>5</sup> PFU/mouse of HKx31. **(A and C)** Mice were weighed daily and the percentage of weight loss was determined relative to their original body weight at day 0. Mean weight loss ( $\pm$  SEM) from 5 mice are shown. **(B and D)** At day 5 post-infection, mice were killed and virus titres in homogenates prepared from nasal tissues or lungs were determined by plaque assay using MDCK cells. Mean titres ( $\pm$  SD) from 5 mice are shown. The dashed line indicates the limit of detection for the plaque assay. A two-tailed unpaired Student's t-test was performed to compare the % of weight loss and viral titres between wild-type and MARCH8 KO mice. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

evidence that a lack of endogenous MARCH8 can alter weight loss and virus replication following TRT infection with IAV.

Next, mice were inoculated via the intranasal route with  $10^4$  or  $10^5$  PFU of IAV in a total volume of 5  $\mu$ L, representing an upper respiratory tract (URT) infection. With certain strains of IAV, including HKx31, an URT inoculation of mice allows the virus to replicate in the nasal tissues and then “travel” to replicate in the lungs [251]. Compared to TRT infections, weight loss was very mild in both WT and MARCH8 KO mice even following inoculation with  $10^5$  PFU (**Figure 6.2A/C**), with a trend for enhanced weight loss in MARCH8 KO mice, which was significant at one day in each experiment only. When examining virus titres, IAV replicated efficiently in nasal tissues following inoculation with either  $10^4$  or  $10^5$  PFU of HKx31, but with no significant differences noted in the three experiments (**Figure 6.2B/D/F**, left panels). At day 5 p.i., lung titres were somewhat lower than at the equivalent time following TRT infections, however titres in MARCH8 KO mice were significantly higher (**Figure 6.2B**, right panel). At day 7 p.i., no significant differences were noted in lung virus titres between WT and MARCH8 KO mice irrespective of inoculum dose used (**Figure 6.2D/E**, right panels).

Together, these data indicate that the TRT model would be most appropriate for further studies to characterize responses to IAV infection in WT versus MARCH8 KO mice. Moreover, as animals having lost  $\geq 20\%$  of their original body weight must be euthanised to comply with the University of Melbourne’s Animal Experimentation and Ethics guidelines, we have focussed on studies using  $10^3$  PFU of HKx31 for all subsequent studies.



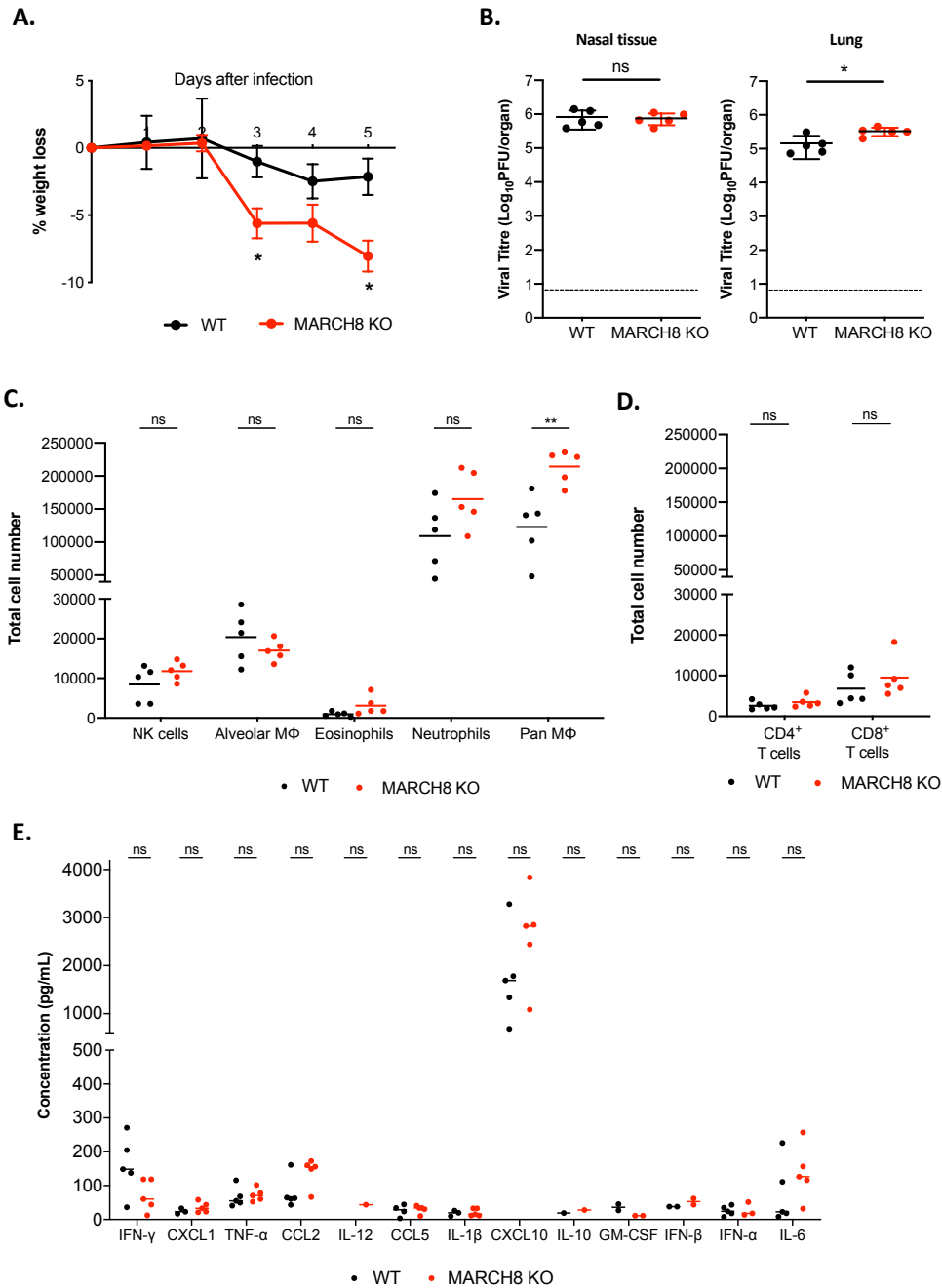
**Figure 6.2. Weight loss and virus replication in the airways following an upper respiratory tract (URT) infection of wild-type and MARCH8 KO mice with IAV.** C57BL6 WT or MARCH8 KO mice were inoculated intranasally with 5  $\mu$ L of PBS containing 10<sup>4</sup> or 10<sup>5</sup> PFU/mouse of HKx31. (**A**, **C** and **E**) Mice were weighed daily and the percentage weight loss was determined relative to their original body weight at day 0. Mean weight loss ( $\pm$  SEM) from 5 mice are shown. At day 5 (**B**) and 7 (**C** and **D**), mice were killed and virus titres in homogenates prepared from nasal tissues or lungs were determined by plaque assay using MDCK cells. Mean titres ( $\pm$  SD) from 5 mice are shown. The dashed line indicates the limit of detection for the plaque assay. A two-tailed unpaired Student's t-test was performed to compare the % of weight loss and viral titres between wild-type and MARCH8 KO mice. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.



### 6.2.2. Characterizing the course of infection following TRT infection of wild-type and MARCH8 KO mice with $10^3$ PFU of IAV strain HKx31.

To characterize TRT infection following IAV infection of mice with  $10^3$  PFU/mouse, we focussed on analysis at **(i)** 5 days p.i. (early time point at peak of viral replication where innate responses predominate), **(ii)** 7 days p.i. (intermediate time point corresponding to and early influx of effector T cells), and **(iii)** 10 days p.i. (peak of effector T cell responses, corresponding to viral clearance from the lower respiratory tract). We assessed weight loss and infectious virus titres in nasal tissues, as well as using flow cytometry to analyse leukocyte populations and soluble inflammatory mediators in bronchoalveolar lavage (BAL) samples at these different times p.i.

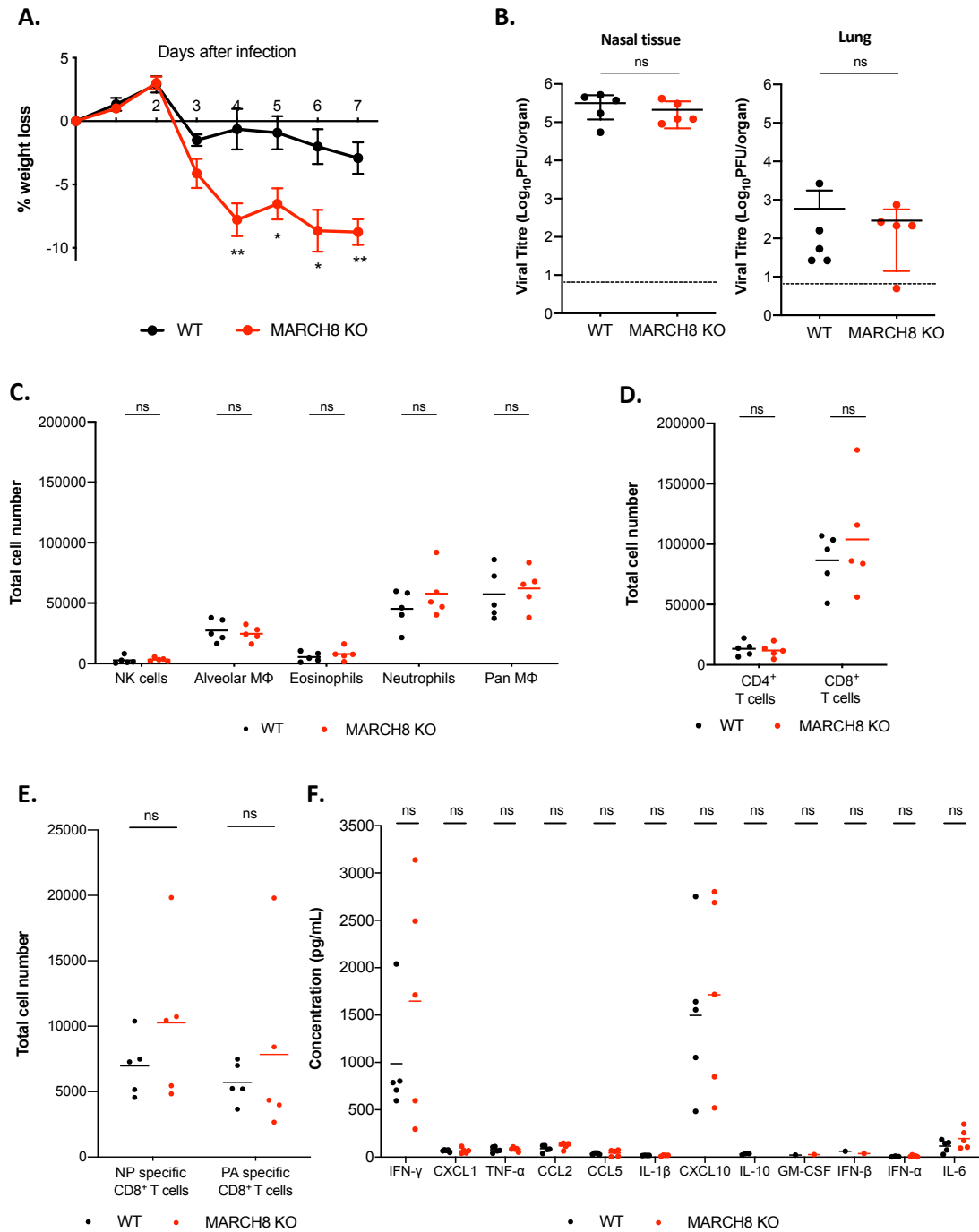
First, we assessed weight loss and virus titres in mice 5 days after intranasal TRT infection with  $10^3$  PFU of HKx31. As seen in **Figure 6.3A**, MARCH8 KO mice showed enhanced weight loss compared to WT mice and this was significant at days 3 and 5 p.i. Moreover, while virus titres were similar in nasal tissues at day 5 p.i., titres were significantly higher in lung tissue homogenates from MARCH8 KO compared to WT controls (**Figure 6.3B**). To characterize the different immune cell populations, cells isolated from the BAL fluid were stained with antibodies targeting cell-surface markers (as described in Materials and Methods) and analysed by flow cytometry. As predicted, the majority of immune cells (CD45.2<sup>+</sup>) in BAL fluid at day 5 p.i. were innate immune cells, including natural killer (NK) cells, macrophages (M $\phi$ ) and neutrophils (**Figure 6.3C**). Numbers of 'pan' M $\phi$  (CD11b<sup>+</sup>, Siglec-F<sup>-</sup>, CD64<sup>+</sup>) were significantly higher in BAL fluid from MARCH8 KO mice, whereas there was no significant difference in numbers of alveolar M $\phi$  (CD11b<sup>+</sup>, Siglec-F<sup>+</sup>, CD11c<sup>+</sup>, CD64<sup>+</sup>), neutrophils (CD11b<sup>+</sup>, Ly6G<sup>+</sup>), NK cells



**Figure 6.3. Airway inflammatory responses following a TRT infection of wild-type and MARCH8 KO mice with IAV at day 5 post-infection.** C57BL6 WT or MARCH8 KO mice were inoculated intranasally with 50  $\mu$ L of PBS containing  $10^3$  PFU/mouse of HKx31. **(A)** Mice were weighed daily, and the percentage weight loss was determined relative to their original body weight at day 0. Mean weight loss ( $\pm$  SEM) from 5 mice are shown. **(B)** At day 5 p.i., mice were killed and virus titres in homogenates prepared from nasal tissues or lungs were determined by plaque assay using MDCK cells. Mean titres ( $\pm$  SD) from 5 mice are shown. The dashed line indicates the limit of detection for the plaque assay. **(C/D/E)** Before removal of lungs, bronchioalveolar lavage (BAL) was performed on all animals. BAL cells were stained with antibodies against specific markers and flow cytometry was used to identify and quantify populations of **(C)** innate immune cells and **(D)** T cells. **(E)** Cell-free BAL fluid was analysed by flow cytometry using a multiplex CBA assay to detect 13 different inflammatory cytokines and chemokines. Data are representative of 2 independent experiments. A two-tailed unpaired Student's t-test was performed to compare the % of weight loss, viral titres, numbers of immune cell populations and cytokines and chemokines released between wild-type and MARCH8 KO mice. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

(CD3<sup>-</sup>, NKp46<sup>+</sup>) or eosinophils (CD11b<sup>+</sup>, Siglec-F<sup>+</sup>, CD11c<sup>-</sup>, CD64<sup>-</sup>). In addition to innate cells, we also assessed T cell populations in BAL fluid. As expected, only very low numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were detected in BAL fluid at this early time point, with no significant differences between WT and MARCH8 KO mice (**Figure 6.3D**). We also used a multiplex cytometric bead array (CBA) assay to determine levels of inflammatory cytokines and chemokines in cell-free BAL fluid at day 5 p.i. Analysis of individual mediators indicated that day 5 responses in BAL were dominated by C-X-C Motif Chemokine Ligand 10 (CXCL10) followed by interferon (IFN)- $\gamma$ , C-C Motif Chemokine Ligand (CCL2) and interleukin 6 (IL-6), with levels of interleukin 12 (IL-12) and interleukin 10 (IL-10) generally below detection. When comparing each mediator from WT or MARCH8 KO mice, no significant differences were noted for any chemokine or cytokine tested (**Figure 6.3E**), however a tendency to record higher levels was observed in the BAL samples from MARCH8 KO mice, especially for CXCL10.

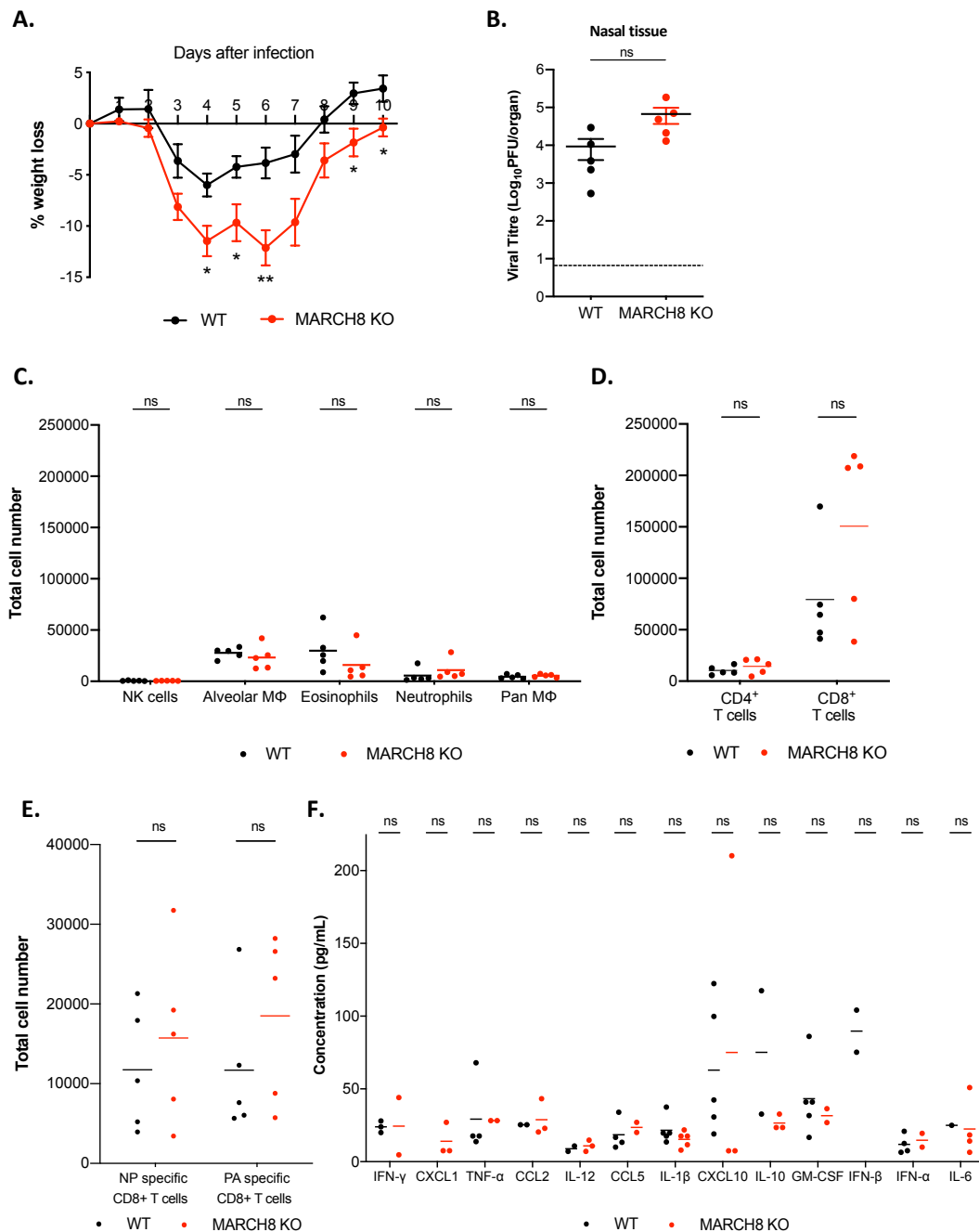
Next, we assessed weight loss and virus titres in mice 7 days after intranasal TRT infection with 10<sup>3</sup> PFU of HKx31. Although MARCH8 KO mice showed a significant enhancement in weight loss between days 4-7 p.i. (**Figure 6.4A**), there were no significant differences in viral titres detected in homogenates from nasal or lung tissues at day 7 p.i. (**Figure 6.4B**). Note that titres of infectious virus in lungs at day 7 p.i. were ~100-fold less than at day 5 p.i. (**Figure 6.3B**), consistent with partial clearance of infectious IAV from the lungs. While numbers of most innate immune cell populations were reduced at day 7 compared to day 5 p.i. (compare **Figure 6.3C** and **Figure 6.4C**), numbers of CD3<sup>+</sup>, CD8<sup>+</sup> T lymphocytes showed a marked increase in BAL fluid from WT and MARCH8 KO mice (**Figure 6.4D**). To further characterize CD8<sup>+</sup> T cells, we used



**Figure 6.4. Airway inflammatory responses following a TRT infection of wild-type and MARCH8 KO mice with IAV at day 7 post-infection.** C57BL/6 WT or MARCH8 KO mice were inoculated intranasally with 50  $\mu$ L of PBS containing  $10^3$  PFU/mouse of HKx31. **(A)** Mice were weighed daily and the percentage of weight loss was determined relative to their original body weight at day 0. Mean weight loss ( $\pm$  SEM) from 5 mice are shown. **(B)** At day 7 p.i., mice were killed and virus titres in homogenates prepared from nasal tissues or lungs were determined by plaque assay using MDCK cells. Mean titres ( $\pm$  SD) from 5 mice are shown. The dashed line indicates the limit of detection for the plaque assay. Before removal of lungs, bronchioalveolar lavage (BAL) was performed on all animals. BAL cells were stained with antibodies against specific markers and flow cytometry was used to identify and quantify populations of **(C)** innate immune cells, **(D)** T cells and **(E)** IAV-specific CD8<sup>+</sup> T cells. **(F)** Cell-free BAL fluid was analysed by flow cytometry using a multiplex CBA assay to detect 13 different inflammatory cytokines and chemokines. Data are representative of 2 independent experiments. A two-tailed unpaired Student's t-test was performed to compare the % of weight loss, viral titres, numbers of immune cell populations and cytokines and chemokines released between wild-type and MARCH8 KO mice. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

tetramers to identify IAV-specific CD8<sup>+</sup> T cells. Tetramers mimic the peptide-MHC complex, allowing the identification of T cells that can recognize specific antigens presented in the context of MHC. In this case, we used tetramers containing MHC-I loaded with a peptide derived from the viral NP (NP<sub>366-374</sub>), or the PA (PA<sub>224-233</sub>), as these represent the immunodominant peptides during the primary CD8<sup>+</sup> T cell response to this virus in C57BL/6 mice [252]. As seen in **Figure 6.4E**, similar numbers of NP- and PA-specific CD8<sup>+</sup> T cells were detected in WT and MARCH8 KO mice. High levels of CXCL10 and IFN- $\gamma$  were observed in the cell-free BAL samples from WT and MARCH8 KO mice (**Figure 6.4F**). Although no significant differences were observed, MARCH8 KO BAL samples showed a tendency to contain higher levels of IFN- $\gamma$ .

Finally, we assessed weight loss and virus titres in mice 10 days after intranasal TRT infection with 10<sup>3</sup> PFU of HKx31. By this time, both WT and MARCH8 KO mice had recovered from weight loss associated with IAV infection, reaching a body weight similar to that recorded on day 0 of the experiment (**Figure 6.5A**). Again, MARCH8 KO mice showed a tendency to lose more weight during IAV infection and this was significant on days 4, 5, 6, 9 and 10 p.i. Consistent with recovery from IAV infection, no virus could be detected in lungs (<0.85 log<sub>10</sub> PFU/mL) from either WT or MARCH8 KO mice at day 10 p.i. (data not shown). However, infectious virus was still detectable in nasal tissues from WT and MARCH8 KO mice (**Figure 6.5B**), although titres were markedly lower compared to days 5 and 7 p.i., consistent with viral clearance. Interestingly, there was a trend for higher titres in nasal tissues from MARCH8 KO mice compared to WT mice, however this difference was not significant. When we assessed cellular inflammation in BAL fluid at day 10 p.i. (**Figure 6.5C**), we observed an overall reduction in innate immune cells

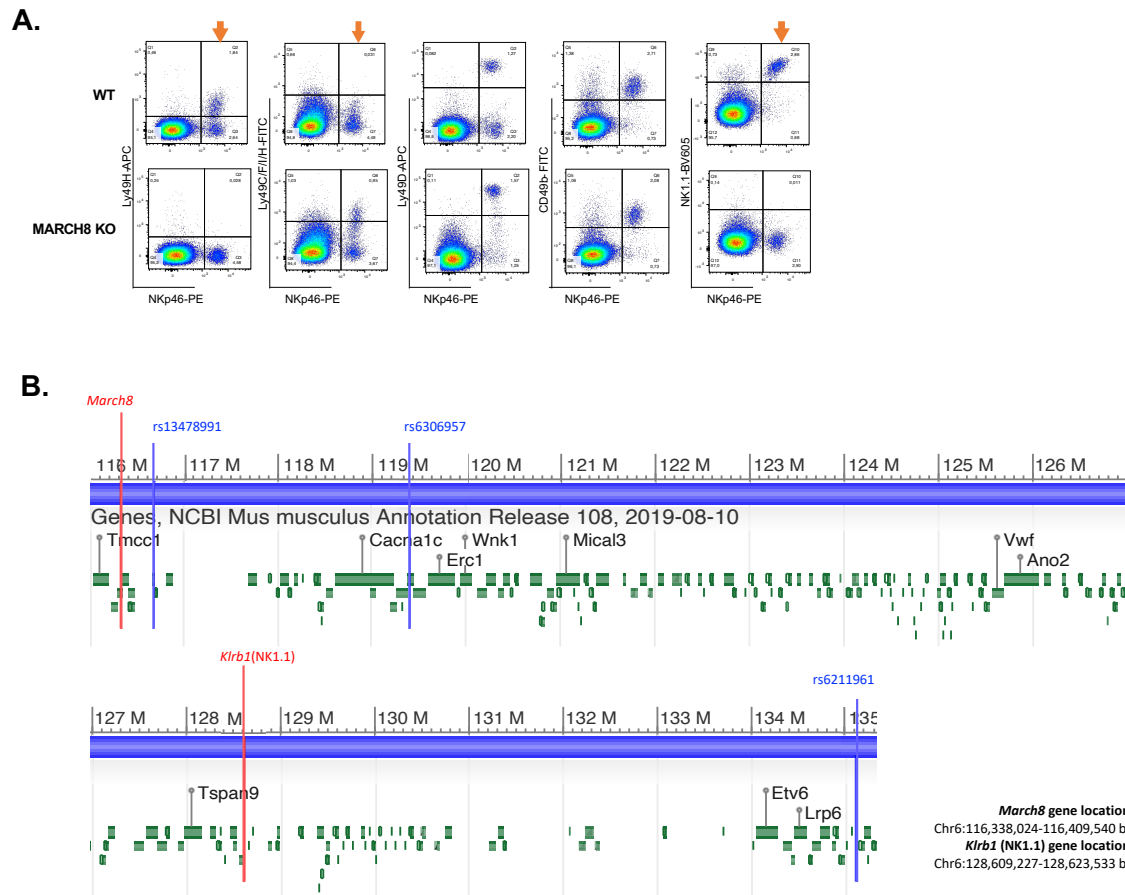


**Figure 6.5. Airway inflammatory responses following a TRT infection of wild-type and MARCH8 KO mice with IAV at day 10 post-infection.** C57BL6 WT or MARCH8 KO mice were inoculated intranasally with 50  $\mu$ L of PBS containing  $10^3$  PFU/mouse of HKx31. **(A)** Mice were weighed daily, and the percentage of weight loss was determined relative to their original body weight at day 0. Mean weight loss ( $\pm$  SEM) from 5 mice are shown. **(B)** At day 10 p.i., mice were killed and virus titres in homogenates prepared from nasal tissues or lungs were determined by plaque assay using MDCK cells. Mean titres ( $\pm$  SD) from 5 mice are shown. The dashed line indicates the limit of detection for the plaque assay. Before removal of lungs, bronchioalveolar lavage (BAL) was performed on all animals. BAL cells were stained with antibodies against specific markers and flow cytometry was used to identify and quantify populations of **(C)** innate immune cells, **(D)** T cells and **(E)** IAV-specific CD8<sup>+</sup> T cells. **(F)** Cell-free BAL fluid was analysed by flow cytometry using a multiplex CBA assay to detect 13 different inflammatory cytokines and chemokines. Data are representative of 2 independent experiments. A two-tailed unpaired Student's t-test was performed to compare the % of weight loss, viral titres, numbers of immune cell populations and cytokines and chemokines released between wild-type and MARCH8 KO mice. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

compared to days 5 and 7 p.i. (**Figures 6.3C** and **6.4C**, respectively). As expected, CD8<sup>+</sup> T cells predominated in the BAL fluid at day 10 p.i., with no significant differences noted between WT and MARCH8 KO mice (**Figure 6.5D**), nor were any significant differences detected in the number of NP- or PA-specific CD8<sup>+</sup> T cells (**Figure 6.5E**). When examining soluble inflammatory mediators, low levels of each cytokine and chemokine measured were observed, consistent with recovery from IAV infection (**Figure 6.5F**).

### **6.2.3. Differences in NK cell markers expressed by wild-type and MARCH8 KO mice.**

The functionality of NK cells depends on a variety of activating and inhibitory cell-surface receptors (reviewed in [253]). Specific receptors expressed by NK cells, such as CD49b, NK1.1 or NKp46, are also often used as markers for the recognition of these cells by flow cytometry. For our studies, specific antibodies were used to identify different immune cells in BAL fluid after IAV infection (see Material and Methods). In initial studies, we used an antibody against NKp46 to identify NK cells in the BAL fluid of virus-infected mice, however when other markers were used to corroborate our results, we found that NK cells from WT and MARCH8 KO mice showed differences in the cell-surface expression of particular NK cell markers. Although NK cells isolated from the spleen of both groups of mice expressed similar levels of Ly49D and CD49b, only cells from WT mice expressed Ly49H and NK1.1. Moreover, only NK cells from MARCH8 KO mice stained with an antibody against Ly49C/F/I/H (**Figure 6.6A**).



**Figure 6.6. Differential expression of cell-surface receptors in NK cells from WT and MARCH8 KO mice.** (A) Single-cell suspensions from spleens isolated from C57BL/6 wild-type or MARCH8 KO mice were stained with anti-CD3 mAbs and specific antibodies against different NK cell receptors (x- and y-axes) and analysed by flow cytometry. Dot plots from CD3<sup>+</sup> cells are shown. The orange arrows indicate distinctive positive populations of NK cells between WT and MARCH8 KO mice. Data shown are from a single experiment. (B) Genome map of murine chromosome 6 from position 116,000,000 bp to position 135,300,000 bp. The location of the genes coding for MARCH8 and NK1.1 is shown in red. The location and rsID of the 3 SNPs found in the genome of MARCH8 KO mice are shown in blue.



Considering that MARCH8 KO mice were generated by crossing chimeric mice carrying the MARCH8 mutation (generated by injecting MARCH8 mutant R1 stem cells on a 129 background into blast cysts from C57BL/6 mice) with CAG-Cre mice (C57BL/6 background) and then backcrossing to C57BL/6 [222], mouse linkage marker set (MLMS) analysis was undertaken to confirm the genetic background of the MARCH8 KO mice. The MLMS panel contains over 550 SNPs with an average distance of 3-5cM across the mouse genome and provides approximately 300 informative markers between common mouse crosses (Australian Genome Research Facility, AGRF, Melbourne, Australia). MLMS analysis identified five single nucleotide polymorphisms in MARCH8 KO compared to C57BL/6 mice, including three on chromosome 6 (rs13478991, rs6306957 and rs6211961, **Figure 6.6B**) which is where MARCH8 is located. This indicates that genetic material from the original mouse strain used to generate the MARCH8 mutant (strain 129) remained in the MARCH8 KO mice used in our studies. Moreover, the presence of the three SNPs in the vicinity of the NK1.1 locus is consistent with our inability to detect Ly49H and NK1.1 in MARCH8 KO mice by flow cytometry, as these are not expressed by NK cells from 129 strain mice (reviewed in [254, 255]).

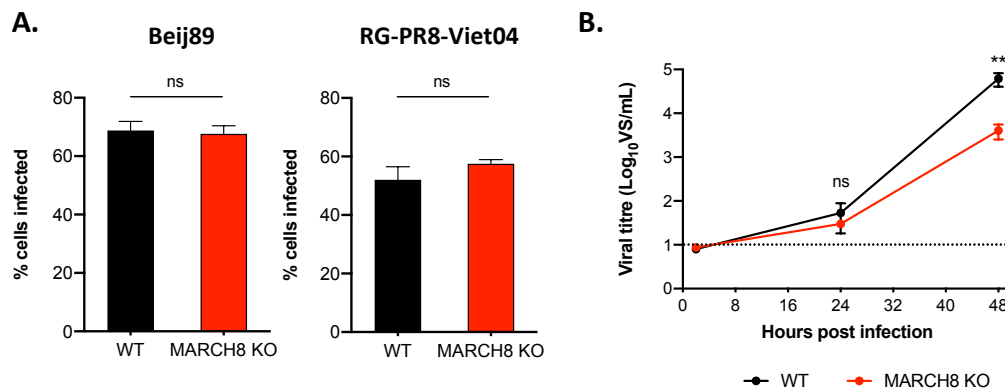
#### **6.2.4. *In vitro* infection of primary macrophages from wild-type and MARCH8 KO mice with IAV.**

Airway epithelial cells are the main target of IAV infection in the respiratory tract. Macrophages (M $\phi$ ) are also susceptible to IAV, however viral replication is abortive, and newly synthesised viral progeny are not released from infected cells. Although the

mechanism underlying abortive replication of seasonal IAV in M $\phi$  is largely unknown, it has been associated with a block during virus assembly and/or budding in murine M $\phi$  [232]. Interestingly, certain strains of highly pathogenic avian influenza viruses (HPAI) are able to bypass this block and replicate productively in M $\phi$  [256]. In regard to MARCH8, human monocyte-derived M $\phi$  (hMDM) have been reported to express high levels of MARCH8 and knockdown or knockout of MARCH8 in hMDM resulted in increased infectivity of virions released from HIV-1-infected hMDMs [210].

Therefore, peritoneal exudate cell (PEC) M $\phi$  from naïve wild-type and MARCH8 KO mice were seeded into 8-well chamber-slides, cultured overnight and then infected with seasonal IAV (strain Beij89, H3N2, MOI = 10 PFU/cell) or with a reassortant virus expressing HA/NA from HPAI H5N1 (strain Viet04) on a PR8 backbone (RG-PR8-Viet04, H5N1, MOI = 5 PFU/cell). These virus strains were chosen as previous studies in our laboratory confirm that the Beij89 strain of IAV does not replicate productively in PEC M $\phi$  whereas RG-PR8-Viet04 does (personal communication, Dr Sarah Londrigan, The University of Melbourne). Moreover, the MOI were chosen to give ~50-60% infection of PEC M $\phi$  with each virus strain. At 8 hr p.i., the percentage of NP-positive PEC M $\phi$  was determined by immunofluorescence microscopy. As seen in **Figure 6.7A**, no major differences were observed in the percentage of NP-positive PEC M $\phi$  from WT or MARCH8 KO mice infected with each virus strain, indicating both populations of M $\phi$  were equally susceptible to the early stages of IAV infection.

To evaluate the role of MARCH8 during late stages of infection, PEC M $\phi$  were infected with IAV strains Beij89 or RG-PR8-Viet04 at a low MOI of 0.01 PFU/cell. After 1 hr, cells were washed and incubated with serum-free media containing TPCK-treated trypsin to



**Figure 6.7. *In vitro* infection of peritoneal macrophages from wild-type and MARCH8 KO mice with different IAV.** Macrophages (M $\phi$ ) isolated from the peritoneal cavity of WT and MARCH8 KO mice were seeded into 8-well chamber slides and culture overnight. **(A)** M $\phi$  were washed and then infected with Beij89 at MOI 10 PFU/cell or with RG-PR8-Viet04 at MOI 5 PFU/cell for 1 hr at 37°C. After washing, macrophages were cultured an additional 7 hr and then washed, fixed, permeabilized and stained with anti-NP mAb and DAPI. The percentage of infected cells was determined by immunofluorescence microscopy. Data represent the mean percentage of cells expressing NP ( $\pm$  SD) from triplicate samples (3 independent fields per sample). **(B)** Macrophages were washed and infected with RG-PR8-Viet04 at MOI 0.01 PFU/mL for 1 hr at 37°C. After washing, macrophages were cultured in the presence of exogenous trypsin (1  $\mu$ g/mL) to facilitate multi-cycle virus replication. Supernatants harvested at 2, 24 and 48 hr p.i. were clarified by centrifugation (3000 rpm, 5 min) and titres of infectious virus determined by ViroSpot assay using MDCK cells. Mean virus titres from triplicate samples ( $\pm$  SD) are shown. The dashed line indicates the limit of detection of the ViroSpot assay. A two-tailed unpaired Student's t-test was performed to compare % of infected cells and viral titres. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ , ns = not significant.

allow for multicycle virus growth. Cell supernatants were harvested at 2, 24 and 48 hr after infection, and titres of infectious virus were determined by ViroSpot assay. Cell supernatants from WT and MARCH8 KO PEC M $\phi$  infected with IAV Beij89 did not contain detectable infectious particles at any time-point tested, consistent with the abortive replication described for seasonal IAV in M $\phi$  (data not shown). As expected, RG-PR8-Viet04 did replicate productively in PEC M $\phi$  from both WT and MARCH8 KO mice. Interestingly, higher virus titres were detected in supernatants of M $\phi$  from WT mice, compared to MARCH8 KO mice (**Figure 6.7B**), suggesting that the absence of MARCH8 had an impact in the ability of IAV to replicate in murine macrophages.

### 6.3. Discussion

Previous chapters of this thesis demonstrated that MARCH8 displays potent antiviral activity against influenza viruses *in vitro*. In this chapter we aimed to validate the role of MARCH8 as an antiviral restriction factor for IAV through evaluation of the role of endogenous MARCH8 during IAV infection *in vivo*. Mice are the most convenient and widely used animal model for studying different aspects of influenza virus infection including morbidity, pathogenesis and immunity. Moreover, inbred strains of mice can be used to generate knockout mice which specifically lack expression of a particular host protein. Thus, to investigate the role of MARCH8 during IAV infection *in vivo*, we initially compared the susceptibility of WT C57BL/6 and MARCH8 KO mice to infection with a mouse-adapted strain of IAV.

Preliminary studies tested different inoculum doses and compared TRT (**Figure 6.1**) versus URT (**Figure 6.2**) infection models in WT and MARCH8 KO mice, examining weight loss over time as well as virus titres in nose and lung. Based on these studies, the evidence for endogenous MARCH8 in limiting IAV infection was more compelling following TRT infection. Therefore, subsequent studies focussed on the TRT infection model only. Note that additional studies performed by both us ( $10^3$  PFU/mouse HKx31) and our collaborators ( $10^4$  PFU/mouse HKx31) confirmed that while MARCH8 KO mice showed enhanced weight loss following TRT infection, MARCH1 KO mice did not (data not shown), consistent with our findings presented in previous chapters (Chapters 3 and 4). Further studies with MARCH1 KO mice have not been performed at this stage.

With a standard inoculum dose ( $10^3$  PFU/mouse) and TRT infection model, we then compared WT and MARCH8 KO mice at different times during IAV infection that broadly correspond to the peak of virus replication (day 5, **Figure 6.3**), generation of early adaptive responses (day 7, **Figure 6.4**) and virus clearance (day 10, **Figure 6.5**), as described in previous studies [257, 258]. Consistent with results using higher inoculum doses, significantly higher titres of virus were recovered from lungs of MARCH8 KO mice at day 5 p.i. (peak), although this was not observed at day 7 (early adaptive) or day 10 p.i. (clearance). No differences were detected in virus titres recovered from nasal tissues at any time point. Of interest, the only differences noted in inflammatory cells were also recorded at day 5 p.i., with pan M $\phi$  significantly higher in BAL from MARCH8 KO mice at this time; all other innate cells and T lymphocyte populations were not significantly different between WT and MARCH8 KO mice at any time point examined. Similarly, we did not record significant differences in any of the soluble mediators examined in BAL

fluid from WT or MARCH8 KO mice at day 5, 7 or 10 p.i. In terms of innate cells, it is well established that NK cells, neutrophils and “pan” macrophages can modulate IAV infections in the mouse model [257, 259-261]. Further studies are required to determine if the increased numbers of “pan” macrophages in BAL fluid from MARCH8 KO mice at day 5 p.i. could be contributing to weight loss and disease or are recruited in an effort to limit exacerbated infection in MARCH8 KO mice.

While we could detect NK cells (CD3<sup>+</sup>, NKp46<sup>+</sup>) in BAL fluids from MARCH8 KO mice, more detailed characterization revealed that NK cells from WT, but not MARCH8 KO mice, expressed cell-surface NK1.1. Due to the close proximity of genes encoding MARCH8 and NK1.1 (approximately 12 million base pairs between *March8* and *Klrb1* (NK1.1) genes) (**Figure 6.6B**), the absence of NK1.1 is likely due to a disruption of the NK locus that has remained despite over 25 backcrosses onto the C57BL/6 background. Consistent with our flow cytometry data using MARCH8 KO mice, NK cells from 129 mice do not express NK1.1 or Ly49H (reviewed in [254, 255]). Although other phenotypic markers, including NKp46, CD49b and Ly49C/D/F/I (**Figure 6.6A**), were expressed on the surface of NK cells from WT and MARCH8 KO mice, we cannot rule out the possibility that NK cells from MARCH8 KO mice may be altered in terms of their functional response to IAV infection. Certainly, NK cells can play a protective role in mice during the early stages of IAV infection [259], and a functional defect in NK cell activity could contribute to the enhanced weight loss and lung viral titres observed in MARCH8 KO mice. Experiments could be designed to compare functionality of NK cells from WT and MARCH8 KO mice (e.g. cytotoxic activity, intracellular cytokine production), or to deplete NK cells from WT and MARCH8 KO prior to and during IAV infection (e.g. using

anti-asialo GM antisera [262]). To date, the MARCH8 KO mice have been reported in only two studies [194, 222], both of which relying on isolation of specific cell subsets (not NK cells) for subsequent *in vitro* studies. However, when this issue was identified, we decided to generate new MARCH8 KO mice using CRISPR/Cas9 technology. This line of mice has now been established and will be used in future experiments to confirm the role of MARCH8 during IAV infection *in vivo*.

One of the best described roles of MARCH8 is its ability to control the turnover of molecules involved in antigen presentation, such as MHC-II and CD86, and therefore modulate adaptive immune responses. It was interesting to note that we observed differences in lung virus titres only at day 5 p.i. (using  $10^3$  and  $10^4$  PFU/mouse inoculum doses), when T cell responses were very low, and not at day 7 or 10 p.i. ( $10^3$  PFU/mouse), when CD8<sup>+</sup> T cell responses dominated inflammatory cells in the BAL. Moreover, we did not observe any significant differences in CD4<sup>+</sup> or CD8<sup>+</sup> T cell responses, including NP- and PA-specific CD8<sup>+</sup> T cell responses at any time point, consistent with no major impact on adaptive cellular immunity, at least in terms of cellular recruitment to airspaces of the lung during the acute phase of infection. Further studies could focus on dissecting antigen presentation via dendritic cells from WT vs MARCH8 KO mice and/or the activation status of CD8<sup>+</sup> T cells in the airways (e.g. intracellular cytokine/granzyme production, cell surface markers of activation such as CD69, defining proportions of naïve, effector memory, central memory and tissue resident CD8<sup>+</sup> T cells), as well as the establishment of immune memory and recall at later time-points (e.g. beyond day 28 p.i.). However, for the purposes of our study, we have demonstrated that MARCH8 KO mice show enhanced susceptibility to IAV infection, as evidenced by increased weight

loss and virus replication in the lungs at day 5 p.i., but with no detectable differences in lung virus titres at days 7 and 10 p.i., corresponding to no major differences in virus clearance. Moreover, considering results from previous chapters implicating MARCH8 in disrupting assembly and/or budding of IAV from infected cells, future studies should focus on defining virus replication at earlier time points (e.g. days 1/3 p.i.). This will allow us to focus on effects of MARCH8 on virus replication prior to recruitment of cellular components of adaptive immunity to the lung.

Although experimental evidence proposes that MARCH8 is expressed at high levels in the lung [177], the particular lung cell subsets with highest expression of MARCH8 are yet to be defined. MARCH proteins are generally expressed at low levels within mammalian cells, complicating detection of the protein using conventional techniques, such as western blot. Therefore, many studies determine mRNA levels of particular MARCH proteins, however this approach is complicated by the finding that most MARCH proteins undergo post-translational modifications that can regulate protein expression levels (reviewed in [227]). Alternatively, indirect approaches can be used to define MARCH expression, such as assessing turnover of known target proteins in different cell types. For example, upregulation of Class II MHC on particular cell subsets in MARCH1/8 KO mice has been used as an indirect measure to define MARCH8 expression in some parenchymal cell subsets such as cortical and medullary autoimmune regulator (AIRE)<sup>+</sup> thymic epithelial cells [222]. In a recent study, our laboratory performed RNA-seq on parenchymal and immune cell subsets from IAV-infected mice [150], noting that MARCH8 expression was higher in airway macrophages than in type II airway epithelial cells (AEC-II), prior to and after IAV infection. Moreover, unpublished data from our



laboratory has demonstrated enhanced cell-surface expression of MHC-II on AEC-II from MARCH8 KO mice compared to WT mice (personal communication, Dr Joel Ma and Prof. Patrick Reading, The University of Melbourne). Considering that IAV replicate productively in AEC, including AEC-II [263], it is tempting to speculate that AEC-II from MARCH8 KO mice might support enhanced IAV replication. However, although we can sort AEC-II from the lungs of naïve mice, our laboratory has not been able to successfully culture these cells to perform *in vitro* infections that could test this hypothesis. Obviously, generation of appropriate primary cultures from MARCH8 KO mice would be an important step towards addressing the importance of endogenous MARCH8 in regulating IAV replication. Currently, we do not know which cells in the nasal tissue express MARCH8, but our findings that IAV replicates to similar levels in both WT and MARCH8 KO mice (even when lung titres were significantly different) suggest that nasal epithelial cells may not express high levels of endogenous MARCH8. Establishment of flow cytometry cell sorting followed by qPCR for murine MARCH8 would allow us to gain more insight as to which airway cells might express the highest levels of murine MARCH8.

Given the possibility that NK cell functionality could be altered in the MARCH8 KO mice used herein, it would be ideal to isolate cell subsets from WT and MARCH8 KO mice and perform *in vitro* infections to assess impact on IAV replication. In this approach, any potential impacts on NK cells should not be relevant. However, for such an approach it is important to be able to generate primary cultures of an appropriate cell type expressing endogenous MARCH8. Human monocyte-derived macrophages (hMDM) and dendritic cells (hMDDC) have been reported to express high levels of MARCH8 and

hMDMs treated with siRNAs or CRISPR/Cas9 to knockdown/knockout MARCH8 released HIV-1 virions with increased infectivity [210]. Additionally, a recent study demonstrated that the abortive replication of IAV in hMDMs was associated with a defect in the assembly process [219]. Given that we could not generate primary AEC-II cultures, we aimed to evaluate the contribution of MARCH8 to abortive replication of seasonal IAV in primary mouse macrophages. Thus, PEC M $\phi$  from WT or MARCH8 KO mice were infected *in vitro* with IAV Beij89, a seasonal IAV strain which undergoes abortive replication in PEC M $\phi$  and RG-PR8-A/Viet04, a reassortant virus expressing the HA and NA from a highly pathogenic avian influenza virus (A/Viet04, H5N1) on a PR8 backbone, which our laboratory has shown to replicate productively in PEC M $\phi$ . As expected, both viruses infected PEC M $\phi$  *in vitro*, and no differences were observed in the percentage of PEC M $\phi$  expressing the viral NP at 8 hr post-infection (**Figure 6.7A**). IAV Beij89 did not grow in PEC M $\phi$  from WT or MARCH8 KO mice, confirming that MARCH8 was not the major restriction factor responsible for its inability to replicate productively in PEC M $\phi$ . As expected, RG-PR8-A/Viet04 did grow in PEC M $\phi$  from WT and MARCH8 KO mice, however we were surprised to observe reduced virus titres released from MARCH8 KO PEC M $\phi$  (**Figure 6.7B**). These results contrast those obtained using human 293T cells with DOX-inducible expression of MARCH8 where virus release was inhibited. Clearly, there are differences between seasonal IAV and HPAI H5N1 in their ability to replicate in macrophages and therefore it would be of interest to assess growth of RG-PR8-Viet04 and other H5N1 viruses in our MARCH8 DOX-inducible system to determine if MARCH8 is able to restrict HPAI H5N1 viral replication. Another possibility may be that PEC M $\phi$  display other antiviral mechanisms that can compensate for the lack of MARCH8. Alternatively, it is also possible that differences exist in antiviral activity of human and

murine MARCH proteins. While qPCR indicates that hMDMs express high levels of MARCH8 [210], we did not see differences in cell-surface expression of MHC-II by PEC M $\phi$  from WT and MARCH8 KO mice (data not shown), suggesting that these cells may not express high levels of MARCH8. Thus, differences in ability to support RG-PR8-Viet04 replication may relate to indirect effects of negligible/low level expression of MARCH8 in WT PEC M $\phi$  compared to those from MARCH8 KO mice. This will be confirmed in subsequent studies using qPCR to examine expression of murine MARCH8 in different cell types susceptible to *in vitro* infection with IAV.



## Chapter 7

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# **General Discussion**

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As we face a pandemic event resulting from the emergence of the SARS-CoV-2 virus, the importance of developing new therapeutic strategies to combat a broad range of respiratory viral infections is particularly evident. Different respiratory viruses utilize a combination of viral and host factors to replicate productively within target cells. Although viruses have developed strategies that allow them to utilise and/or manipulate “proviral” host factors to promote replication, target cells have also evolved mechanisms to limit and control viral infections. As part of cell-intrinsic antiviral immunity, host cells constitutively express or rapidly induce cellular restriction factors with the ability to antagonise viral infections at various steps in the virus replication cycle. As different viruses share common steps in their replication cycle, some cellular restriction factors show broad antiviral activity, whereas others are much more specific for particular viruses (reviewed in [16, 264, 265]). Clearly, defining the ability of putative restriction factors to act against respiratory viruses, as well as understanding the mechanisms by which they do so, is a critical first step towards the development of novel therapeutics. These novel approaches would harness the antiviral activity of particular host factors to provide protection from severe disease against a broad range of respiratory viruses.

Different viruses target particular cell subsets for replication and spread. For example, viruses such as HIV-1 and human T-lymphotropic virus type 1 (HTLV-1) have adapted to replicate productively in immune cells rather than parenchymal cells of the host. In contrast, while airway macrophages and airway epithelial cells are both susceptible to infection by seasonal IAV, viral replication is productive in epithelial cells and tends to be abortive in macrophages (reviewed in [15]). These findings suggest that while

macrophages remain susceptible to infection, they may express host restriction factors that allow them to block later stages of the IAV replication cycle. Of interest, other respiratory viruses such as respiratory syncytial virus (RSV) and rhinovirus also infect both macrophages and epithelial cells, and like IAV, replication is also abortive in macrophages [47, 266]. These findings are consistent with the idea that one or more host factors expressed in macrophages may block replication of different respiratory viruses. Identification of such restriction factors and developing strategies to induce their expression in epithelial cells could represent one avenue towards developing novel broad-spectrum antivirals against respiratory virus infections.

Recently, our laboratory performed RNA-seq analysis of airway macrophages and epithelial cells from mock- and IAV-infected mice, reporting major differences in their transcriptional activity in the steady-state and following virus infection [150]. Further analysis of putative host restriction factors, including MARCH proteins, indicated differential expression patterns between these cell types (Chapter 1, **Figure 1.8**). Given the emerging role of MARCH proteins in modulating other viral infections [191, 210-212], the aim of this thesis was to evaluate the role of MARCH-family proteins as putative restriction factors against influenza and other respiratory viruses.

### **Ubiquitination during respiratory viral infections.**

There is a growing body of evidence demonstrating the importance of ubiquitination in the modulation of viral infections. Ubiquitination and/or ubiquitination-related processes are known to target specific components of many different viruses to enhance

viral replication or to counteract host-mediated antiviral responses (reviewed in [267]). This includes respiratory viruses such as coronaviruses [268], adenoviruses (reviewed in [269], RSV (reviewed in [270]) and influenza viruses. For IAV, many viral proteins require ubiquitination to facilitate efficient viral replication, including the matrix proteins M1 [245] and M2 [271], the nucleoprotein (NP, [272]) and the polymerase complex (PB1, PB2 and PA, [273]). Additionally, many restriction factors utilize ubiquitination in an effort to limit IAV replication via promoting intrinsic antiviral responses. For example, tripartite motif (TRIM)25 [274], and Riplet [275] activate RIG-I through K63-linked ubiquitination. However, other restriction factors target specific viral proteins to promote their degradation. For IAV, TRIM14 [129] and TRIM22 [131] induce ubiquitin-mediated degradation of NP while TRIM32 was reported to mediate K48-linked ubiquitination of PB1, promoting its proteasomal degradation [132]. Hence, while targeting ubiquitination is an attractive strategy to control viral infections, this post-translational modification is also central to many essential cellular processes and modulation of ubiquitination could lead to off-target effects detrimental to cell homeostasis. While components of the ubiquitin system are often dysregulated in cancers and neurodegenerative diseases, to date there are few drugs targeting the ubiquitin system that have been approved by the FDA [276, 277]. While the complexity of the ubiquitin system presents significant challenges, further understanding of ubiquitin-mediated antiviral effects may open up new avenues to develop safe and specific antiviral therapies in the future.



### **The anti-IAV activity of MARCH8: What we know and what we don't.**

In this thesis, we developed experimental approaches to ectopically express different MARCH proteins, allowing us to subsequently evaluate their antiviral activity against influenza viruses, as well as against RSV and human metapneumovirus (hMPV). Studies described in Chapter 3 identified MARCH8 as a restriction factor against IAV and RSV. Interestingly, we found that MARCH8 inhibited these viruses at different steps of their replicative cycle. While MARCH8 did not appear to block IAV entry and early events in replication (as measured by production of newly-synthesized NP, HA, NA and M2 using flow cytometry), it did reduce the infectivity of particles released from infected cells. In contrast, expression of MARCH8 inhibited an earlier step in RSV infection, resulting in a reduced percentage of NP-positive infected cells. This differential activity may reflect differences in the replicative cycle of each virus and therefore differential spatio-temporal interactions between MARCH8 and specific viral/host components targeted by MARCH8.

IAV and RSV add to a growing list of viruses which are modulated by MARCH8 expression. In the case of HIV-1, the mechanisms underlying MARCH8-mediated restriction are broadly defined, with downregulation of cell-surface Env resulting in reduced incorporation into newly synthesized virions, although recognition of the specific host/viral proteins targeted by MARCH8 is yet to be reported [210]. For hepatitis C virus (HCV), MARCH8 acts as a proviral factor mediating K63-linked polyubiquitination of the non-structural 2 (NS2) protein, allowing its interaction with the ESCRT-0 component hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and subsequent recruitment of the ESCRT machinery, a critical step during HCV envelopment

[212]. Moreover, this allowed for subsequent identification of particular K residues in NS2 targeted for MARCH8-mediated ubiquitination (K27, K172, K173 and K212). While understanding MARCH8-mediated restriction of RSV will be the topic for future research, subsequent studies in this thesis have focussed on gaining insight into MARCH8-mediated restriction of IAV, including efforts to identify viral targets of MARCH8-mediated ubiquitination.

The E3 ligase activity of MARCH8, and therefore its ability to ubiquitinate target proteins, was critical for its ability to block the late stages of IAV infection. MARCH8 expression was associated with a reduced infectivity per particle of IAV released from infected cells, and lower levels of several viral proteins appeared to be incorporated into the released virions. Although these findings were reminiscent of MARCH8-mediated activity against HIV-1, where downregulation of cell-surface Env correlated with its reduced incorporation into nascent virions [210], the IAV HA, NA and M2 proteins were not downregulated from the surface of IAV-infected cells. These findings led us to speculate that MARCH8 was modulating the assembly and/or budding of newly-formed IAV particles. Given that the E3 ligase activity of MARCH8 was critical for its antiviral activity, we focused on the identification of viral proteins that might be targeted by MARCH8-mediated ubiquitination. Mutant viruses were generated with one or more specific lysine (K) to arginine (R) substitutions in the cytoplasmic tail (CT) of all viral proteins known to interact with the plasma membrane, namely HA, NA, and M2, and also throughout the M1 protein. However, mutant viruses were not able to overcome MARCH8-mediated antiviral activity. Although we were able to rule out ubiquitination of the CT of HA, NA and M2, it is still possible that MARCH8 ubiquitinates residues in

M1. A recent study using crystal structures of the N-terminal domain of M1, together with models of full length M1 from the orthomyxovirus infectious salmon anemia virus (ISAV), proposed structures of IAV M1 protein in solution and also interacting with a lipid bilayer [278]. Based on these models, it could be speculated that multiple K residues from the N-terminal domain of IAV M1 might also be in close proximity with MARCH8, and therefore potential targets for ubiquitination. These include residue K21, the only mutant we were unable to rescue in our IAV reverse genetic experiments. Of interest, while we detected similar levels of wild type M1 and M1-K21R proteins by flow cytometry after transient transfection of 293T cells (data not shown), we could not rescue infectious particles after the transfection with the additional 7 plasmids required to generate recombinant viruses. These results are certainly consistent with an association between the ubiquitination of M1 and IAV assembly, budding and/or release. In future studies, we could generate recombinant viruses carrying multiple IAV M1 mutations as well as viruses carrying mutations other than K-to-R substitutions at residue 21. As the IAV M1 protein is the central component during viral assembly, interacting with the viral glycoproteins (HA, NA and M2), vRNPs and the plasma membrane, we could investigate if MARCH8 is altering interactions between the different viral components and/or their localization at the cell surface using proximity ligation assays (PLA) [219] and membrane flotation assays [30, 238], respectively. In preliminary studies, we could detect differences in the interactions between certain viral proteins at the surface of IAV-infected cells by PLA (data not shown), suggesting that MARCH8 could potentially modify interactions between viral proteins and therefore virus assembly at the cell surface. Furthermore, given that IAV assembly occurs in lipid

rafts, we could also investigate if MARCH8 expression disrupts these microdomains at the cell surface.

MARCH1 and MARCH8 are generally expressed in different cell types, but are reported to target identical proteins, including CD86 and transferrin receptor [177]. Moreover, while MARCH1/8 appear to reduce infectivity of nascent HIV-1 virions by similar mechanisms [210, 211], it was striking to observe that MARCH8, but not MARCH1, could mediate potent restriction of IAV strain Beij89. Although we are yet to identify the viral/host protein/s targeted by MARCH8 during IAV infection, these findings are consistent with recognition only by MARCH8, perhaps due to its subcellular distribution, levels of expression or intrinsic biochemical features, including specific motifs in its sequence. To gain mechanistic insight into specific features of MARCH1/8 that might determine differential regulation of IAV infectivity, we designed chimeric MARCH proteins substituting different domains/regions of MARCH1 into a MARCH8 backbone. We did not identify a chimeric protein where the ability to block late stages of IAV infection was completely abrogated, although the chimeric protein expressing all cytoplasmic domains from MARCH1 was less potent in its ability to inhibit release of infectious IAV. Until we identify the specific host/viral protein targeted by MARCH8, we can only speculate as to why this chimeric protein showed a reduced potency of IAV restriction. It is possible that differences in the polarity and/or charge of the amino acids exchanged might alter the strength of interactions between the chimeric protein and protein/s targeted by MARCH8. Alternatively, it is possible that the distribution of this chimeric protein is different compared to wild type MARCH8, thereby altering the particular subcellular compartment where MARCH8 would usually encounter the target

protein. It is also possible that the chimeric protein is producing suboptimal ubiquitination of the target protein, therefore delaying its turnover. Whatever the case, our results indicate that multiple regions of MARCH8 are responsible for its antiviral activity against IAV, suggesting a complex interplay between E2(s), MARCH8, the target protein(s), and possibly other host proteins to act as adaptor molecules relevant to interactions between MARCH8 and the specific virus/host protein targets.

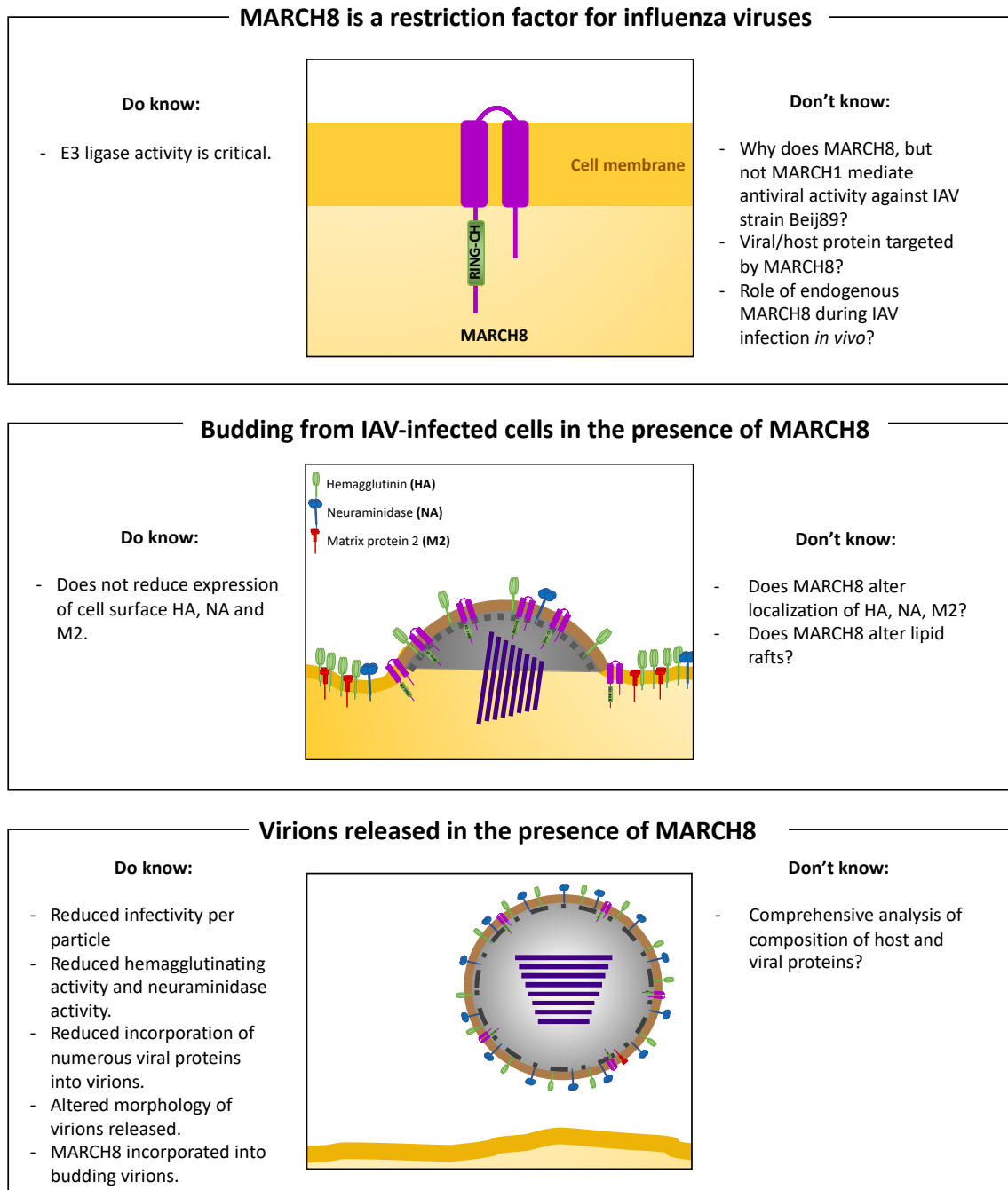
Multiple host proteins have been identified that can play important roles during the late stages of IAV infection, including trafficking of vRNPs and viral glycoproteins to the plasma membrane and also particle assembly and budding (reviewed in [18]). Of interest, host proteins such as RACK1, CD81 and F1Fo ATPase have been associated with virus assembly and budding, playing roles in the induction of membrane curvature as well as in the ‘pinching-off’ of budding particles [279-281]. It will be particularly important to evaluate the role of CD81 in MARCH8-mediated IAV antiviral activity, given that CD81 is a known target of MARCH8-mediated downregulation [182]. Furthermore, knockdown of CD81 resulted in elongated virions budding from the surface of IAV-infected cells, most likely due to an impairment at the final scission step [281]. This is of particular interest, given that our transmission electron microscopy studies showed that virions released from MARCH8-expressing cells clearly displayed a more filamentous morphology. It is also of interest to note that MARCH8 was incorporated into viral particles released from MARCH8-induced cells, suggesting that putative host protein/s targeted by MARCH8 may also be incorporated. Therefore, evaluating the protein composition of the viral particles released by mass spectrometry will allow us to confirm results from western blotting for viral proteins, as well as identify host proteins which

may serve as putative targets for MARCH8-mediated ubiquitination. Alternatively, co-immunoprecipitation experiments could also be performed in an effort to identify host proteins interacting with MARCH8 in virus-infected cells.

A number of studies have described the antiviral activity of MARCH proteins against HIV-1 [191, 210, 211]. While MARCH8 has been shown to be proviral during flavivirus infections [212], we now report that MARCH8 also mediates antiviral activity against RSV and IAV at an early and a late stage of the virus replication cycle, respectively. While we have investigated the anti-IAV activity in some detail, various questions still remain to be answered. These have been summarized in **Figure 7.1**. Identification of the host/viral protein targets of MARCH8 will be particularly informative and may provide insights regarding its ability to modulate infections by other enveloped respiratory viruses, including parainfluenza viruses and coronaviruses.

### **Challenges of working with MARCH-family proteins.**

Particular features of MARCH-family proteins presented a number of challenges when attempting to assess their ability to modulate respiratory virus infections. While transient transfection could be performed with varying efficiency, all attempts to generate cell lines with constitutive high-level expression of MARCH proteins were unsuccessful. Over time, we noticed increased cell death and/or differences in growth rates in cell lines with prolonged expression of particular MARCH proteins, which may relate to their central role in governing various aspects of cell homeostasis. Others have reported that MARCH8 overexpression in different human cells, including A549 cells,



**Figure 7.1. Overview of studies investigating the anti-IAV activity of MARCH8 described in this thesis.** Research described in this thesis has addressed aspects of (i) the MARCH8 protein itself and its ability to restrict IAV infection, (ii) budding of IAV from the surface of IAV-infected cells in the presence of MARCH8, and (iii) characteristics of virions released from infected cells in the presence of MARCH8. The figure summarises findings from the thesis, as well as important questions yet to be answered.

inhibited cell proliferation and promoted apoptosis [228], consistent with our findings. We therefore focused on utilizing a DOX-inducible system, where we could control protein expression more definitively. Compared to transient transfections with constitutive high level expression of MARCH proteins, the DOX-inducible system appeared to result in lower levels of protein expression, where immunoprecipitation in conjunction with western blot was required to detect MARCH expression. This combination of controlled, albeit reduced levels, of MARCH protein expression was used for the bulk of studies performed in this thesis.

Another major issue working with MARCH-family proteins relates to difficulties in accurate quantitation of the level of cellular protein expression. MARCH proteins are tightly regulated post-translationally and detecting protein expression using conventional techniques is not an easy task. To date, most of the information available regarding endogenous expression of different MARCH proteins is derived from qPCR analysis of mRNA. This was particularly problematic when attempting to quantitate endogenous MARCH8 expression during IAV infection, as qPCR detected only very low levels of mRNA expression in the cell lines tested (293T and A549 cell lines). When looking for alternative cell types with increased levels of endogenous MARCH8, we detected very low levels of expression in human monocytes-derived macrophages (data not shown), which is in contrast to other published studies examining MARCH8-mediated restriction of HIV-1 [210]. We are yet to examine MARCH8 expression in particular murine cell subsets that might be relevant to respiratory virus infection, but this will be pursued in the future, particularly due to the availability of a MARCH8 KO mouse. Studies performed in this thesis that examined the susceptibility of MARCH8 KO



mice to IAV infection were encouraging, as mice lacking endogenous MARCH8 showed increased viral titres in the lungs early (day 5) after infection. However, our studies also revealed an issue regarding the genetic background of the NK locus, which is located in the vicinity of the MARCH8 gene, thereby complicating interpretation of our results. This was due to the fact that we could not completely establish that the NK cell activity had not been compromised in these particular MARCH8 knockout mice. We have now used CRISPR/Cas9 to generate a new MARCH8 KO mouse which will be a first step towards complementing the results obtained using our overexpression system *in vitro* and confirming our preliminary data on the role of MARCH8 in modulating IAV infection *in vivo*. Given that a mouse model of RSV is also well described, we will also be able to investigate the ability of endogenous MARCH8 to modulate RSV infection *in vivo*. Currently, there are no published studies addressing the role of endogenous MARCH8 in modulating viral infection in a mouse model of infection, so these studies will be particularly informative.

### **Concluding remarks.**

The study of host restriction factors offers new opportunities for the development of therapeutic strategies to control viral infections of the airways. Studies presented in this thesis have demonstrated that MARCH8 is a potent restriction factor against influenza viruses, acting to block a late stage in the virus replication cycle via a mechanism that appears to be distinct to the antiviral activity of different MARCH proteins against HIV-1. Clearly, additional studies are required to definitively identify the host/viral proteins

recognised by MARCH8 that govern its ability to restrict influenza virus replication, as well as to define the role of MARCH8 in modulating IAV infection *in vivo*. However, the studies presented in this thesis define and characterize the anti-IAV activity of MARCH8 and add to a growing body of evidence that MARCH8 plays a central role in modulating many viral infections.



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## Appendix

Publications generated during this thesis:

- **Villalón-Letelier, F.**; Brooks, A. G.; Saunders, P. M.; Londrigan, S. L.; Reading, P. C., Host Cell Restriction Factors that Limit Influenza A Infection. *Viruses* **2017**, 9, (12), 376.
- **Villalón-Letelier F.**; Reading, P.C., Unraveling the role of the MOV10 RNA helicase during influenza A virus infection. *Biochem J* **2019**, 476, (6), 1005-8.
- Meischel, T.; **Villalon-Letelier, F.**; Saunders, P. M.; Reading, P. C.; Londrigan, S. L., Influenza A virus interactions with macrophages: Lessons from epithelial cells. *Cellular microbiology* **2020**, 22, (5), e13170
- Bedford JG, Infusini G, Dagley LF, **Villalon-Letelier F**, Zheng MZM, Bennett-Wood V, Reading PC and Wakim LM. Airway Exosomes Released During Influenza Virus Infection Serve as a Key Component of the Antiviral Innate Immune Response. *Front. Immunol* **2020**, 11:887.

