The role of AKT during IL-3-dependent survival signalling

Submitted by Benjamin Green, B. Sc (Hons)

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Abstract

The activation of AKT in response to cytokine receptor signalling promotes protein synthesis, cellular growth and proliferation. AKT is also thought to play a central role in maintaining cell viability by repressing apoptosis pathways, via direct or indirect regulation of the Bcl-2 protein family. To determine the role of AKT in interleukin-3 signalling, IL-3 dependent myeloid cell lines derived from mice lacking AKT1, AKT2 or AKT3 were generated. The viability of these cell lines indicated that each gene was redundant for both proliferation and survival pathways; however the deletion of AKT1 specifically, rendered cells less tolerant to low IL-3 concentrations. Conversely, the over-expression of constitutively active AKT1 or AKT2 in WT cells was sufficient to delay apoptosis in response to IL-3 withdrawal, however was not sufficient to induce proliferation or maintain long-term survival in the absence of IL-3. AKT1 prolonged survival of BIM- or BAD-deficient cells, but not cells lacking PUMA, indicating that AKT1-dependent repression of apoptosis was in part dependent on PUMA and independent of BIM or BAD. Furthermore, the over-expression of either AKT isoform required p53 but not FOXO3a to prolong viability after IL-3 withdrawal. Supporting data was observed whereby AKT over-expression is sufficient to phosphorylate and stabilise the p53 inhibitor MDM2. These results suggest that AKT1 promotes survival in response to IL-3 by phosphorylating and stabilising MDM2, thereby repressing p53 activity that would otherwise transcribe PUMA to promote apoptosis.
Declaration

This is to certify that

i) The thesis comprises only my original work towards the degree of Doctor of Philosophy except where indicated in the Preface

ii) Due acknowledgement has been made in the text to all other material used

iii) The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices

Signed

Benjamin Green
Preface

All research performed in this thesis has been performed by the author unless otherwise stated. The author would like to thank the following people for their contributions:

For the provision of the Akt−/− mice the author would like to thank Dr Brian Hemmings. For the generation and provision of the inducible lentiviral system, the author would like to thank Dr John Silke. For the generation and provision of the p53-eGFP reporter construct the author would like to thank Dr Anissa Jabbour. Furthermore, Dr Jabbour generated several of the cell lines utilised throughout the project.

The author would like to thank Anissa Jabbour for assistance with many of the figures provided by offering troubleshooting and advice as well as aiding with interpretation of results. Thank you to Chris Rifkin for performing independent confirmation in figures 4.2 and 4.3, which has been included in the statistical calculations of the figures provided. Although the author performed the experiments numerous independent times, the replicates provided by Dr Jarrod Sandow in figure 4.5 were included as they present the data the most clearly. Thank you to Dimitra Masouras for performing and analysing qRT-PCR in figure 4.7. RNA isolation was performed by the author. Thank you to Dr Rachel Lee for performing the experiments in figure 5.8

A special thank you to Dr Anissa Jabbour for assistance with technical difficulties throughout the project. Dr Jabbour’s help was invaluable and in no small part led to the completion of this thesis. A special thank you to Drs Paul Ekert, Richard Pearson and Anissa Jabbour for discussion and assistance with the project as well as time and effort spent on analysis and interpretation of results.
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Without doubt however, the most important thank you I would like to pass on is that to my supervisor Dr Paul Ekert. Volumes could be filled with adjectives describing how we spent the last seven years; tolerance, friendship, supportive—plus too many more to write. The most profound mark of respect I can show however is as follows; never have I met somebody who deserves the title mentor more than you do. Thank you very much.

To my ever-suffering family; to Daniel and Noah, thank you for staying out of my way for seven years. To my Mum and Dad, without your constant support and drive I could not have finished. At times, a PhD can drive people crazy, and our relationship might have strained, but never broken—testament to the strength and love you two have provided.

To my children, dear Riley, you have been an absolute nuisance, and I thank you for every second of it. To my un-named second child, by provoking your mother you indirectly got me into all new trouble, but I still cannot wait to meet you at the end of the year. I love you both very much.

Finally, to my ever-suffering, ever-supporting wife. Dear Maja, I am in the position of finishing this thesis because of you. Again, too much has to be said to write here, but we have been through so much, a new relationship, a wedding, children and a never-ending thesis. I love you so much, and nothing, even years of thesis stress, has changed my love for you. You deserve the biggest thank you of all.
Publications

Research in this thesis contributes to the following publications:


Table of Contents

ABSTRACT .......................................................................................................................... 1
DECLARATION ................................................................................................................... 2
PREFACE ............................................................................................................................ 3
ACKNOWLEDGEMENTS ..................................................................................................... 4
PUBLICATIONS ................................................................................................................ 5
TABLE OF CONTENTS ....................................................................................................... 6
LIST OF TABLES AND FIGURES ..................................................................................... 11
ABBREVIATIONS ............................................................................................................... 13
GROWTH FACTORS ........................................................................................................ 15
1. INTRODUCTION .......................................................................................................... 17

1.1 CYTOKINE SIGNALLING ........................................................................................... 17
    1.1.1 The Roles and Functions of Cytokines .............................................................. 17
    1.1.2 Cytokine Receptors .......................................................................................... 20
    1.1.3 βc cytokine receptor family .............................................................................. 24
    1.1.4 Signal Transduction ......................................................................................... 30

1.2 PROTEIN KINASE B / AKT ...................................................................................... 35
    1.2.1 AKT Structure and Activity .............................................................................. 35
    1.2.2 Isoforms of AKT .............................................................................................. 37
    1.2.3 Roles and Functions of AKT .......................................................................... 39
    1.2.4 The PI-3K/AKT Axis in Cancer ...................................................................... 43

1.3 APOPTOSIS ................................................................................................................ 46
    1.3.1 Bcl-2 proteins and Intrinsic Apoptosis ............................................................. 46
    1.3.2 The Regulation of Bcl-2 Family by IL-3 ............................................................ 50
    1.3.3 The Regulation of Bcl-2 Family Members by AKT ........................................ 51

1.4 PROJECT AIMS ......................................................................................................... 55
    1.4.1 Aim 1: To elucidate the role of AKT during IL-3 signalling ............................ 55
1.4.2 Aim 2: To determine which Bcl-2 family proteins are required for AKT to maintain survival after IL-3 withdrawal ................................................................. 55

1.4.3 Aim 3: To determine how AKT regulates the Bcl-2 family of proteins to maintain survival after IL-3 withdrawal ................................................................. 56

2 MATERIALS AND METHODS .............................................................................. 57

2.1 CELL LINES AND CULTURE ......................................................................... 57
2.2 CLONING ........................................................................................................ 57
2.3 LENTIVIRAL PRODUCTION AND INFECTION .............................................. 60
2.4 INTRACELLULAR STAINING ......................................................................... 60
2.5 IMMUNOBLOTTING ........................................................................................ 60
2.6 VIABILITY AND CLONOGENIC ASSAYS ...................................................... 62
2.7 CELL CYCLE ANALYSIS .............................................................................. 62
2.8 REAL TIME PCR ............................................................................................ 62
2.9 GENERATION OF CLONES EXPRESSING THE 4-OHT INDUCIBLE ΔPH-AKT OVER-EXPRESSION LENTIVIRUS ................................................................. 62
2.10 STATISTICS AND STATISTICAL ANALYSIS .............................................. 65

3. AKT IS RESPONSIBLE FOR MAINTAINING VIABILITY AT LOW IL-3 CONCENTRATIONS ................................................................................................. 67

3.1 WT FDMs DO NOT HAVE DISTINCT SURVIVAL AND PROLIFERATIVE PATHWAYS IN RESPONSE TO CYTOKINE TITRATION ......................................................... 67
3.2 THE GENERATION OF AKT+/− IL-3 DEPENDENT CELL LINES INDICATES EACH AKT ISOFORM IS REDUNDANT FOR IL-3 DEPENDENT SIGNALLING .............................................. 70
3.3 THE DELETION OF EACH AKT ISOFORM DOES NOT ALTER APOPTOTIC KINETICS AFTER IL-3 WITHDRAWAL ................................................................. 70
3.4 AKT1 IS REQUIRED TO MAINTAIN VIABILITY AT LOW IL-3 CONCENTRATIONS ................................................................. 72
3.5 ΔPH-AKT OVER-EXPRESSION INCREASES SENSITIVITY TO LOW IL-3 CONCENTRATIONS FOR SURVIVAL SIGNALLING ................................................................. 74
3.6 AKT INHIBITOR VIII DEPHOSPHORYLATES AKT AT S473, EFFECTIVELY SHUTTING DOWN AKT ACTIVITY ................................................................. 77
3.7 AKT INHIBITOR VIII INDUCES APOPTOSIS TO THE SAME EXTENT AS PI3K INHIBITORS ................................................................. 79
3.8 AKT INHIBITOR INDUCED APOPTOSIS IS MOST POTENT AT LOW IL-3 CONCENTRATIONS ................................................................. 81
3.9 **Over-expression of ΔPH-AKT1 is insufficient to prevent apoptosis caused by PIK-75** ................................................................. 83
3.10 UO126 dephosphorylates p42/p44, effectively shutting down MEK activity .. 85
3.11 MEK inhibition induced apoptosis does not require AKT1 or p53 ............ 85
3.12 **The over-expression of ΔPH-AKT is not sufficient to promote cell-cycling but delays G1 arrest after IL-3 starvation.** ......................... 88
3.12.1 *The over-expression of ΔPH-Akt1 increases Forward Scatter characteristics of cells on flow cytometry* ............................................... 89
3.13 ΔPH-AKT over-expression is insufficient to promote cell cycling and proliferation independently of IL-3........................................... 92
3.14 **Conclusions** ........................................................................................................... 94

4. **AKT1 regulates PUMA to maintain viability after IL-3 withdrawal** ................................................................. 96

4.1 **Puma is required for ΔPH-AKT1, but not ΔPH-AKT2 to maintain viability after IL-3 withdrawal.** ............................................................ 98
4.2 **Puma is required for AKT1 to maintain clonogenic potential after IL-3 withdrawal** ............................................................. 101
4.3 **Deregulation of AKT over-expression is not the cause of apoptosis after prolonged IL-3 deprivation** ...................................... 106
4.4 **Puma expression after IL-3 withdrawal is down-regulated by AKT over-expression.** ............................................................... 106
4.5 **PUMA, but not Mcl-1, is regulated by ΔPH-AKT1 over-expression in Bax/\(^{-}\)/Bak/\(^{-}\) cells after IL-3 starvation** .......................... 110
4.6 **Transcription of PUMA, but not Mcl-1, is regulated by ΔPH-AKT1 over-expression after IL-3 withdrawal** ............................. 113
4.7 **Conclusions** ........................................................................................................... 113

5. **AKT regulates P53, controlling PUMA transcription, to maintain viability after IL-3 withdrawal** ............................................................. 116

5.1 **P53 is required for ΔPH-AKT to maintain viability after IL-3 withdrawal.** 116
5.2 **P53 is required for AKT to maintain clonogenicity after IL-3 withdrawal.** 117
5.3 AKT INHIBITION INDUCED APOPTOSIS REQUIRES P53, BUT NOT PUMA .......................... 120
5.4 AKT INHIBITION DECREASES RELATIVE CLONOGENICITY OF WT AND \( p53^{−/−} \) CELLS, ALBEIT TO DIFFERENT EXTENTS ................................................................. 121
5.5 AKT INHIBITION, BUT NOT MEK INHIBITION REGULATES PUMA VIA A P53 DEPENDENT MECHANISM ........................................................................................................ 124
5.6 AKT INHIBITION DOES NOT PROMOTE EXPRESSION OF THE PUMA-eGFP REPORTER CONSTRUCT ........................................................................................................ 127
5.7 DELETION OF PUMA OR P53 PROVIDES A SIMILAR SURVIVAL ADVANTAGE IN LOW IL-3 CONCENTRATIONS AS AKT OVER-EXPRESSION ........................................ 129
5.8 DELETION OF PUMA OR P53 PROLONGS CLONOGENIC VIABILITY IN LOW IL-3 CONCENTRATIONS COMPARED TO WT CELLS ..................................................... 129
5.9 CONCLUSIONS .................................................................................................................. 132

6. AKT PHOSPHORYLATES MDM2 IN ORDER TO PREVENT P53-DEPENDENT TRANSCRIPTION ........................................................................................................ 133

6.1 IL-3 RESTIMULATION RESULTS IN AKT ACTIVATION AND MDM2 PHOSPHORYLATION ..... 133
6.2 ΔPH-AKT1 OVER-EXPRESSION IS SUFFICIENT TO PHOSPHORYLATE AND STABILISE MDM2 ... ................................................................................................................................. 135
6.3 INHIBITION OF MDM2-p53 COMPLEXING INDUCES APOPTOSIS IN WT, BUT NOT \( p53^{−/−} \) CELLS ......................................................................................................................... 135
6.4 AKT OVER-EXPRESSION IS INSUFFICIENT TO PROMOTE VIABILITY AFTER IL-3 WITHDRAWAL IN THE PRESENCE OF NUTLIN-3 .................................................. 138

6.4.1 AKT1 over-expression is insufficient to promote viability after prolonged IL-3 withdrawal in the presence of Nutlin-3 ................................................................. 138
6.5 AKT1 IS NOT REQUIRED FOR NUTLIN-3 INDUCED APOPTOSIS ........................................... 141
6.6 CONCLUSIONS .................................................................................................................. 143

7. DISCUSSION ......................................................................................................................... 144

7.1 LIMITING IL-3 CONCENTRATIONS DO NOT RELIABLY SEPARATE SURVIVAL AND PROLIFERATIVE SIGNALS. ....................................................................................... 144
7.2 AKT1 IS REQUIRED FOR CELL SURVIVAL IN LIMITING IL-3 SIGNALLING CONCENTRATIONS 145
7.3 ΔPH-AKT over-expression requires PUMA to maintain survival in the absence of IL-3 .......................................................... 147

7.3.1 AKT is not sufficient to maintain Mcl-1 expression .............................. 148

7.4 AKT regulates p53-dependent PUMA transcription .................................. 149

7.5 AKT phosphorylates and stabilises MDM2 to inhibit p53 dependent transcription ................................................................. 153

8. IMPLICATIONS .................................................................................. 156

9. CONCLUSIONS .................................................................................. 157

10. REFERENCES ..................................................................................... 159

11. APPENDICES .................................................................................... 177

11.1 siRNA ............................................................................................. 177
List of tables and figures

| Figure 1.1 | Haematopoiesis | 19 |
| Figure 1.2 | Structure of Type-1 cytokine receptor subgroups | 23 |
| Figure 1.3 | A schematic illustration of the βc chain | 27 |
| Table 1 | βc chain tyrosine residues | 28 |
| Figure 1.4 | IL-3 dependent survival pathways | 32 |
| Figure 1.5 | Current perspectives on AKT activation | 36 |
| Figure 1.6 | The role of Akt in normal cell cycling | 41 |
| Figure 1.7 | The role of AKT in protein synthesis | 42 |
| Figure 1.8 | Bcl-2 family members used in this thesis and their interactions | 48 |
| Figure 1.9 | Bcl-2 family regulation by AKT to promote survival | 54 |
| Figure 2.1 | Schematic map of the HoxB8 retrovirus | 58 |
| Figure 2.2 | Schematic map of the double vector inducible lentiviral system | 59 |
| Table 2 | A list of antibodies utilised | 61 |
| Figure 2.3 | HA-Akt over-expression is induced by 4-OHT | 64 |
| Figure 2.4 | Phosphorylated (active) Akt is induced by 4-OHT even in the absence of IL-3 | 66 |
| Figure 3.1 | Cell cycle withdrawal occurs independently of apoptosis | 69 |
| Figure 3.2 | Deletion of each Akt isoform does not alter apoptotic kinetics | 71 |
| Figure 3.3 | Akt1 is required for survival signalling at low concentrations of IL-3 | 73 |
| Figure 3.4 | Induced Akt over-expression increases survival in low IL-3 concentrations | 76 |
| Figure 3.5 | AKT inhibitor VIII dephosphorylates Akt at S473 | 78 |
| Figure 3.6 | AKTi VIII and LY294002 promote apoptosis in the presence or absence of IL-3 | 80 |
| Figure 3.7 | Cells cultured in low concentrations of IL-3 are most sensitive to apoptosis induced by Akt inhibition | 82 |
| Figure 3.8 | Over-expression of Akt is insufficient to prevent Pi-3k inhibition induced cell death | 84 |
| Figure 3.9 | UO126 decreases Erk phosphorylation | 86 |
| Figure 3.10 | Mek inhibition induced apoptosis is not dependent on p53 or Akt1 | 87 |
| Figure 3.11 | Induced Akt over-expression does not promote S-phase transition independently of IL-3, but delays S-phase exit after IL-3 withdrawal | 90 |
| Figure 3.12 | Induced Akt1 over-expression increases the forward scatter profile of cells independently of IL-3 | 91 |
| Figure 3.13 | Induced Akt over-expression does not promote S-phase re-entry or proliferation independently of IL-3 | 93 |
| Figure 4.1 | Determining the requirements for Akt dependent survival. | 97 |
| Figure 4.2 | Akt over-expression requires Puma to prolong survival after IL-3 withdrawal | 99 |
| Figure 4.3 | Akt over-expression requires Puma to prolong clonogenic potential after IL-3 withdrawal | 103 |
| Figure 4.4 | Prolonged IL-3 deprivation does not affect induction of Akt over-expression | 107 |
| Figure 4.5 | Puma and Mcl-1 expression is delayed by Akt over-expression | 108 |
| Figure 4.6 | Puma expression is delayed by Akt1 over-expression | 112 |
| Figure 4.7 | Puma transcription is repressed by Akt over-expression | 114 |
| Figure 5.1 | Akt over-expression requires p53 to prolong survival after IL-3 withdrawal | 118 |
| Figure 5.2 | Akt over-expression requires p53 and FoxO3a to prolong clonogenic potential after IL-3 withdrawal | 119 |
| Figure 5.3 | p53<sup>−/−</sup> cells are resistant to apoptosis stimulated by Akt inhibition | 122 |
| Figure 5.4 | p53<sup>−/−</sup> cells have reduced clonogenicity caused by Akt inhibition | 123 |
| Figure 5.5 | AKT VIII and UO126 suggest no crosstalk between the Akt and Mek/Erk pathways | 125 |
| Figure 5.6 | AKTi VIII does not promote p53 reporter GFP expression | 128 |
| Figure 5.7 | The deletion of either p53 or Puma increases sensitivity to low IL-3 concentrations | 130 |
| Figure 5.8 | The deletion of either p53 or Puma increases relative clonogenicity of cells at multiple IL-3 concentrations | 131 |
| Figure 6.1 | IL-3 restimulation promotes Akt, Gsk3 and Mdm2 phosphorylation | 134 |
| Figure 6.2 | AKT1 over-expression is sufficient to phosphorylate and stabilise Mdm2 | 136 |
| Figure 6.3 | Inhibition of Mdm2 induces apoptosis in the presence of IL-3 in WT but not p53<sup>−/−</sup> cells | 137 |
| Figure 6.4 | The protective effect of Akt1 over-expression is blunted by Nutlin-3 | 139 |
| Figure 6.5 | The protective effect of AKT1 over-expression in the absence of IL-3 is blunted by MDM2 inhibition | 140 |
| Figure 6.6 | Mdm2 inhibition induced apoptosis does not require Akt1 | 142 |
| Figure 7.1 | Model of IL-3 dependent Akt signalling | 155 |
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>N/A</td>
</tr>
<tr>
<td>4-OHT</td>
<td>4-HydroxyTamoxifen</td>
</tr>
<tr>
<td>AKTi</td>
<td>AKT selective 1/2 isozyme Inhibitor VIII</td>
</tr>
<tr>
<td>AML</td>
<td>Acute Myeloid Leukaemia</td>
</tr>
<tr>
<td>APAF</td>
<td>Apoptotic Protease Activating Factor</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2 Antagonist of cell Death</td>
</tr>
<tr>
<td>BAK</td>
<td>Bcl-2 Antagonistic Killer protein</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl-2 Associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell Lymphoma 2</td>
</tr>
<tr>
<td>Bcl-w</td>
<td>Bcl-2 like 2</td>
</tr>
<tr>
<td>Bcl-X</td>
<td>Bcl-2 like 1</td>
</tr>
<tr>
<td>BH domain</td>
<td>Bcl-2 Homology domain</td>
</tr>
<tr>
<td>Bid</td>
<td>BH-3 Interacting domain Death agonist</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2 Interacting Mediator of cell death</td>
</tr>
<tr>
<td>BOK</td>
<td>Bcl-2 related Ovarian Killer protein</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin Dependent Kinase</td>
</tr>
<tr>
<td>c-FMS</td>
<td>McDonough Feline Sarcoma Viral (v-FMS)</td>
</tr>
<tr>
<td>c-KIT</td>
<td>N/A</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony Stimulating Factor</td>
</tr>
<tr>
<td>Cyt-c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>Epo</td>
<td>ErythroPOietin</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal Regulated Kinases</td>
</tr>
<tr>
<td>FDMs</td>
<td>Factor Dependant Myeloid Cells</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FLT-3</td>
<td>Fms-reLated Tyrosine kinase 3</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>FoxO3a</td>
<td>Forkhead Transcription Factor O3a</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony Simulating Factor</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoetic Stem Cell</td>
</tr>
<tr>
<td>iAKT</td>
<td>Inducible AKT</td>
</tr>
<tr>
<td>IL-3α</td>
<td>IL-3 specific alpha chain</td>
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<tr>
<td>IL-X</td>
<td>InterLeukin X</td>
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<tr>
<td>JAK</td>
<td>JAnus Kinase</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<td>Myeloid Cell Leukaemia</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony Simulating Factor</td>
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<tr>
<td>Mdm2</td>
<td>Mouse Double Minute 2</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial Outer Membrane Permeabilisation</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PH domain</td>
<td>Plekstrin Homology Domain</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3 Kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Dipospho(4,5)-inositol</td>
</tr>
<tr>
<td>PIP3</td>
<td>Triphospho(3,4,5)-inositol</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and TENsin homologue</td>
</tr>
<tr>
<td>Puma</td>
<td>p53 Upregulated Mediator of Apoptosis</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
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<td>SCF</td>
<td>Stem Cell growth Factor</td>
</tr>
<tr>
<td>SH domain</td>
<td>Src Homology domain</td>
</tr>
<tr>
<td>Tpo</td>
<td>ThromboPOietin</td>
</tr>
<tr>
<td>WT</td>
<td>WildType</td>
</tr>
<tr>
<td>βc</td>
<td>Beta common chain</td>
</tr>
<tr>
<td>γc</td>
<td>Gamma common chain</td>
</tr>
<tr>
<td>ΔPH- myr-AKT</td>
<td>Pleckstrin Homology domain Deleted, MYRistoylated, over-expressed AKT</td>
</tr>
</tbody>
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# Growth Factors

The following is a list of growth factors mentioned within the thesis, including a brief description of their role and function.

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Role and Description</th>
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<tbody>
<tr>
<td>IL-3</td>
<td>Interleukin-3 supports production of a range of haematopoietic cells, mostly of the myeloid lineage. Cellular functions include promoting proliferation and differentiation and the repression of apoptosis.</td>
</tr>
<tr>
<td>IL-7</td>
<td>As with IL-3, IL-7 supports development of a range of haematopoietic cells, although these cells are mostly of the lymphoid lineage.</td>
</tr>
<tr>
<td>Epo</td>
<td>Erythropoietin is responsible for promoting red blood cell production.</td>
</tr>
<tr>
<td>Tpo</td>
<td>Thrombopoietin promotes maturation of platelets inversely proportional to the number of circulating platelets.</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony Stimulating Factor promotes the differentiation of cells into granulocyte/macrophage lineage.</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony Stimulating Factor promotes differentiation of immature cells towards macrophages.</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony Simulating Factor similar to M-CSF, this growth factor promotes differentiation of immature cells into Granulocytes.</td>
</tr>
<tr>
<td>c-KIT/SCF</td>
<td>Mast/Stem Cell Growth Factor promotes proliferation of Stem cells.</td>
</tr>
<tr>
<td>c-FMS/CSF</td>
<td>Colony Stimulating Factor promotes the production, differentiation, and function of macrophages.</td>
</tr>
<tr>
<td>FLT-3</td>
<td>Fms-Related Tyrosine Kinase 3</td>
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<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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1. Introduction

1.1 Cytokine Signalling

Cytokines are growth factors specifically associated with cells of the haematopoietic lineage, which transduce a variety of signals. They are an essential part of the mechanisms that communicate ‘messages’ from neighbouring cells and the cellular environment that indicate whether cells should proliferate, differentiate, or commit programmed suicide by initiating the intrinsic cell death programs, apoptosis [1, 2]. As the life span of the majority of myeloid cells is typically a few days, haematopoiesis must be continuously regulated to ensure that organisms can maintain a steady number of cells. Haematopoiesis must also remain plastic enough to meet increased demand, for example, at times of mounting an inflammatory response or when responding to reduced cell numbers such as during hypertrophy caused by anaemia or during recovery from cytotoxic cancer treatments [3, 4].

The function of cytokines as messengers, allows a cell to interpret the extracellular environment, of which they are a part. In healthy bone marrow for instance, Interleukin-3 (IL-3) controls a number of cellular responses including maintaining stem cell survival and proliferation. When IL-3 is no longer detected by these stem cells, they activate their intrinsic apoptotic response [5-7].

One archetypal role of IL-3 is during pathogenic invasion. In response to pathogen recognition, T-cells release large amounts of IL-3 into the bloodstream. Haematopoietic Stem Cells (HSCs) are stimulated by these increased cytokine levels, responding by increasing peripheral white blood cell numbers [8, 9]. The processes regulated by cytokines include those typically deregulated during tumourigenesis. As such, cytokine insensitivity has been described as one of the ‘hallmarks of cancer’ [10].

1.1.1 The Roles and Functions of Cytokines

Cytokines as a collective are most notable for their capacity to create the entire haematopoietic system from a single renewable, multipotent Haematopoietic Stem Cell. The differentiation patterns and proliferative cycle of the haematopoietic system relies on tight regulation and control of hierarchical, successively more mature blood cells [11]. For example, cells of the myeloid and lymphoid lineage differentiate due to
the cytokine signals involved; Interleukin-7 (IL-7) directs cells towards a lymphoid fate whilst IL-3 provides the stimulus for myeloid progenitor differentiation. The generation of a mature blood cell would therefore require a very specific combination of growth factors at specific phases of maturation. Figure 1.1 highlights the hierarchical structure of the haematopoietic system, and some of the specific cytokines required to generate each indicated cell type [12, 13].

The first cytokine to be purified and cloned was Erythropoietin (EPO) [14, 15]. The isolation of EPO helped researchers understand how red blood cells were consistently maintained at a basal level, even after copious bleeding. Later evidence highlighted the kidneys role in regulating transcription of EPO, its release into the blood stream, and its detection by the bone marrow, which responds by increasing red blood cell production [16, 17].

Platelet levels were reasoned to be regulated by a similar mechanism, although the purification of Thrombopoietin (TPO) proved more difficult [18]. Unlike EPO, TPO was found to be constitutively expressed, yet degraded by circulating platelets. Therefore, platelet production is inversely proportional to the levels of circulating platelets, leading to a tightly regulated system of thrombopoiesis [19].

Whilst EPO and TPO were cytokines responsible for promoting cellular proliferation, Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)[20, 21], Macrophage Colony Stimulating Factor (M-CSF)[22] and Granulocyte Colony Stimulating Factor (G-CSF)[23] were initially observed to increase the tendencies of bone marrow cells to become macrophages and granulocytes. As such, it was suggested that these growth factors were instrumental in directing cellular determination. This contradiction of observed behaviours inevitably led to the question of what the specific functions of cytokines are.
The role of Akt during IL-3 survival signalling

Figure 1.1: Haematopoiesis
An illustration of the haematopoietic system deriving from a single haematopoietic stem cell. Included are some of the cytokines responsible for the promotion of growth or differentiation of the indicated lineages. Interleukin-3 is highlighted in red to give an indication of cell types it regulates, as this thesis will focus heavily on IL-3 signalling.
Clinical uses of cytokines

A healthy haematopoietic system tightly regulates cytokine signalling to maintain a base-line immune response in case of pathogenic assault. When a pathogen is detected, a sudden and significant increase in cytokine signalling promotes growth/differentiation of the required white blood cells to combat infection [24-26]. Unfortunately, patients suffering from immunosuppression are incapable of initiating this response. For example, during the treatment of Acute Myeloid Leukaemia (AML) the cytotoxic effects of chemotherapies result in the ablation of diseased cells, but may also cause toxic cell death in healthy cells as well [27-29]. By targeting the rapidly dividing oncogenic sub-populations, chemotherapeutics often induce cell death in the healthy HSCs in turn. This results in myelosuppression, a lowering of the patient’s white cell count exposing patients to an increased risk of serious infection from plethora of pathogens that would otherwise be harmless. Recombinant cytokines such as Filgrastim have been utilised to boost white cell production in patients undergoing chemotherapy. Filgrastim, the trade name of commercially produced recombinant G-CSF, is given to immunosuppressed patients to promote growth and differentiation of the remaining Haematopoietic stem cells to support white cell proliferation [30-34].

It should be noted that in some cases cytokine treatment has been directly utilised to combat tumour growth. For example, recombinant interferon has been used to promote the immune response towards tumour antigens. By promoting proliferation of healthy white blood cells, particularly natural killer cells, interferon treatment can slow the growth of diseased tumour cells. Obviously, these compounds cannot be used against haematological malignancies [35-38].

Cytokine Receptors

Cytokine receptors form multiple non-covalent bonds with their associated ligand. As these bonds are relatively weak, multiple surfaces of the ligand and receptor must come into contact to initiate signal transduction, requiring a high degree of coordination and specificity. The exposure of certain amino acid side chains allows ligands to lock into receptor binding domains, thereby initiating an activation event, such as a conformational change of the receptor complex in the cytosolic region.
Cytokine receptors are grouped into two different classes; the type-I class possessing a WSXWS domain, or the type-II class without the WSXWS domain [39-41].

1.1.2.1 Receptor Tyrosine Kinases

The receptor tyrosine kinases (RTKs) are kinases possessing a trans-membrane domain. Certain RTKs that have been identified as potent proto-oncogenes include; c-KIT[42, 43], c-FMS[44] and Fms-Like Tyrosine Receptor-3 (Flt-3)[45, 46]. Further RTKs include Platelet Derived Growth Factor Receptor (PDGF-R), Fibroblast Growth factor Receptor (FGF-R), the erbB gene family of Epidermal Growth Factor Receptors (EGF-R) and the Insulin Receptor.

As an example, the model RTK Platelet-Derived Growth Factor (PDGF) and its conjugate receptor consists of two distinct sub-units, α and β, capable of associating as either homo- or heterodimers. The binding of the signalling ligand assists in dimerising the receptor complex, therefore bringing the cytoplasmic tails into close proximity. Trans-phosphorylation results in tyrosine residues accepting phosphate groups, leading to either activation of the catalytic kinase domains of RTKs, or serving as recognition sites for substrates. The recognition domain for downstream substrates is highly specific, allowing the docking and activation of certain substrates, for example those sharing the Src Homology-2 (SH-2) or SH-3 domain. The phosphorylation of these kinase domains therefore acts as a highly specific signalling switch for initiating a cascade of events for amplifying the extracellular signal [47, 48].

1.1.2.2 Type-I cytokine receptors

Type-I cytokine receptors include many of the Interleukin receptors, the EPOR, GM-CSFR, G-CSFR and TPOR [49]. Four cysteine residues are conserved close to the transmembrane domain, which are believed to maintain the structural integrity of the receptor complex. Similarly, a W-S-X-W-S sequence is conserved which provides a recognition site within the receptor complex, where X could be any amino acid. This site is thought to orientate support kinases/proteins at the cytoplasmic domain of the receptor. It is also possible that peptides binding directly to the WSXWS sequence serve to allosterically inhibit further activity of the receptor complex [50-53].
Many of these receptors share common components responsible for structure and activity as is shown in figure 1.2. These common components determine the subgroup of cytokine receptor; those sharing the $\beta_{\text{common}}$ peptide, sharing the $\gamma_{\text{common}}$ peptide or sharing the gp130 peptide. The combination of these shared subunits with ligand specific subunits forms the very specific activation topology for each receptor. For example, the IL-3, IL-5 and GM-CSF receptors share the $\beta_c$-chain signalling peptide, however the $\alpha$-chain of each receptor is unique to each ligand. The $\alpha_{\text{IL-3}}/\beta_c$ receptor for instance binds to IL-3 with very high specificity and affinity, but does not allow for receptor activation by GM-CSF [54-56].

The oligomerisation of the ligand and receptor subunits brings key regions of the receptors into proximity. These regions provide docking sites for supporting cytoplasmic proteins such as Janus Kinase 2 (Jak2). It should be noted that many of the Type-I cytokine receptors lack intrinsic kinase activity and therefore rely on cytoplasmic kinases such as Jak2 [57]. Once the entire signalling complex is assembled, Jak2 initiates the signalling cascades, although the precise mechanism by which these steps occur is not well understood.

**1.1.2.3 Type-II cytokine receptors**

Type-II cytokine receptors encompass a catch-all family of receptors that do not share the WSXWS domain. Although they serve a similar purpose to the type-I cytokine receptors, only short sequences of homology exist between various family members. This group of receptors includes the Interferon-$\alpha$, -$\beta$ and -$\gamma$ receptors [41], however, as the central theme of this thesis focuses on the $\beta_{\text{common}}$ family of receptors these will instead be focused on.
Figure 1.2: Structure of Type-1 cytokine receptor subgroups

An illustration of some of the common elements of the type-1 cytokine receptor. Three sub-groups are identifiable, those that share a common β peptide, those that share a common γ peptide and those that share the gp130 peptide (43).
1.1.3 βc cytokine receptor family

The IL-3, IL-5 and GM-CSF (also referred to as CSF2) receptors consist of a high specificity unique α-chain and the high affinity βc-chain responsible for signal dissemination [7, 54-56]. The unique α-chain of each receptor complex is critical to the receptors specificity for each ligand. Although the βc-chain is absolutely required for all signal transduction, there are clinical associations between alpha chain expression and disease. In AML, for example, the over-expression of the IL-3 α-chain in the leukaemic stem cell population is associated with a poor prognosis [58], and as such is being explored as a potential chemotherapeutic target [59].

The common βc-chain on the IL-3, IL-5 and GM-CSF receptors interacts with all three ligands, promoting some degree of overlap between their regulation of hematopoietic cell signalling [60]. GM-CSF is produced by a number of different cell types, including activated T cells, B cells, macrophages, mast cells, endothelial cells and fibroblasts, in response to cytokine or immune and inflammatory stimuli [61]. IL-5 is a T cell-derived factor that promotes the proliferation, differentiation and activation of eosinophils. In mice, IL-5 is also a growth and differentiation factor for B cells [62, 63]. Meanwhile, IL-3, also known as mast cell growth factor, is a pleiotropic factor produced primarily by activated T cells. IL-3 can stimulate the proliferation and differentiation of pluripotent hematopoietic stem cells, as well as various lineage committed progenitors [64, 65].

As the majority of this thesis will focus on murine cells, one further receptor should be mentioned which utilizes the βc-chain. The murine genome encodes a βIL-3 subunit, which transcribes a beta-chain that only binds to the αIL-3 chain and the IL-3 ligand. This IL-3 specific beta-chain thus forms a unique receptor capable of IL-3 signalling independently of the βc-chain. Dissecting the survival pathways specifically activated by IL-3 may therefore prove problematic when using murine models, as it is unknown whether the IL-3 specific receptor complex signals via the same pathways as the IL-3 receptor sharing the βc-chain. Whilst our understanding of the βc-chain is lacking, it is evident that the deletion of the βIL-3 subunit does not alter normal IL-3 signalling [66]. Furthermore, deletion of the βc-chain results in the disruption of all IL-3 signalling [7, 66, 67]. This indicates that the not only is the βIL-3 subunit at most,
redundant, for normal survival signalling, but it is insufficient to promote normal IL-3 signalling in the absence of the βC-subunit.

One intriguing question concerns the specific signals conveyed by three unique cytokines when their signalling domains are essentially identical. Presumably the α-chain plays a key component in specifying the specific signal broadcast, although the mechanisms involved remain elusive [68, 69]. It is possible however, that the different signals promoted by each cytokine are related to the cell type expressing each receptor. For example, the IL-3 receptor is predominately expressed on stem cells or myeloid progenitor cells. Does it then follow that IL-3 promotes the proliferation and differentiation of these stem cells, or do stem cells express IL-3 receptors and therefore respond specifically to the IL-3 ligand? Similarly, do more mature cells express GM-CSF receptors, or does GM-CSF promote cellular maturation?

These cytokines also contribute to the differentiation and function of leukocyte subpopulations and have clinical importance in protective immunity and in the pathophysiology of a spectrum of immunologic diseases that are as diverse as allergy and asthma, pulmonary alveolar proteinosis, neurodegenerative diseases, and malignancies. Delineating the biology of these cytokines is enabling the development of new strategies for diagnosing and treating these diseases and modulating immune responses [70].

1.2.3.1 βc-chain structure and function

Homology based analysis of the βC-chain showed two Fibronectin III (FN III) homology domains and the common WSXWS motif of type-1 cytokine receptors. Further features of the βc-chain are the ‘Box1’ and ‘Box2’ domains, a consensus region for JAK2 docking. Mutational analysis of the Box domains indicates that JAK2 binding is critical for both survival and proliferation signalling from the βc-chain [71-73]. The solving of the crystal structure of the GM-CSF receptor allowed further significant insight into the functioning of the βc-chain [57, 72, 74].

Although tyrosine phosphorylation is the key initiating event in βc-chain signalling, the βc polypeptide has no intrinsic tyrosine kinase activity. Instead, the oligomerisation of α-chain, βc-chain and ligand brings key regions of the receptors into proximity, supporting the docking of JAK. Multiple activated receptor complexes are
then capable of trans-phosphorylating further tyrosine residues of the βc-chain [57]. Eight tyrosine residues have been identified as being specifically phosphorylated upon receptor activation. Each has the capacity to act as a docking site for adaptor molecules involved in the initiation of specific signalling pathways (Figure 1.3).

Mutational analysis has helped dissect the role these residues play in signalling. Most of these experiments involve over-expression of βc-chain bearing tyrosine mutations in varying combinations in cells that are dependent on either GM-CSF or IL-3 for survival and proliferation, such as Ba/F3 cells. Simultaneous mutation of all eight tyrosine residues disrupted GM-CSF mediated proliferation in Ba/F3 cells, but critically, survival signalling remained unaffected. As each mutation was repaired in succession, three residues, T577, T612 and T695, were sufficient to restart proliferative signalling. This suggests that not only these residues are responsible for proliferation, but that no tyrosine residues are required for survival signalling [75, 76]. Further validation of this effect comes from the observation that when increasingly larger fragments of the βc-chain were deleted from the C’ terminus, receptor signalling, specifically proliferative signalling, was not affected until T695, T612 and T577 were lost [77]. Supporting this is the observation that at limiting GM-CSF concentration, phosphorylation of S585 is sufficient to maintain cellular viability, but not proliferation (Table 1) [78, 79].
The βc-chain shares several features of the type-1 cytokine receptors such as the WSXWS domain near the plasma membrane, as well as the JAK docking regions known as the Box1 and Box2 domains. Also highlighted are the phosphorylatable tyrosine residues and the Serine 585 residue that may play a role in survival signalling. Numbering in this figure includes a 16 amino acid leader sequence cleaved during protein folding.

Figure 1.3: A schematic illustration of the βc-chain

The βc-chain shares several features of the type-1 cytokine receptors such as the WSXWS domain near the plasma membrane, as well as the JAK docking regions known as the Box1 and Box2 domains. Also highlighted are the phosphorylatable tyrosine residues and the Serine 585 residue that may play a role in survival signalling. Numbering in this figure includes a 16 amino acid leader sequence cleaved during protein folding.
<table>
<thead>
<tr>
<th>βc-chain region/Mutation</th>
<th>Function/Result</th>
<th>Activated Pathways</th>
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<tr>
<td>F1Δ (37aa duplication from extracellular region)</td>
<td>Cytokine Independence</td>
<td>Presence of Cytokine</td>
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<td>Viability</td>
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<td>Differentiation</td>
<td>RK1/2</td>
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<td>Absence of Cytokine</td>
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<td>V449E</td>
<td>Cytokine Independence</td>
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<td>Differentiation:</td>
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<td>Macrophages</td>
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<td>Granulocytes</td>
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<td>S585</td>
<td>Viability</td>
<td>I-3K/AKT</td>
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<td>Y612</td>
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<td>Macrophage Differentiation</td>
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Table 1: The following table is a list of ‘important’ residues or domains within the β-common chain, including the cellular function each serves and the pathways activated by these domains. Functions were determined by mutational analysis (as described in indicated references). Asterisk indicates a hypothesised/untested function.
Given the amount of data accumulated regarding βc-chain signalling, surprisingly little is understood about its specific signalling mechanisms. Gaps in our understanding include; the nature of the multimeric complexes making up an active receptor, the abilities of a shared signalling domain to propagate esoteric signals, the specific adaptor proteins required for the activation of downstream pathways and the pleiotropic nature of these receptor complexes. One promising model proposed engaging these queries is the so-called binary switch model. The model is based on the observation that the multiple phosphorylation sites on the βc-chain are activated at differing cytokine concentrations [78, 79]. The model was not without precedent, as previous research had indicated alternate empirical outcomes after growth factor titration [83]. In such circumstances, very high concentrations of cytokine enforced lineage differentiation, less cytokine promoted proliferation, whilst minimal cytokine levels were sufficient only to maintain viability. The absence of cytokine resulted in the initiation of apoptosis.

The authors of the research proposing the binary switch theory showed that when relatively high cytokine concentrations were detected by the receptor complex, threonine phosphorylation was observed, specifically at the T577 residue. Notably, S585 phosphorylation was absent at high cytokine concentrations. Consistent with the aforementioned literature, the phosphorylation of T577 was associated with proliferative signalling. When GM-CSF was titrated to lower concentrations, T577 was dephosphorylated, whilst S585 was phosphorylated. Concurrently, cells withdrew from cell cycle and stopped proliferating, although survival remained unaffected. When GM-CSF was completely withdrawn from cultures, both T577 and S585 residues were dephosphorylated, and cells underwent apoptosis. This led the authors to conclude that two distinct pathways are activated by the βc-chain. A proliferative signal (which also maintained survival) was activated in high cytokine concentrations by the phosphorylation of the T577 residue, hypothesised to be due to the activation of the Phosphoinositide-3 Kinase (PI3K)/AKT pathway. Sub-optimal concentrations of cytokine led to S585 phosphorylation that stimulated survival only, hypothesised to be due to Mitogen Activated Protein Kinase (MAPK) signalling.

The authors further suggested that the application of this model might shed light onto how certain populations of cancer stem cells evade chemotherapy. If a
The role of Akt during IL-3 survival signalling

A subpopulation of cancer cells has activated these ‘survival only’ pathways, they may be resistant to chemotherapeutic agents that target proliferating cells or those with high metabolic activity. The ability of these cells to evade frontline chemotherapeutic strategies could then lead to oncogenic recurrence [84].

1.1.4 Signal Transduction

Cytokine receptors sharing the βC-chain require members of the JAK family kinases for activation. When the docking domain for JAK is disrupted in the βC-chain, the resultant receptor is inactive [85]. Indeed JAK2, another tyrosine kinase family member, is required for normal haematopoiesis, and its deletion in murine models leads to complete loss of the haematopoietic system and complete loss of response to cytokines including IL-3 [86]. When Jak2 was re-expressed, IL-3 signal transduction was restored.

It is in this role, as a master regulator of cytokine signalling, that frequent mutations have been described in JAK2 leading to oncogenic transformation. The JAK2 V617F mutation for instance has almost a 90% occurrence rate in polycythaemia vera, which frequently progresses to myeloproliferative disorders including AML [87]. This mutation constitutively activates JAK2, and therefore activates all downstream pathways. There is however, a possibility that further checks and balances regulating these signals may supersede the constitutively activated signal caused by the mutation. This leads to the interesting possibility that tumour suppressors regulating the downstream pathways may reverse the effect of constitutively active JAK2. We could then use this understanding of these oncogenic kinase pathways to utilise chemical inhibitors that would prevent the transforming signal without significant toxicity to normal cells.

The hierarchical nature of the pathways lying downstream of JAK2 leads to the activation of numerous parallel pathways, many of which contain potent proto-oncogenes. For example, lying downstream of JAK2 is the RAS/RAF pathway, which activates the MAPK pathways, including the p38/JNK pathway and the MEK/ERK pathway. Furthermore, JAK2 promotes activation of lipid-activated pathways, such as the PI3K/AKT pathway. The large number of pathways activated by a single receptor allows for both amplification of the signal, as well as fine adjustment of the
The role of Akt during IL-3 survival signalling

downstream effects. For example, activation of a receptor may promote signalling via both the MEK/ERK pathway and the PI3K/AKT pathway, each regulating different processes. Indeed, various pathways will often regulate the same processes, or even the same genes and proteins [88]. Figure 1.4 shows a schematic representation of the hierarchical nature of IL-3 receptor activation; however, even this diagram does not do justice to the complexity of the IL-3 signalling pathway.

1.1.4.1 Mitogen Activated Protein Kinase

MAP Kinases are a family of proteins that are regulated by growth promoting signals, Mitogens, to facilitate cellular signalling. These pathways frequently lie downstream of cytokine receptors and JAK2 signalling. Many pathways are activated by these signals, initiating parallel, cascading phosphorylating events to regulate numerous cellular processes. For example, both the p38/JNK pathway and the canonical MEK/ERK pathway are capable of regulating CDK7/9 [89], oftentimes by the same stimuli. This means that both pathways are responsible for regulating identical proteins from identical stimuli, demonstrating the overlap and potential redundancy of these signalling pathways. The parallel signalling and complex nature of the network of MAPK pathways contributes to the regulation of cellular processes such as maintaining survival in response to IL-3. Whilst these pathways will not be a focus of this thesis, it is important to remember that these pathways may signal parallel to, or even interfere with AKT signalling. One such example is the frequently discussed ‘crosstalk’ between the PI3K/AKT pathway and the MEK/ERK pathway [90-92].

1.1.4.2 The MEK/ERK pathway

The MEK/ERK pathway was the first MAPK pathway described to be activated in a hierarchical manner. Certain substrates of the MEK/ERK signalling axis have been identified as particularly potent oncogenes, including c-Fos, c-Myc and c-Jun [93]. Although not the main focus of this thesis, MEK/ERK activation is a well recognised feature of cytokine receptor activation and, in the context of the βc-chain family of receptors, contributes to cellular proliferation, cell cycle checkpoint progression and growth. There are also some indications MAPK may regulate intrinsic apoptosis pathways by regulating the abundance of key members of the Bcl-2 family of proteins.
Figure 1.4: IL-3 dependent survival pathways

An illustrative example of the activation of survival pathways downstream of the IL-3 receptor. Due to a lack of intrinsic kinase activity, the βc-chain recruits JAK2 upon activation to propagate the cytokine signal. JAK2 is responsible for the activation of numerous signalling pathways, which promote cellular survival. These pathways include RAS/RAF dependent pathways, STAT transcription factors and MAP Kinase pathways such as p38/JNK and the MEK/ERK pathway. PI3K is also activated by JAK2, however, it functions as a lipid kinase; phosphorylating inositol rings within the plasma membrane. The phosphoinositol rings act as binding domains for the activation of further survival kinase pathways including PKA, PKB (AKT) and PKC. Further studies have suggested that the predominant means by which AKT inactivates apoptosis is through phosphorylation of the chaperone protein 14-3-3, inhibition of the BH-3 only protein BAD, and thus activation of Bcl-2 family members promoting survival. This will be further covered in chapter 1.3.
This led to attempts to identify pathways involved with survival and proliferation sharing roles and potentially substrates with the MEK/ERK pathway [94]. One such example is the postulated ‘crosstalk’ between the MEK/ERK pathway and the PI-3K/AKT pathway. These studies hint that negative feedback loops result in increased reliance on one pathway when the other is blocked. As these pathways share common elements this may be one mechanism by which tumour cells can evade attempts to inhibit survival and proliferative signals [95].

1.1.4.3 Phosphoinositol Signalling

A curious alternative mechanism for protein kinase signalling is the utilisation of lipid molecules that make up the plasma membrane. The plasma membrane consists of a wide variety of lipid molecules, including those with an inositol ring on the cytoplasmic surface. These inositol rings are bound to the lipid bilayer at the first carbon position. A plain inositol ring would have hydroxyl groups bonded to each carbon. Phosphoinositol signalling replaces these hydroxyl groups with phosphate groups, serving as an anchorage and activation site for downstream substrates [96].

For example, an inositol ring phosphorylated at the fourth carbon site would be phospho(4)-inositol. If a lipid kinase were to then phosphorylate the fifth carbon molecule, by way of replacing the hydroxyl group with a phosphate group, the inositol ring would be referred to as diphospho-(4,5)-inositol. Therefore the specific role of PhosphoInositol-3 Kinase (PI3K) is to convert the third carbon hydroxyl group to a phosphate group creating triphospho(3,4,5)-inositol. It is this regulation of phosphorylated inositol rings that allows for highly specific regulation of phosphoinositol signalling cascades [97-99].

Using this method, numerous proteins are activated by the unique structures generated by the phosphorylation of the membranous inositol rings. For example, the protein kinase AKT requires PI3K to prepare triphospho(3,4,5)-inositol for activation. This will be covered more in the next chapter.

Indeed, the PI3K/AKT signalling axis is often regarded as a single signalling axis; PI3K is responsible for the regulation of AKT, whilst AKT requires PI3K for activation. While there is limited truth to this simplistic model, more recent evidence suggests that not only does PI3K regulate multiple pathways parallel to AKT, but that
AKT can be activated independently of PI3K [100, 101]. Furthermore, as expected for a pathway regulating such critical cellular processes, tight regulation of the pathway is required. It is in this role that Phosphatase and Tensin Homologue (PTEN) has been observed deactivating the PI3K signal, and has been described as a potent tumour suppressor [102-104].
1.2 Protein Kinase B / AKT

AKT, also known as Protein Kinase B (PKB), is a Serine/Threonine kinase involved in a number of growth factor signalling pathways. AKT was originally identified in 1987 as the driving oncogene in retrovirally transformed murine Thymoma [105]. Subsequently cellular AKT expression was identified in many different tumour types, with particularly high prevalence in breast, prostate and colon cancers. Early research suggested AKT was a prominent component of growth factor signalling [106-108]. Further studies identified key roles of AKT in cellular metabolism [109], glucose transport [110, 111], proliferation and the inhibition of apoptosis [101].

1.2.1 AKT Structure and Activity

AKT possess two major domains, the catalytically active kinase domain and the regulatory Plekstrin Homology (PH) Domain. The kinase domain features four phosphorylatable residues, although it is the S473 and T308 residues critical for AKT activity (Figure 1.5) [112].

The PH Domain acts like a flap. When AKT is inactivated, the PH domain folds over the hydrophobic region of the kinase domain, hiding the key S473 and T308 residues [113]. AKT is indirectly regulated by PI3K [114]. When PI3K is activated, PIP(4,5)_2 is converted to PIP(3,4,5)_3. This particular isomer of phosphoinositide serves as a specific binding site for the PH domain. Two simultaneous steps then occur,

1. The PH domain opens out to bind to PIP(3,4,5)_3 exposing the catalytically active region of AKT.
2. AKT translocates from the cytoplasm to the plasma membrane, where the PH domain anchors the entire protein [115, 116].

This translocation brings AKT into proximity of the kinases responsible for phosphorylating and activating AKT. These kinases include PDK1, PDK2 and the mTOR2 complex (suggesting a possible positive feedback loop, see section 3.5.3)
Figure 1.5: Current perspectives on AKT activation

This figure (from Matheny, 2009 (98)) displays the crystal structure of Akt1, and also shows the key phosphorylatable residues responsible for Akt activity. This thesis will mostly reference the residues as T308 and S473 (Akt1), but there is little difference in the surrounding domains on each Akt isoform, and importantly, antibodies targeting S473 will also detect S474 (Akt2) and S472 (Akt3).
When cells dependant on growth factors for survival are grown in the presence of Wortmannin or Ly294002, specific inhibitors of PI3K, cells undergo apoptosis, indicating that the PI-3K / AKT pathway plays a role promoting survival in response to growth factors [117-120].

As an arbiter of apoptosis, proliferation and metabolism, it is not surprising that the PI3K / AKT pathway has been implicated in oncogenesis, and the presence of activating mutations is commonly found in PI3K [121, 122]. The myristoylation of AKT is an artificial method to promote constitutive AKT expression, however, there are relatively few clinical AKT oncogenic mutations described to date. Nonetheless, AKT expression is believed to work in conjunction with the Bcl-2 family of proteins to prevent apoptosis leading to neoplasms.

### 1.2.2 Isoforms of AKT

Three isoforms of AKT, encoded by three distinct genes, have been identified, varying in function and expression pattern, and in some instances, the substrates targeted. This selectivity of the substrates is notable as all three isoforms have as much as 80% identity. Numerous groups have sought to determine the specific roles of AKT1, AKT2 and AKT3, with the most definitive results coming from gene-deleted mice. Whilst AKT1 and AKT2 are expressed ubiquitously, they are believed to possess different functions and substrates; AKT1 functions to inhibit apoptosis and drive cell cycle and proliferation [123, 124], whilst AKT2 has a role in glucose transport and metabolism [125, 126]. AKT3 shares similar functions to AKT1, however is expressed primarily in neuronal tissues and the testes [127]. Studies of AKT knockout mice suggest that many of roles of AKT may be redundant, or perhaps meaning that other AKT isoforms may compensate for the loss of each AKT gene. As explained below however, as there are phenotypes associated with each individual AKT knockout isoform (albeit relatively minor phenotypes), there must be some isoform specific functions that are not compensated for by the remaining AKT isoforms.

#### 1.2.2.1 AKT1

*Akt1*−/− mice have a subtle increase in perinatal mortality although homozygous deleted animals can also survive to adulthood and breed. These mice also display a reduction in body weight [128-130], although, intriguingly, post-natal survivors do not
display any more subsequent severe phenotypes. One suggestion is that AKT1 may therefore have a role in regulation of *in-utero* cellular growth, or even placental growth. AKT1 is a critical mediator in the responses to cytokines and growth factors. Initially this survival signalling role was identified in response to serum activated signalling [108], however, further evidence suggests AKT1 specific roles in NFKB signalling [131], PDGF signalling [132] and in response to cytokine signalling [133]. Critically mice possessing only a single AKT1 gene, and with no AKT2 or AKT3 (*Akt1*+/−; *Akt2*−/−; *Akt3*−/−), are viable with only a minor diminutive phenotype and severe glucose homeostasis dysfunction. Curiously, *Akt1*−/−; *Akt2*−/− or *Akt1*−/−; *Akt3*−/− mice do have a perinatal lethal phenotype, suggesting there is a non-redundant role of the AKT family, although what this role is, remains unclear [134-136].

1.2.2.2 AKT2

*Akt2*−/− mice, unlike the *Akt1*−/− mice, are not susceptible to embryonic lethality, but have an obese, hypoglycaemic diabetic phenotype, suggesting that there is a heavy reliance on AKT2 for glucose transport [137, 138]. AKT2 has more commonly been associated with metabolic pathways and glucose transportation than with the direct regulation of survival; although some would argue that whilst it is the duty of AKT1 to regulate survival by directly modifying the traditional apoptotic regulators such as the Bcl-2 family, AKT2 likewise is responsible for maintaining survival by maintaining metabolic pathways. These pathways, if switched off, would otherwise indirectly promote apoptosis.

1.2.2.3 AKT3

The role of AKT3 is more homologous to that of AKT1 than AKT2; that is its function is more often associated with direct regulation of the apoptotic machinery than glucose transport and metabolism. AKT3 is primarily expressed in neuronal tissue and the testes, yet displays up to ten times the activity as AKT1 [130]. *Akt3*−/− mice exhibit a reduction in brain weight resulting from decreases in both cell size and cell number, but maintain normal glucose homeostasis and body weights [127, 139]. Taken together, this suggests that the AKT3 isoform may perform a similar role as AKT1 in specific tissues where AKT1 activity is insufficient to maintain the requisite signalling.
1.2.3 Roles and Functions of AKT

Signalling via cytokine receptors has long been known to regulate the Bcl-2 family proteins, repressing them to promote survival [140]. The deregulation of this process, leading to an imbalance of Bcl-2 family proteins favouring survival, is a part of the oncogenic process [10]. Therefore, researchers studying AKT in the past decade have tried to identify the mechanisms by which IL-3 signalling is regulated by AKT. Early research suggested that AKT had prominent roles in both proliferation and survival pathways, whilst more recent investigations have described roles of AKT in the regulation of protein translation through the promotion of ribosomal biogenesis [141-144]. Furthermore, recent evidence has shown that AKT (AKT2 in particular) has a major role in the regulation of metabolism by promoting glucose transportation [145-147]. Chemical inhibition of the AKT pathway leads to initiation of apoptosis. Paradoxically, deletion of each AKT isoform does not disrupt the survival pathways. This suggests that either every tested chemical inhibitor of AKT has off target toxic effects, or the mechanism by which AKT promotes cytokine dependent signalling is more complex than a linear pathway.

1.2.3.1 Survival Signalling, AKT and apoptosis.

One of the most frequently cited models illustrating the way AKT functioned as the engagement between cytokine receptor activation and apoptosis pathways was described by Datta et al. This model proposed that AKT directly regulated Bcl-2 family members such as BAD to regulate apoptosis [148]. In this instance, these Bcl-2 family members were direct substrates of AKT. Although discussed later, it is worth mentioning at this point that there are several problems with this model. Firstly, there must AKT dependent and AKT independent survival pathways, since the deletion of any individual AKT gene does not prevent the survival of cytokine dependent cells in mice or ex vivo [149]. Further, the deletion of the Bcl-2 family protein phosphorylated by AKT, BAD, does not make cells resistant to IL-3 deprivation induced apoptosis. The potential substrates of AKT that may regulate survival are not restricted to the Bcl-2 family members [150], but may also include regulation of the transcription factors responsible for the expression of the Bcl-2 family members [151-153], or even regulation of the proteasome to prevent degradation of key survival proteins [154].
Thus it remains an important and open question for further research; what are the ways AKT can regulate the activity of apoptosis pathways in the context of IL-3 receptor (or other cytokine receptors) signalling?

**1.2.3.2 AKT, Proliferation and Cell Cycling**

Early research identified an increase in proliferation when AKT was over-expressed in immortalised cells, providing a clue to the oncogenic nature of AKT. Specifically, AKT plays an integral part in the progression through cell cycle checkpoints. For instance, the AKT substrate GSK3β is known to inhibit the Cyclin D/CDK4/6 complex from passing into S-phase. When GSK3β is phosphorylated by AKT, CDK4/6 allows the cell to progress. Further still, by phosphorylation of MDM2, AKT represses p53 and p21 activity, both capable of inhibiting the Cyclin E/CDK2 pathway (Figure 1.6). This suggests that AKT at least plays a hand in directly promoting proliferation [155, 156].

**1.2.3.3 Protein Synthesis**

AKT has long been described as a universal regulator of translation, and recent studies have identified the key mechanisms by which AKT controls ribosomal biogenesis. Activated AKT phosphorylates, and thereby inhibits, the Tuberous Sclerosis (TSC) proteins, which would otherwise impede the formation of the mTOR complex (mTORC) 1/2. This large multimeric compound is responsible for phosphorylation/activation of S6 Kinase (S6K or p70) a critical component of the 40S subunit of the ribosome (Figure 3.3) [141-144, 157, 158]. Although there is a much more complex network of signalling pathways involved in the regulation of translation downstream of AKT, this thesis will not discuss this in further detail.
Figure 1.6: The role of AKT in normal cell cycling
An example of some of the substrates that AKT regulates to promote cell cycling (adapted from Xu, 2012 (137))
Figure 1.7: The role of AKT in protein synthesis

One model as to how AKT regulates protein synthesis through the activation of mTOR, which results in S6 phosphorylation. This promotes ribosomal construction and further protein synthesis.
The role of Akt during IL-3 survival signalling

1.2.3.4 Glucose Metabolism

AKT has also been shown to play a crucial role in regulating glucose importation into the intracellular environment. In response to IGF receptor signalling for instance, AKT has been shown to promote expression, translocation and activation of the GLUT4 receptor [146, 159, 160]. The protein AS160, when unphosphorylated is responsible for shutting down glucose transportation by preventing the glucose receptor/transport molecule GLUT4 from translocating to the plasma membrane. When metabolism is stimulated, AKT is responsible for phosphorylating, and therefore shutting down, AS160 activity [161, 162]. Although this process is more involved, it is not further discussed in this thesis.

1.2.4 The PI-3K/AKT Axis in Cancer

AKT is known to play a prominent role in oncogenesis and this recognition underpins much of the research interest in AKT and AKT inhibitors. Indeed, when first discovered, viral AKT, presumably hijacked from their mammalian hosts, was found to cause aggressive Thymomas in murine models. Genetic analysis of this viral oncogene identified the three homologous mammalian isoforms. It was found that when over-expressed in immortalised cells, c-Akt promoted growth, increased metabolite consumption and promoted rapid proliferation. One further notable role of AKT was identified, the capacity to maintain survival, specifically in response to cytokines [108, 163]. Therefore, due to AKT regulating several pathways critical to oncogenesis, it was quickly proposed as a proto-oncogene. In fact, early genetic and histological studies identified AKT deregulation / over-activation in a wide variety of cancers [164, 165]. However, there was one outstanding piece of evidence cementing AKT as a particularly potent oncogene; if AKT regulates the majority of cellular processes disturbed during oncogenesis, where were all of the activating mutations to be expected appearing regularly in the clinic?

Indeed, current perspectives on AKT pathophysiology suggest that the role of AKT in promoting oncogenic transformations may be over-stated [166]. In fact, it was not until 2007, that Carpten et al. described the first activating point mutation of AKT found the clinic [167]. The mutation described was the E17K mutation, which prevented the AKT hinge region in the Pleckstrin Homology domain from correctly
folding over and hiding the catalytic kinase domain, thus preventing deactivation of the molecule. However, immediately after publication, numerous rebuttals were published suggesting that any immortalization caused by the E17K mutation was a combination of an artefact of over-expression and utilization of an already partially immortalised cell line [168-176].

Mutations of the p110 subunit of PI3K have been found in high frequencies in tumours, particularly colorectal, breast and prostate cancers. Two common activating mutations, the E545K and the H1047R mutations, have been observed with varying frequencies in patient samples [177-179]. Knock-in E545K or H1047R cell lines suggest these mutations are capable of promoting growth factor independent survival and proliferative signalling [180]. Similarly, over-expression of the p110 subunit promotes proliferation, survival and cellular mobility.

Of particular interest are the cells possessing a knock-in mutation of the E17K mutation, which were compared to cells possessing H1047R mutations in the PI3K p110α gene [181]. Under these circumstances, there was a substantial increase in the capacity of H1047R to immortalise cells compared to the E17K mutation. This suggests that even mutations in the same pathway as AKT are more ‘oncogenic’ than AKT mutations.

### 1.2.4.1 Targeting the PI3K/AKT pathway using therapeutic inhibitors

Today, inhibitors of PI3K are being utilised to target tumour cells and the activating mutations associated with the p110 subunit. These new compounds are being developed as exciting new therapies for cancer patients. It is notable then, that AKT inhibitors have performed comparatively poorly in clinical trials to date. For example, Perifosine© is an alkylphosphocholine responsible for disrupting AKTs anchorage to PIP₃. Under laboratory conditions, this drug successfully induced apoptosis in both in-vitro and in-vivo conditions in numerous tumour types. Recent Phase III clinical trials however, have been terminated as “it was highly unlikely that the trial would achieve a significant difference in progression-free survival [182].”

Indeed, novel inhibitors of the AKT/PI3K pathways that have proven most successful in clinical trials have been found to simultaneously inhibit multiple parallel pathways to induce apoptosis in transformed cells. One such example is the PI3K
inhibitor PIK-75. This inhibitor possesses highly potent anti-oncogenic properties that led to promising new therapeutic strategies. Initially described as a PI3Kα inhibitor, further investigation proved that a secondary target of the drug were the Cyclin Dependent Kinases 7 and 9. Indeed, on closer inspection, it was observed that the potency of PIK-75 was due to a significant decrease in the expression of the anti-apoptotic Bcl-2 family member Mcl-1, an effect that was evidently not caused by PI3K inhibition alone [183, 184]. Thus, to date, AKT/PI3K inhibition alone has not proven to be a particularly promising treatment option.

1.2.4.2 PTEN

A relatively recent discovery was of the role of the phosphatase PTEN in PI3K/AKT signalling. Greater than 90% of sufferers of Cowden’s Disease have a PTEN mutation. Cowden’s syndrome is characterised by developmental abnormalities and a significantly increased susceptibility to a range of cancers. PTEN is a phosphatase that reverses the phosphorylation of the phosphoinositol carbon-3 residue performed by PI3K, and therefore disrupts AKT signalling. It is in this role that PTEN has therefore been described as a tumour suppressor. Interestingly, despite the very low frequency of AKT mutations present in tumours, and the moderate number of PI3K mutations presenting in clinics, some researchers have suggested that the loss of PTEN occurs in up to 80% of prostate cancers [185], as well as high frequencies of the mutation in colorectal, bladder & breast cancers, gliomas, and leukaemias. Taken together, this suggests that perhaps it is PTEN that is the arbiter of AKT activity.
1.3 Apoptosis

The work presented in this thesis will focus on the engagement between Akt and apoptosis pathways, and therefore it is important to review key features of the molecular regulation of apoptosis. During the normal development of multicellular organisms, it is the pre-determined fate of many cells to die. Proteins controlling this ‘programmed’ cell death, known as apoptosis, are widely conserved. The programmed death ultimately results in phagocytosis of the dead cell, which prevents inflammation, leakage of toxins and the continued uptake of nutrients.

Homeostasis between cellular proliferation and apoptosis is of critical importance to an organism, and imbalance between survival and death may contribute to a variety of diseases. Increased levels of apoptosis may account for cell loss in diseases such as Alzheimer’s and AIDS. The evasion of apoptosis is one mechanism whereby a cell may accumulate mutations in its DNA, ultimately leading to neoplasms [186].

In general, apoptosis occurs via two main, distinct pathways; the extrinsic pathway, involving so-called death receptors of the TNF-superfamily, or the intrinsic pathway, which results in the permeabilisation of the outer mitochondrial membrane and leakage of activators of proteases. Intrinsic apoptotic stimuli include DNA damage or growth factor withdrawal [187].

The survival of many cells, such as haematopoietic cells, depends on exogenous signals from growth factors. When these signals are lost, these cells often activate their intrinsic cell death pathways. The molecular links between loss of growth factors and engagement of the cell death pathway remain contentious.

1.3.1 Bcl-2 proteins and Intrinsic Apoptosis

Cell death controlled by the intrinsic apoptotic pathway, including growth factor withdrawal, is regulated by a class of proteins known as the Bcl-2 family of proteins. Bcl-2 was the first family member to be described after it was identified as being over-expressed in 80% of B-cell lymphomas, the result of a genetic translocation; t(14:18) [188]. It was later demonstrated that the oncogenic potential conferred by Bcl-2 was not due to an increase in proliferation, but a survival advantage due to the blocking of apoptosis in these cells. The formal demonstration that Bcl-2 was an oncogene was
from experiments showing that Bcl-2 co-operated with genes such as c-myc, and the combination of these genes could promote the formation of lymphoma [140]. Bcl-2 therefore led to the description of an alternative type of oncogene - one that inhibits apoptosis.

The Bcl-2 family proteins display varying degrees of homology with Bcl-2, based on the presence of particular motifs known as the Bcl-2 Homology (BH) domains. The sub-classes are divided based on their roles in apoptosis and the domains with which they share homology with Bcl-2 [189, 190].

The pro-apoptotic class of proteins function to initiate apoptosis, which is characterised by permeabilisation of the outer mitochondrial membrane, resulting in with the release of factors such as cytochrome-c (cyt-c). Cytochrome c, together with an adaptor molecule Apoptotic Protease Activating Factor 1 (APAF-1) and the cysteine protease caspase-9, form a complex referred to as the apoptosome. This complex functions to activate other caspases, notably caspase-3 and caspase-7. These proteases, by cleaving their substrates at specific aspartate residues, result in the typical morphological changes associated with apoptosis; membrane blebbing and internucleosomal DNA cleavage. However, the key point at which cells commit to apoptosis is mitochondrial outer membrane permeabilisation (of MOMP).

The precise means by which MOMP occurs remains controversial. However, what is undisputed is that two pro-apoptotic Bcl-2 family members, BAX and BAK, are absolutely required for apoptosis via the intrinsic pathway. Accordingly, $Bax^{+/+}/Bak^{+/+}$ cell lines are unable to undergo apoptosis and no MOMP occurs in these cells. Normal MOMP can proceed in $Bax^{-/-}$ or $Bak^{-/-}$ cells indicating either protein is sufficient for intrinsic apoptosis to occur [191]. MOMP occurs when BAX and BAK undergo conformational changes from an inactive state to an active state and oligomerise. The other Bcl-2 family member’s function, through their protein-protein interactions with one another, to either repress or promote the conformational changes and oligomerisation associated with Bax and Bak activation (Figure 1.8).
**Figure 1.8: Bcl-2 family members used in this thesis and their interactions**

Three sub-groups of Bcl-2 family members are separated by function and homology to Bcl-2. The multi-BH pro-apoptotic proteins BAX and BAK promote apoptosis by perforating the mitochondrial outer membrane. Pro-survival proteins inhibit this function by binding to Bax and Bak. The BH-3 only proteins share only a single domain of homology with Bcl-2 (domain #3), and antagonise specific pro-survival proteins to allow Bax and Bak to initiate apoptosis. (with regards Dr Mark Guthridge for this figure)
1.3.1.1 Anti-apoptotic Bcl-2 family members

Bcl-2, Mcl-1, Bcl-x and Bcl-w are the anti-apoptotic Bcl-2 family members as they contain multiple BH domains. These proteins structurally resemble BAX and BAK as they have a hydrophobic groove, which is the key interaction domain. They function to repress apoptosis in part by directly binding to and inhibiting BAX and BAK oligomerisation and activation. They also bind to the other class of Bcl-2 family member, the BH3-only proteins (see next section) [192, 193]. By binding to BAX and BAK and preventing their oligomerisation, the anti-apoptotic family members prevent apoptosis from occurring. When over-expressed, Bcl-2 and Bcl-XL prevent cells undergoing apoptosis, even upon removal of growth factor [194-197]. One critical anti-apoptotic member is Mcl-1. Mcl-1 is often expressed in haematological malignancies, and has been an obstacle in targeting the survival pathway during chemotherapy [197-199]. When GM-CSF is removed from cells Mcl-1 is rapidly degraded, a process retarded when GM-CSF is re-added to the cells [200]. This is of particular interest as the GM-CSF receptor and IL-3 receptor both signal through the βc-chain. Furthermore, Mcl-1 is critically required for normal haematopoiesis [201, 202].

1.3.1.2 Pro-apoptotic Bcl-2 family members

The two most critical members of this group of proteins are BAX and BAK, as it has been shown that cells lacking both of these are incapable of undergoing intrinsic apoptosis [149, 203]. Other related proteins such as BOK have a similar function, although are not as critical to the induction of apoptosis [204]. It is these two proteins that ultimately act as the arbiters of cell death by oligomerising at the outer mitochondrial membrane, which is therefore permeabilised allowing mitochondrial proteins such as cytochrome-c to spill into the cytosol and from the apoptosome [205]. As this step results in the loss of mitochondrial integrity, this is the irreversible step leading to the caspase cascade and cell death. It is by binding to and antagonizing these proteins that the Bcl-2 anti-apoptotic proteins maintain survival, however, it is also possible that the BH-3 only subgroup may bind to these proteins directly in order to induce apoptosis [206].
1.3.1.3 BH3-only proteins

Members of a subfamily of the pro-apoptotic Bcl-2 proteins display homology with only one of the four conserved sites within Bcl-2, designated the BH-3 site, however show little other similarity with other class members. One model by which these proteins are suggested to function is by binding to anti-apoptotic proteins via the BH-3 domain, therefore suppressing the anti-apoptotic proteins function. The BH-3 domain is a hydrophobic domain shared by all Bcl-2 family members, which allow the Bcl-2 family members to interact in a highly specific conformation. The anti-apoptotic Bcl-2 family members usually bind the pro-apoptotic members to prevent their oligomerisation to permeabilise the mitochondria. The indirect model of the BH-3 only proteins suggests that by preferentially binding to the anti-apoptotic Bcl-2 members, the BH-3 only proteins free up BAX and/or BAK to oligomerise, and therefore induce apoptosis.

Some BH-3 only members, such as BID and BIM, may also directly bind to and activate BAX and BAK [190, 207]. The agonistic activities of the BH-3 only proteins allow BAX and/or BAK to oligomerise and permeabilise the outer mitochondrial membrane. BH-3 only family members include BAD, BID, BIM and PUMA.

1.3.2 The Regulation of Bcl-2 Family by IL-3

The deprivation of growth factor is sufficient to inhibit survival signals and promote apoptosis. IL-3 is one such growth factor used to promote survival and proliferation in myeloid cells [208]. Many transformed cell lines are available for IL-3 withdrawal studies, making IL-3 a prototypic model for growth-factor signal transduction. Such cell lines however, are often derived from tumours and as such, exhibit great genetic differences between themselves and normal cells.

One of the more commonly accepted models describing the molecular link between withdrawal of growth factor signalling and the initiation of apoptosis revolves around the BH-3 only protein, BAD. This will be covered further in chapter 1.3.3.1. If BAD is critical to IL-3 withdrawal stimulated apoptosis then this model predicts that Bad−/− cells should be resistant to IL-3 withdrawal. However, once generated these cells were as sensitive to IL-3 withdrawal as wildtype cells [149, 209]. This suggests that BAD has, at most, a redundant role in the regulation of apoptosis stimulated by
IL-3 withdrawal. The same researchers implicated a different BH-3 only protein in the initiation of apoptosis, as Puma−/− exhibited significant protection from IL-3 withdrawal.

1.3.3 The Regulation of Bcl-2 Family Members by AKT

It has long been suggested that the mechanism by which AKT regulates survival is through the regulation of the Bcl-2 family [148, 210-212]. However, despite numerous attempts, no unified conclusion has been formed at identifying the precise apoptotic machinery regulated by AKT. The following are several competing (or perhaps co-operating) mechanisms suggested within the literature. Figure 1.9 illustrates several of these competing models describing the mechanism by which AKT regulates the Bcl-2 family proteins.

1.3.3.1 The regulation of BAD by AKT

Datta and Zhoa proposed a model which suggests that AKT is responsible for phosphorylating BAD at the S99 and S118 residues [148, 213]. It was shown that phosphorylated BAD bound the chaperone protein 14-3-3 [148]. 14-3-3 then sequestered BAD in the cytosol where it was unable to bind to anti-apoptotic Bcl-2 family proteins, in particular, Bcl-XL and Bcl-2. In the absence of IL-3, unphosphorylated BAD is able to bind Bcl-2 and Bcl-XL, and as a result, the pro-apoptotic BAX and BAK oligomerise, translocate to the mitochondrial outer membrane and initiate caspase activity. The binding of anti-apoptotic proteins to BAX and BAK prevents their oligomerisation and therefore, inhibits apoptosis (for detailed review, see [214]).

Unfortunately, this model does not address the observation that Bad−/− cells underwent apoptosis to a similar degree as WT cells when treated with specific PI3K inhibitors [149, 209]. Again, this suggests that the role of BAD is at least redundant for PI3K/AKT dependent survival signalling. Therefore, a new model is required to describe the molecular link between IL-3 receptor deactivation, and the engagement of the apoptotic machinery.

1.3.3.2 The regulation of Mcl-1 by AKT

The regulation of Mcl-1 by AKT is a relatively new model proposed by Maurer et. al. [154]. This model utilises the well-documented phosphorylation of Glycogen Synthase Kinase 3β (GSK3β). Maurer suggests that the function of GSK3β is to
phosphorylate/ubiquitinate the anti-apoptotic protein Mcl-1, leading to the rapid proteasomal degradation of Mcl-1 and the initiation of apoptosis. Under this theory, AKT would phosphorylate and deactivate GSK3β, retarding this process thus preventing degradation of Mcl-1. This elegant theory is unique as it involves the proteasome for AKT dependent survival signalling. Furthermore, as previously mentioned, Mcl-1 is a critical regulator of haematopoietic cell survival. Therefore, it is likely that any key process promoting survival will in some way tie in with Mcl-1 regulation.

1.3.3.3 **The regulation of BIM by AKT**

Dijkers et al. proposed another model of AKT dependent survival. By determining the genes regulated by the transcription factor FOXO3a during cytokine signalling, BIM was identified as being transcriptionally upregulated after cytokine withdrawal. Critically, it was further shown that this transcription could be inhibited by AKT. This suggested that the apoptotic stimulus upon cytokine withdrawal was the transcription of BIM by FOXO3a, which would otherwise be phosphorylated and inhibited by AKT [215, 216].

1.3.3.4 **The regulation of PUMA by AKT**

Ekert et al. [149] explored the regulation of the Bcl-2 family members using conditionally immortalised cytokine dependent cells and observed a partial dependence on the pro-apoptotic protein PUMA to regulate cytokine withdrawal stimulated apoptosis. The upregulation of PUMA was mimicked using inhibitors responsible for blocking the PI-3K/AKT pathway. Further analysis of this pathway suggested that the upregulation of PUMA was dependent on the transcriptional activity of p53 [217, 218]. Therefore, the group concluded that the initiation of apoptosis depended, in part, on the activation of p53 to transcribe PUMA. Interestingly, the same research group identified a possible parallel mechanism by which PUMA is directly regulated by AKT. This model proposed that phosphorylation of the S9 residue results in rapid targeted degradation of PUMA by the proteasome. The region surrounding the S9 residue was identified as an AKT recognition sequence, and thus one of the proposed kinases responsible for this phosphorylation was AKT [219]. It should be
The role of Akt during IL-3 survival signalling

mentioned however, that this theory was recently disregarded in favour of an IKK dependent mechanism of S9 phosphorylation.
Figure 1.9: Bcl-2 family regulation by AKT to promote survival

An illustration of four proposed models by which AKT regulates survival downstream of cytokine signalling.
1.4 Project Aims

Given the abundance of research conducted on the role of AKT during survival signalling, there is surprisingly little consensus on the mechanisms by which AKT promotes survival. The overall aims of this project are to identify the pathways AKT regulates during survival signalling using a novel IL-3 dependent cell line known as Factor Dependent Myeloid Cells (FDMs). As these cell lines are derived from murine haematopoietic progenitors, we can generate an appreciable panel of cells deleted of potential AKT substrates or regulators to identify the requirements for AKT signalling during IL-3 stimulated survival. Specifically, this project will be divided into three specific aims.

1.4.1 Aim 1: To elucidate the role of AKT during IL-3 signalling

Utilizing the unique tools described, this thesis will attempt to determine what roles AKT plays after IL-3 stimulation or withdrawal. AKT has been shown to affect numerous pathways including survival, proliferation, cellular growth, etc. Using novel experimental tools and methods, the research will determine whether AKT is required for certain cellular functions in response to IL-3 signalling. As numerous parallel pathways are known to signal similar cellular functions, the project will seek to identify which functions are regulated by AKT alone.

1.4.2 Aim 2: To determine which Bcl-2 family proteins are required for AKT to maintain survival after IL-3 withdrawal

Using murine cell lines lacking potential AKT substrates, particularly Bcl-2 family proteins, the research will attempt to identify whether the loss of each protein leads to a loss of the phenotype caused when constitutively active AKT is over-expressed. Further, it will be determined whether there is an isoform specific selection for particular substrates by over-expressing either AKT1 or AKT2 in these experiments. It is reasoned that if a protein required for AKT mediated survival signalling is deleted from a cell, these cells are no longer capable of promoting survival when AKT is over-expressed as it would in wildtype cells.
1.4.3 Aim 3: To determine how AKT regulates the Bcl-2 family of proteins to maintain survival after IL-3 withdrawal

If a substrate of Akt is identified that is critical for regulating AKT dependent survival, the research will attempt to further delineate the pathway utilised by the IL-3/Akt signalling axis to regulate the candidate protein(s). As previously mentioned, many of the Bcl-2 family members are capable of being regulated by numerous mechanisms. Assuming a specific substrate of AKT is identified, a combination of genetic and chemical blocks of the required pathways may inform us further on the specific role of AKT.
2 Materials and Methods

2.1 Cell lines and culture

Factor Dependant Myeloid cells (FDMs) of indicated genotypes were generated from gene-deleted mice by infecting haematopoietic cells derived from E14.5 embryos with HoxB8 retrovirus. Briefly, Ψ2 fibroblasts expressing the HoxB8 retrovirus (figure 2.1) were co-cultured with E14.5 foetal livers in low glucose DMEM (Invitrogen) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) (JRH Laboratories) and 0.25 ng/mL murine rIL-3 (0.5 ng/mL IL-3 in Akt1−/−, Akt2−/−, Akt3−/− and corresponding WT cell lines) (R&D systems). Cells were kept at 37°C at 5% CO2. After 10 days, cells were centrifuged at 300 x g for five minutes and replated into fresh IL-3 supplemented media. It should be noted that no coefficient of infectivity was determined as only HoxB8 infected are transformed in the presence of IL-4. Ψ2 fibroblasts and 293T cells were maintained in high glucose DMEM (Invitrogen) supplemented with 10% (v/v) FCS.

2.2 Cloning

Murine AKT1 or AKT2 cDNAs encoding myristoylated AKT, lacking a Plekstrin Homology (PH) domain, together with an N-terminal HA tag (myr-HA-AKT1(ΔPH) or myr-HA-AKT2(ΔPH)) (gift from D James, Garvin Institute of Medical Research) or eGFP was cloned into the pF5xUAS-SV40-puromycin lentiviral vector (figure 2.2). Cells were co-infected with GEV16 and pF5xUAS-SV40. Infected cells were selected using hygromycin (400µg/ml) and puromycin (4µg/ml). Coefficient of infectivity was not determined as only infected clones capable of resisting hygromycin/puromycin induced death were harvested. Clones were selected and tested for expression by intracellular staining. Expression of HA-myr-Akt or eGFP was induced with 1µM 4-hydroxytamoxifen (4-OHT) for 24 hours before all experiments (figure 2.1). This construct will be henceforth expressed as iΔPH-Akt, or iAKT.
Figure 2.1: Schematic map of the HoxB8 retrovirus
This diagram is a map of the key features of the HoxB8 retrovirus utilised for conditional immortalization of FDM cells. The virus is based on the pMSCV retrovirus, with the murine HoxB8 cDNA cloned into the multiple cloning site using the XhoI/BglII restriction sites. These sites are lost after gene insertion.
Figure 2.2: Schematic map of the double vector inducible lentiviral system

This diagram is a map of the key features of the 4-Hydroxy-Tamoxifen inducible lentiviral expression system. This system utilises an Ubiquitin Promoter element to transcribe the GEV16 transcription factor. GEV16 is a fusion protein possessing the GAL4 transcription factor, the VP16 binding domain, and a heavily modified Oestrogen receptor (ERt2). 4-Hydroxy-Tamoxifen binds to the oestrogen receptor, allowing the GEV16 fusion protein to translocate to the nucleus. Within the nucleus, the VP16 binding domain binds to the 5xUAS promoter region on the second lentivirus, which in turn allows the GAL4 transcription factor to promote transcription of the gene of interest. The over-expressed product of this system is referred to in this thesis as ΔPH-Akt, or iAKT.
2.3 Lentiviral production and infection

293T cells were grown to 70% confluence before being transfected with a 5:3:2 mixture of pCMVδR.8, GEV16 or pF5xUAS-SV40 and VSVG plasmids using Effectene as per manufacturer’s instructions (Qiagen). Viral supernatant was harvested at 24 and 48 hours and either used fresh or stored at -80°C. Lentiviral infection of FDM cells was done by plating FDM cells at 50% confluence in viral supernatant, supplemented with 10% (v/v) FCS, 0.25 ng/mL rIL-3, with 5 µg/mL polybrene (Sigma Aldrich). Cells were then centrifuged for 90 mins at 2500 rpm at 30°C. Cultures were left overnight before fresh media was added. After one week, cells were selected with either 400 µg/mL Hygromycin (Roche) for seven days or 4 µg/mL Puromycin (Sigma) for four days. Clones were selected by growing aliquots of cells in DMEM containing 0.5 ng/mL and 20% FCS in semi-solid media of DMEM, 10% FCS, 0.5 ng/ml IL-3 and 0.3% soft-agar.

2.4 Intracellular staining

To detect ΔPH-Akt expression, cells were induced for 24 hours with 100µM 4-OHT for 24 hours before being washed in PBS and fixed with 80% methanol on ice for 20 min. Cells were washed in PBS again and 1:200 anti-HA (Covance) was added to cells in antibody buffer (10% Saponin, .027% FCS in PBS). Cells were incubated for 1 hour on ice before being washed and 1:100 anti-mouse-PE was added in antibody buffer. Cells were incubated for 1 hour at room temperature before being washed, resuspended in PBS and analysed by LSRII. Comparative histograms were generated using FCSExpress.

2.5 Immunoblotting

Cell lysates were generated using RIPA buffer (150mM NaCl, 50mM TrisHCl pH 7.4, 0.5% NaDOC, 0.1% SDS, 1%NP40) or Onyx Buffer (20mM TrisHCl pH 7.4, 135mM NaCl, 1.5 MgCl₂, 1mM EDTA, 10% Glycerol, 1% Triton-X 100) with a Protease Inhibitor Cocktail (Roche) and Phosphatase Inhibitors (2.5mM sodium pyrophosphate, 1mM β-glycerol phosphate, 1mM Na₃VO₄) at a cell density of 5x10⁴ cells/µL of buffer. Lysates were boiled in the presence of loading buffer (2% SDS, 10% Glycerol, 60mM Tris pH 6.8, 5% 2-mercaptoethanol and 0.01% bromophenol blue) for 5 minutes before being resolved on 12% or 15% SDS-PAGE gels. Proteins were detected after western
The role of Akt during IL-3 survival signalling

Blotting by chemiluminescence using ECL reagent (Thermo Scientific) or ECL Plus reagent (Amersham). The following antibodies were used:

<table>
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<tr>
<th>Name</th>
<th>Company</th>
<th>Clonality</th>
<th>Species</th>
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Table 2: A list of antibodies utilised
2.6 Viability and clonogenic assays

Viability was determined using Propidium Iodide (when indicated, Annexin-V antibody (1:1000) was also used) exclusion as assessed by flow cytometry. At the end-point of the experiment, cells were washed in PBS and Propidium Iodide (Sigma) added at 1 µg/mL in PBS. Cells were analysed using a Becton Dickson LSRII and FACSDiva. Clonogenic Assays were performed as follows; briefly, 500, 2500 or 12500 cells from viability assays were plated in low glucose DMEM supplemented with 20% FCS and 0.5 ng/mL IL-3 and 0.3% soft agar. After 14 days the number of colonies was then counted.

2.7 Cell cycle analysis

Cell-cycle analysis was performed by staining nuclear DNA of fixed cells with PI (using hypotonic solution) followed by FACS analysis as previously described.

2.8 Real Time PCR

RNA was extracted from 10^6 cells using the RNeasy RNA extraction kit (Qiagen), as per manufacturer’s instructions. 500ng of RNA was reverse transcribed using MMLV RVTase H minus mutant (Promega) and random primers (Promega) following manufacturer’s instructions. TaqMan gene expression assays using FAM (6-carboxy-fluorescein) labeled probes (Applied Biosystems) were used to quantitate Puma (Bbc3, Mm00519268_m1) or Mcl1 (Mm00725832_s1) mRNA levels and compared to two reference genes, Hmbs (Mm01143545_m1) and Polr2a (00839502_m1) using the Viia7 Real-Time PCR system (Applied Biosystems). The ΔΔCT method was used to calculate mRNA levels relative to control genes.

2.9 Generation of clones expressing the 4-OHT inducible ΔPH-Akt over-expression lentivirus

FDM cell lines were generated from murine foetal livers of various genetic backgrounds. Each cell line was then infected with both inducible construct plasmids and selected using the requisite selection agent, either puromycin or hygromycin. These cell lines were then cultured in media containing 0.5ng/mL IL-3, 20% FCS and 0.3% soft agar. After 10-14 days, single colonies were picked and further cultured to select clonal populations. Before any clones expressing the inducible lentiviral
The role of Akt during IL-3 survival signalling

construct were utilised, each clone was tested for the inducible, uniformed over-expression of the gene of interest. Figure 2.1 shows representative histograms of $\text{Bax}^{-/-}\text{Bak}^{-/-}$ cells expressing the eGFP, ΔPH-Akt1 or ΔPH-Akt2 over-expression constructs or the parental cell line. Each clone was cultured in either the presence or absence of 4-OHT for 24 hours in the presence of IL-3, before haemaglutannin (HA) expression was determined by flow cytometry (figure 2.3). These histograms demonstrate the induction of over-expression by the addition of 4-OHT to the culture media increases the expression of HA compared to uninduced cells. Importantly, as the eGFP over-expression construct does not possess a HA tag, there is no increase in relative fluorescence in eGFP over-expressing cells. Furthermore, uninduced cells have increased relative fluorescence compared to parental cell lines, suggesting there is ‘leakiness’ to the inducible construct allowing HA expression independently of 4-OHT. As such, it is likely that these cells are still over-expressing HA, although at much lower levels than induced cells.

Cells were also cultured in the presence or absence of 4-OHT for 24 hours before the indicated cells were starved of IL-3. After 0, 24 and 48 hours, cells were lysed and resolved by SDS-PAGE (figure 2.4). This data shows that the over-expression of AKT is induced by 4-OHT, but not dependent on the presence of IL-3. The phosphorylation of the S473 residue is indicative of active AKT. Low over-expression of AKT and pAKT can be observed in cells cultured in the absence of 4-OHT, further suggesting leakiness of the inducible over-expression system independently of 4-OHT.
Figure 2.3: HA- ΔPH-Akt over-expression is induced by 4-OHT
All cell lines expressing the inducible lentivirus construct were tested for uniform inducible HA. Representative $\text{Bax}^{-/-}\text{Bak}^{-/-}$ cell lines expressing eGFP, ΔPH-AKT1 and ΔPH-AKT2 are shown (note: the eGFP construct does not possess an HA tag). Cells were induced with 4-OHT for 24 hours in the presence of IL-3. Cells were then fixed, permeabilised and stained, before being analysed using flow cytometry.
Cells were also cultured in the presence or absence of 4-OHT for 24 hours before the indicated cells were starved of IL-3. After 0, 24 and 48 hours, cells were lysed and resolved by SDS-PAGE (figure 2.2). This data shows that the over-expression of AKT is induced by 4-OHT, but not dependent on the presence of IL-3. The phosphorylation of the S473 residue is indicative of active AKT. Low over-expression of AKT and pAKT can be observed in cells cultured in the absence of 4-OHT, further suggesting leakiness of the inducible over-expression system independently of 4-OHT.

2.10 Statistics and Statistical Analysis

All experiments have been tested in multiple independent experiments, with at least three independent clones (unless otherwise stated). Not every independent clone was tested in every experiment, although some clones were tested over multiple experiments. The standard error of the mean (S.E.M.) was determined as \( \text{S.E.M.} = \frac{\text{SD}}{\sqrt{N}} \), where \( N \) = number of independent clones. Calculations and Graphic analysis was performed using the following programs; Microsoft Excel (2007), Graphpad Prism (4.0), DeNovo Software FCS Express (version 3) and BD Biosciences FACSDiva (2.0).
Figure 2.4: Phosphorylated (active) AKT is induced by 4-OHT even in the absence of IL-3

WT cells expressing the inducible ΔPH-Akt 1 construct were stimulated with 4-OHT for 24 hours before being plated in the presence or absence of IL-3 and 4-OHT. At the indicated time points, cells were lysed and resolved by SDS-PAGE. This blot shows AKT induction is dependent on 4-OHT and independent of IL-3. In the absence of 4-OHT less AKT is detected, although some background expression of the construct is evident.
3. AKT is responsible for maintaining viability at low IL-3 concentrations

To determine the processes regulated by AKT in response to IL-3 signalling, two main approaches were used. The first was to utilise a lentiviral system to over-express AKT in a manner that could be induced by the addition of 4-Hydroxy Tamoxifen to culture media. The AKT constructs expressed were constitutively active, by virtue of deletions of the negative regulator Plekstrin-Homology domain. Therefore, when AKT expression is enforced, it is an active (phosphorylated) form of Akt (see figures 2.3 and 2.4). Thus, by maintaining AKT activation after removal of IL-3, the functions that active AKT maintain can be determined. The second approach was to generate a novel tool, Akt^{-/-} FDM cells (see Chapter 2.1). By observing the differences in response to IL-3 titration or withdrawal compared to WT cells, it is possible to identify the functions specifically requiring each AKT isoform.

3.1 WT FDMs do not have distinct survival and proliferative pathways in response to cytokine titration

The model cell lines used in these experiments are haematopoietic progenitor cells derived from E14.5 fetal livers of C57Bl6 mice that have been infected with retrovirus to express the homeobox gene, HoxB8. It had previously been established that these cell lines absolutely require IL-3 for survival and proliferation [149, 220, 221]. These cells are referred to as FDM cells, for Factor Dependent Myeloid cells.

In studies of the activation of the human GM-CSF receptor Guthridge et.al, had shown that the β-common chain peptide transduced distinct survival and proliferative signals depending on the cytokine concentrations that were used to stimulate cells [79, 222]. Using the GM-CSF dependent human TF-1 cell line, they showed that a concentration of GM-CSF could be identified at which the majority of cells were viable, but were no longer proliferating. Therefore, it was sought to determine whether wildtype IL-3 dependent FDM cells could also be cultured in concentrations of IL-3 that specifically promoted viability but not proliferation. Determining this ‘survival-only’ concentration would provide a model for the interrogation of downstream signalling events that may specifically promote viability over proliferation.
Eight independently generated cell lines were cultured at the indicated range of concentrations of IL-3 for 72 hours. The cells were then analysed for viability by exclusion of Propidium Iodide (PI) and Annexin-V (AV) stain using flow cytometry (FACS) (figure 3.1 A-C). In these same cell cultures, the proportion of cells in S-phase was also determined by transferring cells to a hypotonic PI solution before analysis using FACS. To quantify the difference in concentration of IL-3 required to promote viability but *not* proliferation, an ‘ED<sub>50</sub>’ was determined by establishing the concentration of IL-3 at which half of the cells undergo apoptosis, or exit S-phase. When the results of all independent lines were examined as a pool, (figure 3.1A), the ED<sub>50</sub> of cell cycle withdrawal was approximately 0.5 pM, whilst the ED<sub>50</sub> of apoptosis was 0.1 pM IL-3. It was evident that although a subtle difference between the concentrations of IL-3 that promoted viability and proliferation could be distinguished, these two signalling outcomes from the IL-3 receptor were not completely separated by IL-3 concentration. There was no IL-3 concentration in which the majority of cells were viable but not also dividing. As a difference of at least 10 fold was considered an indicator of significance (one that would approach that observed by Guthridge et al. in the human GM-CSF receptor), these results suggested that cell cycle withdrawal and apoptosis occur at similar IL-3 concentrations. There was however, clonal variation in the response to IL-3 titration. Four clones appeared to have a more significant separation between the concentrations of IL-3 required for viability and proliferation, whereas the other four clones had virtually no such separation. These are shown in figures 3.1 B and C. This data shows that there may be a subset of cells for which IL-3 concentrations can be titrated to maintain viability but not drive cell division. However, this is, at least in FDM cells, subject to clonal variation and is not necessarily an intrinsic feature of IL-3 receptor signalling observed in all cell lines. Thus, the approach to vary IL-3 concentrations would not be sufficiently robust to identify survival-only signalling kinase activation and in particular to detect subtle changes in AKT activation.
Figure 3.1: Cell cycle withdrawal occurs independently of apoptosis

(A) 8 independent WT FDM clones were cultured at the indicated IL-3 concentrations for 72 hours before cells were stained with AV/PI or fixed with a hypotonic-PI solution. Cells were analysed using flow cytometry, and then plotted from minimum to maximum. Data represents the average of 5 independent experiments +/- S.E.M. These results suggest that cells exit S-Phase at similar IL-3 concentrations as cells progress to apoptosis (B) A subset of (A) (N=4) were plotted separately from minimum to maximum. These cell lines exit cell cycle at the same IL-3 concentrations as that at which they undergo apoptosis. (C) A subset of (A) (N=4) were plotted separately from minimum to maximum. These cell lines exit cell cycle at a significantly higher concentration of IL-3 as that at which they undergo apoptosis. These results can neither confirm nor refute whether there are distinct pathways signalling cell cycle progression or apoptosis activated at differing IL-3 concentrations.
3.2 The generation of Akt⁻/⁻ IL-3 dependent cell lines indicates each AKT isoform is redundant for IL-3 dependent signalling

To determine the requirement of each AKT isoform individually, haematopoietic stem cells from the foetal livers of E14.5 Akt¹/¹, Akt²/² and Akt³/³ were harvested. Each of these gene-deleted mice had been previously described [128, 130, 138]. FDM cell lines were generated as described previously (chapter 2.1,). FDMs are usually cultured at a concentration of 0.25 ng/mL IL-3, however it was noted that this concentration of IL-3 was insufficient for consistent generation or maintenance of the Akt¹/¹ FDM cells specifically. At 0.25 ng/mL Akt¹/¹ cells have a high amount of background cell death and the doubling time of these cells was longer than cells derived from heterozygous litter-mates. The concentration of IL-3 these cells were cultured in was doubled (0.5 ng/mL). Under these conditions a higher proportion of Akt¹/¹ cells were viable and cellular proliferation was similar to WT cells. Therefore, cells derived from mice lacking each AKT isoform, including the WT litter-mate controls, were maintained in culture at 0.5 ng/mL IL-3.

Although Akt¹/¹ cell lines required increased IL-3 concentrations to maintain viability, the ability to generate IL-3 dependent FDMs from each of the Akt isoform deleted mice suggests that no Akt isoform is absolutely required for IL-3 dependent survival or proliferation.

3.3 The deletion of each AKT isoform does not alter apoptotic kinetics after IL-3 withdrawal

Having determined that IL-3 dependent Akt⁻/⁻ cell lines are viable in the presence of IL-3, it was next to be determined whether IL-3 withdrawal induced apoptosis might be influenced by the deletion of each AKT isoform. Multiple independent clones of Akt¹/¹, Akt²/², Akt³/³, WT (litter-mates) and Bax⁻/⁻Bak⁻/⁻ cells were starved of IL-3 for the indicated amount of time before viability was determined (figure 3.2).
Figure 3.2: Deletion of each AKT isoform does not alter apoptotic kinetics

Cells of the indicated genotypes were washed of IL-3 and replated into IL-3 deficient media. At the indicated time-points the cellular viability was determined using PI exclusion on FACS. These results show that the deletion of AKT1 (N=6), AKT2 (N=4) or AKT3 (N=3) does not alter the apoptotic kinetics compared to WT (N=4) cells. This data shows that the deletion of each AKT isoform does not alter apoptotic kinetics compared to WT cells.
The withdrawal of IL-3 does not promote apoptosis in IL-3 dependent $Bax^{-/-}$ $Bak^{-/-}$ cells, as was expected. This is because BAX and BAK are absolutely required for apoptosis induced by cytokine deprivation [149]. After 24 hours of IL-3 deprivation, approximately 35% of WT cells remained viable, decreasing to 15% viability after 48 hours. Cells lacking AKT1, AKT2 or AKT3 had similar apoptotic kinetics as WT cells. These results suggest that the deletion of each individual AKT isoform does not accelerate apoptosis after IL-3 starvation.

### 3.4 AKT1 is required to maintain viability at low IL-3 concentrations

As previously mentioned, Guthridge et al described a model of GM-CSF survival signalling that suggested distinct pathways were activated in response to various cytokine concentrations. The initial observation that $Akt1^{-/-}$ FDM cells required higher IL-3 to maintain in culture suggested the possibility that AKT1 may be important for cellular viability and proliferation at limiting IL-3 concentrations. It should be noted that even if our initial results (figure 3.1) suggested that limiting IL-3 concentrations do not, in FDM cells, reliably separate survival and proliferation signals; this would not preclude AKT1 contributing to both survival and proliferation at limiting IL-3 concentrations.

$Akt1^{-/-}$, $Akt2^{-/-}$, $Akt3^{-/-}$ and WT (litter-mate) cells were cultured in a titration of IL-3 for 48 hours before the cell viability was measured. WT cells remained above 75% viability at 0.5 ng/mL IL-3 after 48 hours, as do $Akt1^{-/-}$, $Akt2^{-/-}$ and $Akt3^{-/-}$ cells (figure 3.3). Complete IL-3 withdrawal resulted in less than 25% of WT cells remaining viable. $Akt2^{-/-}$ cells had higher viability than WT cells, whilst $Akt3^{-/-}$ cells had reduced viability, although neither of these results is statistically significant. $Akt1^{-/-}$ cells, in contrast, had significantly reduced viability compared to WT cells.

At low concentrations of IL-3 (5-100 pg/mL) the majority of WT, $Akt2^{-/-}$ and $Akt3^{-/-}$ remain viable (>50%). Strikingly, cells that lacked AKT1 were significantly more susceptible to apoptosis at the same IL-3 concentrations. To ensure this result was truly repeatable, a second parental strain of $Akt1^{-/-}$ cells was generated and the experiment repeated with similar outcomes. The pooled data are shown in Figure 3.3.
Figure 3.3: AKT1 is required for survival signalling at low concentrations of IL-3

At least four individual clones of Akt1/−, Akt2/−, Akt3/− and WT cells were plated in a titration of IL-3. After 48 hours cellular viability was determined by PI uptake using flow cytometry. These results show that cells lacking AKT2 and AKT3 undergo apoptosis at similar concentrations of IL-3 as WT cells. Cells lacking AKT1 have a significantly reduced viability at low concentrations of IL-3 compared to WT cells, which suggests that AKT1 is critical for survival signalling at these IL-3 concentrations. Data represents average of three independent experiments +/- S.E.M.
To quantify the difference in the responses to limiting IL-3, the ED\textsubscript{50} was measured as previously described. In WT cells, the ED\textsubscript{50} was 2.5 pg/mL IL-3. Akt\textsuperscript{2/-} and Akt\textsuperscript{3/-} cells had an ED\textsubscript{50} of 8 and 10 pg/mL respectively, suggesting there was only a slight difference in response to IL-3 titration compared to WT cells. Cells lacking AKT1 on the other hand had an ED\textsubscript{50} of 40 pg/mL IL-3, demonstrating that higher concentrations of IL-3 were required to maintain 50% viability when AKT1 was absent.

These results indicate that the deletion of AKT2 or AKT3 does not affect the concentrations of IL-3 at which cells undergo apoptosis or can maintain viability. Critically, the loss of AKT1 decreases the sensitivity of cells to low IL-3 concentrations, showing that AKT1 is required for IL-3 receptor dependent signalling survival at low IL-3 concentrations.

3.5 ΔPH-AKT over-expression increases sensitivity to low IL-3 concentrations for survival signalling

Several research teams have identified AKT as an integral part of the survival signal maintained by cytokines [148, 154, 216]. Furthermore, the above data suggests that there is a requirement for AKT1 to maintain survival at low IL-3 concentrations. If AKT did play a critical function in maintaining survival at limiting IL-3 concentrations, it was reasoned that the over-expression of constitutively active AKT might maintain survival, even after loss of the upstream cytokine signalling. Multiple independent clones of WT cells expressing the inducible ΔPH-AKT\textsubscript{1}, ΔPH-AKT\textsubscript{2} or eGFP over-expression constructs were generated, as described in the methods section (chapter 2.3). The cells were pre-cultured in the presence or absence of 4-OHT for 24 hours before being plated into a titration of IL-3, to which 4-OHT was re-added to the required cells.

As expected, cells over-expressing eGFP did not have increased viability at any concentration of IL-3 (figure 3.4c).

The induction of ΔPH-AKT\textsubscript{1} over-expression significantly increased the viability of cells cultured at all concentrations of IL-3 (figure 3.4a). In the complete absence of IL-3 however, ΔPH-AKT\textsubscript{1} over-expression was not sufficient to prevent apoptosis from occurring in WT cells, thus ΔPH-AKT\textsubscript{1} was not sufficient to promote viability in the
absence of IL-3. It is noteworthy that there was approximately a 20% increase in cellular viability when ΔPH-AKT1 is over-expressed.

It should be noted that the viability of ΔPH-AKT over-expressing cells (whether in the presence of 4-OHT or not) is greater than cells expressing GFP. This is explained by the ‘leakiness’ of the inducible system, as was demonstrated in the FACS analysis of expression (figure 2.3). Thus, the uninduced cells could still be considered to be over-expressing ΔPH-AKT, albeit at much lower levels than induced cells. When survival in induced and uninduced ΔPH-AKT over-expressing cells was compared to eGFP over-expressing cells, there was a significant increase in viability due to ΔPH-AKT over-expression. The higher viability following ΔPH-AKT over-expression suggested that higher ΔPH-AKT levels promoted increased viability, that is, induced over-expression of ΔPH-AKT promoted higher viability than uninduced ΔPH-AKT over-expression, which promoted in turn higher viability than cells not over-expressing AKT (eGFP over-expression).

ΔPH-AKT2 over-expressing cells also had significantly increased viability at all concentrations of IL-3 (figure 3.4b). As with ΔPH-AKT1, in the absence of IL-3 there was some protection from apoptosis caused by induced ΔPH-AKT2 over-expression, however the majority of these cells still underwent apoptosis. When the cells were cultured in low IL-3 concentrations, ΔPH-AKT2 over-expression significantly increased viability. This suggested that over-expression of ΔPH-AKT1 or ΔPH-AKT2 was sufficient to promote viability in the presence of IL-3.
Figure 3.4: Induced AKT over-expression increases survival in low IL-3 concentrations

Four independent WT cell lines expressing the AKT1 (A), AKT2 (B) or eGFP (C) over-expression construct were induced with 4-OHT for 24 hours before being cultured in a titration of IL-3. Cells were tested for viability after 72 hours by measuring PI uptake using flow cytometry. These results indicate that induced over-expression of AKT1 or AKT2 significantly increases the viability of cells at low IL-3 concentrations when compared to uninduced cells or eGFP controls. Data represents average of three independent experiments +/- S.E.M.
3.6 AKT inhibitor VIII dephosphorylates AKT at S473, effectively shutting down AKT activity

AKT’s capacity to maintain survival signalling, its proliferative effects and its potential to increase cellular metabolism, are thought to contribute to AKT having a role as a potent proto-oncogene. Many AKT inhibitors have been tested in *in vitro, in vivo* and human trials (see chapter 1.2.4 for more information), albeit with somewhat underwhelming efficacy. Some AKT inhibitors such as the compound creatively named AKT isozyme selective inhibitor VIII can be used to dissect the pathways regulated by AKT. Therefore, the AKT VIII specific inhibitor (AKTi), which selectively inhibits the AKT1 and AKT2 isoforms, was tested in two independent WT clones cultured in the presence of 0.25 ng/mL IL-3 and the indicated concentrations of AKTi for 24 hours. Cells were lysed and resolved by SDS-PAGE and probed with antibodies against phosphoAKT (S473 phosphorylation), total AKT and Actin as a loading control (figure 3.5).

Under these conditions AKTi dephosphorylated S473, thereby shutting down AKT activity. As the IL-3 receptor activates the AKT pathway by indirectly phosphorylating AKT S473, it is critical that IL-3 signalling did not supersede the effect of the inhibitor under these conditions. This inhibitor was therefore further utilised to understand the signalling mechanisms of AKT.
**Figure 3.5: AKT inhibitor VIII dephosphorylates AKT at S473**

Two independent WT clones were cultured in the presence of IL-3 at the indicated concentrations of AKT inhibitor VIII (AKTi). After 24 hours, the cells were lysed and resolved by SDS-PAGE. The figure shows a reduction in Akt S473 phosphorylation, indicating diminished AKT activity.
3.7 AKT inhibitor VIII induces apoptosis to the same extent as PI3K inhibitors

As previously mentioned, AKT inhibitors have performed poorly as therapeutic agents. Within the last decade however, our understanding of the pathways regulating AKT, specifically PI3K, have increased to a point where promising new candidate inhibitors are emerging as potential treatments. These include novel PI3K inhibitors and dual kinase inhibitors targeting signalling components either including AKT or upstream/downstream of AKT. An early PI3K inhibitor, Ly294002, did not prove to be particularly effective as an anti-tumour agent. It has however been frequently utilised for research into PI3K/AKT pathway signalling. For example, Ly294002 inhibits IL-3 dependent survival signalling [149]. It was therefore hypothesised that, given AKT signalling is dependent on PI3K, AKT specific inhibitors (AKT inhibitor VIII) would disrupt IL-3 dependent survival signalling similarly to Ly294002. WT FDM cells were cultured in the presence of LY294002 at the same concentrations used in the previous research [149], or with the AKTi at 0, 1 or 5 µM. The cells were plated in either the presence or absence of 0.25 ng/mL IL-3. After 24 hours, the cells were tested for viability using PI exclusion measure by flow cytometry (figure 3.6).

These results indicated that the AKTi was also ineffective at inducing apoptosis in the presence of IL-3, even though the doses of the inhibitor were sufficient to block AKT phosphorylation (see figure 3.6). This was a further indication that the survival signal promoted by AKT was not absolutely required for IL-3 dependent survival, similarly to as seen in figures 3.2 and 3.3, where in Akt−/− cells, AKT may titrate the sensitivity to IL-3, but was not required for cellular survival. This was further supported by the Ly294002 treated cells, which also did not undergo apoptosis in the presence of IL-3. This was similar to as seen in previous experiments using WT FDM cells and AKT inhibitors [166]. When cultured in high concentrations of AKT inhibitors and IL-3, a small decrease in viability was observed, however this was not statistically significant. This may be evidence of some off-target or cytotoxic effects.
Figure 3.6: AKTi VIII and LY294002 promote apoptosis in the presence or absence of IL-3

Three independent WT clones were cultured in the presence or absence of IL-3 at the indicated concentrations of either AKT inhibitor (AKTi) or the PI-3K inhibitor LY294002. After 24 hours the cells were tested for viability by PI exclusion using flow cytometry. These results indicate that in the presence of IL-3 both inhibitors only slightly increase apoptosis at high concentrations, whilst in the absence of IL-3, AKT or PI3K accelerates apoptosis. Data represents average of two independent experiments +/- S.E.M.
When FDM cells were starved of IL-3, approximately 75% of the cells, the vast majority, succumbed to apoptosis within 48 hours. This also occurred in either the presence or absence of the AKT inhibitors. At 1 µM of AKTi, or 25 µM of Ly294002 however, there was evidence of a subtle increase in apoptosis in the absence of IL-3. This result was significant (p < 0.05) when cells were treated with Ly294002, but was not significant in AKTi treated cells. This too suggested that a fraction of the cells detecting a limited IL-3 concentration were highly dependent on the PI-3K/Akt signalling axis.

### 3.8 AKT inhibitor induced apoptosis is most potent at low IL-3 concentrations

In light of the previous results, the efficacy of AKTi on FDMs cultured in limiting IL-3 concentrations was tested. Multiple independent WT cell lines were cultured in either 0.5 ng/mL (high) IL-3, 0.02 ng/mL (Low) IL-3 and 0 ng/mL IL-3. The AKTi was also added to these cells at 0, 1 and 5 µM. After 24 hours, the cell viability was assayed (figure 3.7).

In abundant IL-3, cell viability was approximately 80%. The addition of the AKTi only induced significant apoptosis at a concentration of 5 µM. This indicates that this higher concentration of AKTi was sufficient to promote apoptosis, although at 1µM of AKTi was sufficient to deactivate Akt signalling (figure 3.5) as indicated by Akt phosphorylation on Western blot. It is noteworthy that 1µM AKTi did not induce apoptosis.

In the absence of IL-3, WT cells underwent apoptosis; about 50% of cells were viable after 24 hours. The addition of AKTi in the absence of IL-3 further decreased the viability of these cells, although the majority of these cells had already undergone apoptosis due to IL-3 withdrawal alone.

When cultured at 0.02 ng/mL IL-3, in the absence of AKTi, the majority of WT cells (∼65%) remained viable. Strikingly, even 1µM AKTi was sufficient to induce a significant proportion of cells to undergo apoptosis.

These results indicate that AKT signalling was most critical for maintaining survival at low concentrations of IL-3.
Figure 3.7: Cells cultured in low concentrations of IL-3 are most sensitive to apoptosis induced by AKT inhibition

Three independent WT clones were cultured in the indicated IL-3 concentrations with the indicated AKTi VIII concentrations for 24 hours before cell viability was measured by PI uptake using flow cytometry. These data show that the AKT inhibitor is insufficient to kill cells in the presence of high concentrations of IL-3, although very high AKT VIII concentrations cause a minority of cells to undergo apoptosis. Cells grown in the absence of IL-3 undergo the majority of apoptosis independently of AKT inhibition, although at least half of surviving cells undergo apoptosis due to AKT inhibition. Cells grown in low IL-3 concentrations remain healthy, although low AKTi VIII concentrations are sufficient to kill the majority of cells. Data represents average of two independent experiments +/- S.E.M.
3.9 Over-expression of ΔPH-AKT1 is insufficient to prevent apoptosis caused by PIK-75

PI3K inhibitors, which may also have significant activity against other kinases, induce apoptosis in FDMs in a similar manner to AKT inhibitors (figure 3.6). Newer PI3K inhibitors are not only more specific, with higher affinities for PI3K, but are also capable of targeting individual p110 isoforms. The PI3K inhibitor, PIK-75, for instance is highly specific for p110α, and is capable of rapidly inducing apoptosis even in the presence of IL-3 in WT, but not Bax−/−;Bak−/− FDMs [183, 184]. If the cytokine dependent survival-signalling axis were PI3K/AKT dependent, then one would predict that apoptosis induced by inhibition of PI3K would be prevented by over-expression of artificially activated ΔPH-AKT.

To test this prediction, WT FDMs over-expressing either ΔPH-AKT1 or eGFP were cultured in the presence or absence of IL-3 and/or 4-OHT, in increasing concentrations of PIK-75. It should be mentioned that ΔPH-AKT2 cells were not tested in this experiment as it had previously been observed that AKT1 was the predominant isoform during IL-3 dependent survival signaling (figure 3.4). After 24 hours, cell viability was determined. As expected, cells cultured in the absence of IL-3 succumbed to apoptosis, with only minor protection from ΔPH-AKT1 over-expression. Strikingly, when 100nM of PIK-75 was added, nearly 100% of cells died in as little as 24 hours, independently of the presence of IL-3. Interestingly, when ΔPH-AKT1 is over-expressed, apoptosis is still stimulated by 100nM PIK-75 (figure 3.8). These results suggest that over-expressed, active AKT was insufficient to prevent cell death stimulated by PI3K inhibition.
Figure 3.8: Over-expression of ΔPH-AKT1 is insufficient to prevent PI3K inhibition induced cell death

(A) Three independent WT cell lines expressing the eGFP construct were cultured in either the presence or absence of 4-OHT for 24 hours before cells were washed and replated into the presence or absence of IL-3 at the indicated concentrations of PIK-75. After 24 hours the cells were tested for viability by PI uptake. (B) Four independent WT cell lines expressing the ΔPH-AKT1 construct were cultured in either the presence or absence of 4-OHT for 24 hours before cells were washed and replated into the presence or absence of IL-3 at the indicated concentrations of PIK-75. After 24 hours the cells were tested for viability by PI uptake. These data indicate that PIK-75 induces apoptosis in the presence or absence of IL-3, and that ΔPH-AKT over-expression is insufficient to prevent this. Data represents average of two independent experiments +/- S.E.M.
3.10 UO126 dephosphorylates p42/p44, effectively shutting down MEK activity

Other pathways besides the PI3K/AKT pathway contribute to cytokine dependent survival, including the MEK/ERK pathway. Some evidence indicates significant ‘cross-talk’ between MEK/ERK and the PI3K/AKT pathways [90-92]. Specifically, AKT has been shown to phosphorylate and activate ERK, whilst MEK has been also been shown to phosphorylate AKT. This cross-talk phenomenon is proposed to fine-tune the survival signals. Using the tools at our disposal, we sought evidence of this crosstalk in IL-3 signalling in FDMs.

To determine whether some level of crosstalk exists, a specific MEK inhibitor, UO126, was utilised to deactivate MEK signalling. Two independent WT FDM clones were cultured in the presence of IL-3 and the indicated concentrations of the MEK inhibitor. Cells were lysed after 24 hours and resolved on SDS-PAGE (figure 3.8). Using ERK phosphorylation as a readout of MEK activity, a decrease in MEK activity is observed in both clones.

3.11 MEK inhibition induced apoptosis does not require AKT1 or p53

To determine whether MEK inhibition is sufficient to promote apoptosis, multiple independent cell lines of the indicated genotypes (WT, Akt1−/− and Bax−/−;Bak−/−, as well as p53−/− cells, for reasons which will be revealed in chapter 5) were cultured in the presence or absence of IL-3 at the increasing concentrations of UO126 for 24 hours. Cellular viability was then determined (figure 3.11).

In WT cells cultured in the presence of IL-3, MEK inhibition induced a subtle, yet significant amount of apoptosis, with viability declining from a mean of around 75% to approximately 60%. In the absence of IL-3, MEK inhibition further exacerbated the loss of viability that resulted from IL-3 deprivation, suggesting the MEK/ERK pathway partially contributed to survival signalling. As Bax−/−;Bak−/− cells are resistant to apoptosis caused by MEK inhibition in both the presence or absence of IL-3, MEK induced apoptosis must occur via the intrinsic apoptotic pathway.
Figure 3.9: UO126 decreases Erk phosphorylation

Two independent WT clones were cultured in the presence of IL-3 at the indicated concentrations of UO126. After 24 hours the cells were lysed and resolved by SDS-PAGE. The figure indicates the reduced activity of MEK (as shown by Erk phosphorylation of T202/Y204)
The role of Akt during IL-3 survival signalling

Figure 3.10: MEK inhibition induced apoptosis is not dependent on p53 or AKT1

At least three independent clones of WT, p53−/−, Akt1−/− and Bax−/−;Bak−/− cell lines were grown in either the presence or absence of IL-3 with the indicated concentrations of UO126 (MEKi). After 24 hours, cells were stained with PI and analysed by flow cytometry. Results suggest that cells lacking p53 or AKT1 are not protected from MEK induced apoptosis when compared to WT cells. Data represents average of two independent experiments +/- S.E.M.
Akt1−/− cells underwent apoptosis to a similar degree as WT cells in either the presence or absence of IL-3, or the presence and absence of UO126, indicating that UO126 contributes to apoptosis independently of AKT1. As had been shown previously, p53−/− FDM cells are more resistant to apoptosis induced by IL-3 withdrawal. The MEK inhibitor induced apoptosis in p53−/− cells to the same degree as in WT cells, showing that apoptosis induced by this inhibitor does not require p53. These results indicate that MEK inhibition induced apoptosis functions independently of either AKT1 or p53.

3.12 The over-expression of ΔPH-AKT is not sufficient to promote cell-cycling but delays G1 arrest after IL-3 starvation.

To determine if ΔPH-AKT over-expression was sufficient to promote IL-3 independent proliferation, Bax−/−;Bak−/− cell lines over-expressing either the ΔPH-AKT1, ΔPH-AKT2 or eGFP constructs were generated. These lines were used as they resist apoptosis induced by IL-3 deprivation. Thus, cells could be tested over time in the absence of cytokine. Cells were cultured in the presence or absence of 4-OHT for 24 hours to induce ΔPH-AKT or eGFP expression, before half of the cells were starved of IL-3. At the indicated time-points, cells were analysed to determine the percentage of cells in S-phase. An example of cell cycle analysis is included (figure 3.11a).

When eGFP was over-expressed, approximately 40-50% of cells remained in S-phase at any point (figure 3.11d). Upon IL-3 withdrawal, these cells rapidly exited S-phase, so that after 24 hours the majority of these cells had accumulated in G0/G1. In cells expressing the ΔPH-AKT1 construct, the same percentage of ΔPH-AKT1 over-expressing cells were in S-phase in the presence of IL-3 compared to uninduced cells. Thus, there was no change in the proportion of cells in each phase of the cell cycle by virtue of ΔPH-AKT1 expression, in the presence of IL-3 (figure 3.11b). When IL-3 was removed from cells over-expressing ΔPH-AKT1, there was a significant delay in the rate at which cells exited S-phase. ΔPH-AKT1 over-expression however, was not sufficient to prevent cells exiting S-phase after prolonged IL-3 withdrawal.

A similar percentage of cells over-expressing ΔPH-AKT2 in the presence of IL-3 were in S-phase as control cells (figure 3.11c). Upon IL-3 starvation, there was also a subtle, yet significant delay in S-phase exiting in cells induced to express ΔPH-AKT2
compared to control cells. The effect of ΔPH-AKT2 over-expression on delaying cell cycle exit was less than the effect of ΔPH-AKT1 over-expression.

Together, this data indicates that in the presence of full IL-3 receptor signalling, the over-expression of ΔPH-AKT did not change the proportion of cells in each phase of the cell cycle and did not therefore promote more rapid proliferation. The most noticeable effect was in the absence of IL-3, where cells expressing ΔPH-AKT were slower to undergo cell cycle arrest, although they did all ultimately stop dividing.

3.12.1 The over-expression of ΔPH-Akt1 increases Forward Scatter characteristics of cells on flow cytometry

During the above investigation of the effects of ΔPH-AKT over-expression on proliferation, it was noted that a curious effect was observed in relation to the forward scatter (FSC) characteristics of the cells grown in the various conditions. The FSC parameter is an indirect measure of cellular size/volume. Normally, FDMs starved of IL-3 will shrink, but as the cells used were Bax⁻/⁻Bak⁻/⁻ cells, they would not undergo the other manifestations of apoptosis. Figure 3.12a shows cells shrunk in response to IL-3 deprivation, but strikingly, the cells increased in FSC in response to the addition of 4-OHT. This implies that ΔPH-AKT1 over-expression promotes cellular growth independently of IL-3. These results were quantified by normalizing the median FSC of each condition to cells grown under ‘normal’ conditions, those cultured in the presence of IL-3 but absence of 4-OHT.

These results indicate that control cells shrunk by roughly 60% after IL-3 withdrawal, whilst ΔPH-AKT1 prevented this. Over time however, ΔPH-AKT1 over-expression merely delayed this effect (figure 3.12b), and eventually cells shrunk to comparable levels as uninduced cells. As forward scatter is a measure of cellular volume, this indicates that one role of AKT is to regulate cellular metabolism and growth which is correlated to protein synthesis. This is consistent with the current literature (see chapter 3.5.3), and as this thesis will focus the survival signals maintained by AKT, follow-up investigations were not performed.
Figure 3.11: Induced ΔPH-AKT over-expression does not promote S-phase transition independently of IL-3, but delays S-phase exit after IL-3 withdrawal

(A) A representative cell cycle histogram cultured in the presence of IL-3 and absence of 4-OHT, showing the majority of cells in G₀/G₁. Six BB clones expressing the inducible ΔPH-AKT1 (B), ΔPH-AKT2 (C) or eGFP (D) or constructs were stimulated with 4-OHT for 24 hours before being cultured in the presence or absence of IL-3 and/or 4-OHT. At indicated time points, cells were fixed and stained in a hypotonic PI solution, then analysed by flow cytometry. When IL-3 is withdrawn induced ΔPH-AKT1 over-expression delays the withdrawal of cells from S-phase, however does not prevent cells from exiting S-phase. Similarly, induced ΔPH-AKT2 over-expression subtly delays S-phase withdrawal after IL-3 withdrawal, but does prevent cells from exiting cell cycle. Data represents average of three independent experiments +/- S.E.M.
Figure 3.12: Induced AKT1 over-expression increases the forward scatter profile of cells independently of IL-3

(A) An example of ΔPH-AKT1 over-expression increasing the forward scatter characteristics of cells in a sample experiment. Four independent Bax⁻/⁻;Bak⁻/⁻ clones expressing the inducible ΔPH-AKT1 (B), ΔPH-AKT2 (C) or eGFP (D) constructs were cultured in either the presence or absence of IL-3 and/or 4-OHT. At the indicated time points the forward scatter profile of cells was determined using flow cytometry, with means normalised to cells grown in the presence of IL-3 / absence of 4-OHT. This data shows that ΔPH-AKT1 but not ΔPH-AKT2 or GFP significantly increases the forward scatter of cells, in either the presence or absence of IL-3. Data represents average of three independent experiments +/- S.E.M.
### 3.13 ΔPH-AKT over-expression is insufficient to promote cell cycling and proliferation independently of IL-3

ΔPH-AKT over-expression was not capable of preventing cells exiting S-phase after IL-3 withdrawal; however, it was possible that ΔPH-AKT over-expression was sufficient to initiate entry into S-phase independently of IL-3. Bax⁻/⁻·Bak⁻/⁻ cells expressing the eGFP, ΔPH-AKT1 or ΔPH-AKT2 constructs were starved of IL-3 for 24 hours. Twenty-four hours after the removal of IL-3, the majority of cells were synchronised at the G₀/G₁ phase of cell cycle. IL-3 or 4-OHT was then re-added to the culture media and at the indicated time-points, the percentage of cells in S-phase was determined. The total number of cells was also counted to estimate proliferation.

When restimulated with IL-3, cells rapidly re-entered S-phase. Interestingly, cells expressing the eGFP construct were marginally slower, but not statistically significantly slower, to re-enter cycle compared to cells expressing either ΔPH-AKT construct (figure 3.13a). As expected, by 24 hours 25-40% of the cells were in S-phase indicating a return baseline proliferation (see the percentage of cells in S-phase in Fig 3.12). Furthermore, five days after the re-addition of IL-3, cell number has increased 100 fold (figure 3.13b).

In the cells stimulated with 4-OHT to induce ΔPH-AKT expression, cells remained in G₀/G₁ (figure 3.11a). Prolonged 4-OHT exposure was not sufficient to promote most cells to re-enter S-phase. It was observed that cell numbers in these cultures slowly increased over time; however, this was the same for ΔPH-AKT1, ΔPH-AKT2 and eGFP over-expressing cells (figure 3.11b), showing that this effect is not due to ΔPH-AKT specifically. In addition, the increase in cell number when restimulated with 4-OHT was minimal compared to the increase in cell number when cells are restimulated with IL-3. This increase in cell numbers may have resulted from some residual IL-3 still present in cultures after washing driving a very small number of slowly dividing cells. Another explanation however, would be that the over-expressed ΔPH-AKT was no longer induced after IL-3 withdrawal. It should be reiterated though that previous results indicate ΔPH-AKT expression is not affected by IL-3 signalling (figure 2.4).
The role of Akt during IL-3 survival signalling

Figure 3.13: Induced AKT over-expression does not promote S-phase re-entry or proliferation independently of IL-3

Four independent Bax−/−;Bak−/− clones expressing the inducible AKT1, AKT2 or eGFP constructs were starved of IL-3 for 24 hours before either IL-3 or 4-OHT was re-added. At indicated time-points cells were fixed in a hypotonic PI solution and analysed by flow cytometry, or trypan blue negatively stained cells were counted using a haemocytometer. These results indicate that the re-addition of IL-3 promotes rapid re-entry of cells into S-phase after 24 hours, leading to increased cell number after 5 days. Prolonged induced over-expression of AKT1, AKT2 or eGFP is insufficient to promote cell cycling or to promote proliferation. Data represents average of two independent experiments +/- S.E.M.
3.14 Conclusions

It was hypothesised that should any of the AKT isoforms have a critical role in cytokine dependent survival signalling, or indeed any process required for cell culturing including proliferation, glucose metabolism or protein synthesis, that deletion of the Akt gene would result in cells being highly sensitive to IL-3 deprivation-induced apoptosis, or perhaps not even viable in an IL-3 dependent cell line. Indeed, there is indication that AKT was contributing to the IL-3 receptor mediated survival signal because much higher concentrations of IL-3 were required to maintain Akt1/− cells in culture. However, it is also abundantly apparent that because FDM cells could be generated from each knockout strain, no single AKT isoform is required for IL-3 dependent viability. It may be that the survival role of each AKT isoform is redundant, and that in the absence of one isoform of AKT, there is still sufficient AKT present to replace signalling. Such redundancy and compensation could be tested using compound deleted animals, which were not available at the time. This redundancy may also be due to parallel pathways taking over the role of AKT during IL-3 dependent survival signalling. One possible way to determine whether this is the case is to utilise conditional knockouts.

One pathway proposed to share this cytokine dependent survival role is the MEK/ERK pathway, which has been postulated to co-operate with the PI3K/AKT pathway. Such ‘cross-talk’ as it is described was not evident in FDMs, as apoptosis induced by inhibition of MEK/ERK is not altered by the deletion of AKT1. Furthermore, evidence presented within the next chapter will show that AKT inhibitors do not alter the phosphorylation state of ERK 202/204, nor do MEK inhibitors deactivate AKT (figure 5.5). Whilst the literature does not necessarily corroborate this hypothesis [90-92], the observation presented may only apply in context of the cell line utilised, that is, perhaps non-physiological concentrations of IL-3 as high as required to culture FDM cells may supersede any fine AKT/ERK crosstalk effects.

Interestingly, there does appear to be a specific role for AKT1 in cytokine signalling, as cells cultured in low IL-3 concentrations require AKT1, but not AKT2 or AKT3 for survival. Furthermore, WT FDM cells are highly sensitive to AKT inhibition induced apoptosis and low IL-3 concentrations, but do not undergo appreciable amounts of apoptosis at high IL-3 concentrations. This is further evidence that the
survival role of AKT is redundant in FDMs, unless IL-3 concentrations decrease below a certain threshold.

There is an interesting contrast between AKT1 deletion and over-expression of the constitutively active ΔPH-AKT1. When over-expressed, either ΔPH-AKT1 or ΔPH-AKT2 is sufficient to promote some level of IL-3 independent signalling, both in prolonging survival and progression through cell cycle check points. Importantly however, supra-physiological expression of either AKT isoform neither prevents apoptosis nor maintains proliferation independently of IL-3, indicating that AKT is insufficient to fully transform cells without the co-operation of further oncogenic mutations.

A surprising observation was the incapability of ΔPH-AKT over-expression to compensate for PI3K inhibition. Contrary to the literature [155, 164, 223] there does not appear to be a linear pathway of IL-3 promoting PI3K promoting AKT for survival signalling. However, this is explained by the recent observation that the PI3K ‘specific’ inhibitor PIK-75 also decreases Mcl-1 levels independently of PI3K signalling [184]. This is critical, as FDM cells, as well as many AMLs, are highly dependent on Mcl-1 expression. Therefore, it is more likely that the FDMs are undergoing apoptosis in response to Mcl-1 reduction than PI3K inhibition.

Chapter 3 focused on the requirement of AKT during IL-3 signalling. These results suggest that there is no specific AKT isoform required for survival, proliferation, or indeed any cellular process required for in vitro culture. One critical observation however, was the requirement for AKT1 specifically to maintain survival signalling in low IL-3 concentrations. Chapter 4 will pursue the machinery by which AKT might regulate the survival machinery.
4. **AKT1 regulates PUMA to maintain viability after IL-3 withdrawal**

In the previous chapter it was determined that AKT played a significant role in maintaining cellular viability, primarily when titrating cells in limiting concentrations of IL-3. Although this is not a new concept, the mechanisms by which AKT might regulate the repression of apoptosis pathways are a hotly debated topic within the apoptosis field. There is considerable focus on the mechanisms by which AKT regulates the Bcl-2 family of proteins that control the apoptosis pathways activated by IL-3 deprivation. As figure 1.9 indicates, there are several proposed models, centering on whether AKT controls the activity of the BH3-only proteins. Utilising the novel tools developed, this question of whether AKT regulates survival by regulating candidate BH3-only proteins was addressed.

It was reasoned that if ΔPH-AKT over-expression was capable of preventing or delaying apoptosis caused by IL-3 withdrawal, then the deletion of genes that were critical for ΔPH-AKT to do so would abolish any potential survival advantage, however small, derived from ΔPH-AKT over-expression. This research focused on the BH3-only proteins, as several models suggest these are direct substrates of Akt [148, 154, 215, 216, 224]. The logic of this approach is shown in figure 4.1. When IL-3 is removed from cells, they will undergo apoptosis to a degree that is dictated by their genotype and baseline response [149, 203]. This is in part related to the BH3-only proteins that are utilised. For example, specific BH3-protein expressions are increased in response to IL-3 withdrawal such as BIM or PUMA. ΔPH-AKT over-expression can increase the number of cells surviving by inhibiting the BH3-only protein. If this BH3 only protein is deleted, and if AKT functions to repress its activity, then one would predict that the cell death response to IL-3 will be same with ΔPH-AKT over-expression as without.
Figure 4.1: Determining the requirements for AKT dependent survival.

It was hypothesised that if AKT required a specific BH3 only protein (Green in the figure above) to maintain survival, then any survival advantage caused by AKT over-expression after IL-3 withdrawal would be lost if that BH3-only protein were deleted. In this image, the size of the symbol expresses the relative amount of apoptosis expected. Note that although in the $BH3^{-/-}$ cells less apoptosis is expected despite AKT over-expression not being induced, we expect little change in apoptosis when AKT over-expression is induced, particularly when compared to WT cells.
4.1 Puma is required for ΔPH-AKT1, but not ΔPH-AKT2 to maintain viability after IL-3 withdrawal.

Two independent parental FDM cell lines were generated from mice lacking the following genes; BAD, BIM, PUMA and BAX/BAK double knockouts. From these, at least four independent clones from each genotype were generated, expressing inducible ΔPH-AKT1, ΔPH-AKT2 or eGFP as a control. Each clone was tested for inducible uniform over-expression of ΔPH-AKT or eGFP (see chapter 2.9). Cells were then treated with 4-OHT for 24 hours prior to removal of IL-3 from the cultures. At the indicated time-points cell viability was determined by PI exclusion (figure 4.2) and clonal survival was performed using soft agar assays (see chapter 4.2). Every experiment performed included an eGFP expressing control and matched WT cell lines.

WT cells over-expressing eGFP rapidly underwent apoptosis after IL-3 withdrawal, being less than 50% viable after 24 hours and less than 10% viable after 48 hours. The induced over-expression of ΔPH-AKT1 significantly prolonged viability after IL-3 withdrawal, maintaining cell viability at 70% and 50% after 24 and 48 hours respectively. Prolonged IL-3 deprivation ultimately resulted in apoptosis in ΔPH-AKT1 over-expressing cells, again indicating that ΔPH-AKT1 over-expression alone is not sufficient to prevent IL-3 withdrawal induced apoptosis. WT cells infected with the ΔPH-AKT1 constructs, but which were not induced with 4-OHT, had a viability that was intermediate between eGFP cells and cells induced to express ΔPH-AKT1. This was most likely a result of ‘leaky’ ΔPH-AKT expression and further suggests that the effect of ΔPH-AKT1 over-expression to increase the proportion of viable cells is dose dependent.

Induction of ΔPH-AKT2 over-expression also increased the proportion of cells that were viable at each time point after IL-3 withdrawal, but like ΔPH-AKT1, was not sufficient to completely prevent apoptosis. Cells infected with the ΔPH-AKT2 constructs, but not treated with 4-OHT also had enhanced viability as a result of leaky ΔPH-AKT over-expression. Thus, over-expression of either ΔPH-AKT1 or ΔPH-AKT2 could increase the number of WT cells that remained viable after IL-3 deprivation.
Figure 4.2: AKT over-expression requires PUMA to prolong survival after IL-3 withdrawal

At least three independent cell lines derived from cells of the indicated genotypes were infected with the ΔPH-AKT1 (red lines, left side), ΔPH-AKT2 (red lines, right side) or eGFP (green lines). The cells were cultured in the presence or absence of 4-OHT for 24 hours before being starved of IL-3. At the indicated time-points cellular viability was determined by PI uptake using flow cytometry. These data indicate that ΔPH-AKT1 or ΔPH-AKT2 over-expression prolongs survival after IL-3 withdrawal, but does not prevent apoptosis from occurring. This phenotype does not require BIM or BAD, but ΔPH-AKT1 over-expression requires PUMA to promote survival. Data represents average of at least two independent experiments +/- S.E.M. With thanks to Chris Rifkin for independent confirmation of results.
As previously mentioned, \( \text{Bax}^{-/-}\text{Bak}^{-/-} \) cells do not undergo apoptosis in response to IL-3 withdrawal. eGFP over-expressing cells maintained above 90% viability over the five day period of IL-3 starvation. Therefore, over-expression of \( \Delta \text{PH}\text{-AKT1} \) or \( \Delta \text{PH}\text{-AKT2} \) did not increase survival as measured using flow cytometry. These cells were included as controls, and indicated that the intrinsic apoptosis pathway accounts for virtually all cell death induced by IL-3 deprivation. It was also clear that the increased cell survival that resulted from over-expression of either \( \Delta \text{PH}\text{-AKT1} \) or \( \Delta \text{PH}\text{-AKT2} \) in WT cells was substantially less than that provided by the complete inhibition of apoptosis pathways that result from deletion of both BAX and BAK.

\( \text{Bad}^{-/-} \) cells underwent apoptosis at a similar rate as WT cells when over-expressing eGFP, by 48 hours approximately 10% of cells remained viable. This result confirmed previously published observations [149] and proves that BAD is not required for IL-3 withdrawal induced apoptosis. Induced \( \Delta \text{PH}\text{-AKT1} \) over-expression in these cells significantly increased viability compared to uninduced cells after 48 and 72 hours when compared to cells over-expressing eGFP. This data shows that even if BAD is a substrate of \( \Delta \text{PH}\text{-AKT} \) (see chapter 4.5 and figure 4.5), this function was not required for \( \Delta \text{PH}\text{-AKT} \) to increase viability after cytokine deprivation. A similar effect was observed in \( \Delta \text{PH}\text{-AKT2} \) over-expressing cells. Induced \( \Delta \text{PH}\text{-AKT2} \) over-expression increased viability compared to uninduced cells or eGFP over-expressing cells, indicating \( \Delta \text{PH}\text{-AKT2} \) over-expression maintains viability via BAD independent mechanisms.

\( \text{Bim}^{-/-} \) cells over-expressing eGFP underwent apoptosis with similar kinetics as WT cells did. This result also confirmed previous observations that BIM is not required for IL-3 withdrawal induced apoptosis in FDM cells [149]. Induced over-expression of either \( \Delta \text{PH}\text{-AKT1} \) or \( \Delta \text{PH}\text{-AKT2} \) significantly increased viability after IL-3 withdrawal compared to uninduced \( \Delta \text{PH}\text{-AKT} \) expressing cells. Furthermore, uninduced cells had significantly increased viability compared to eGFP over-expressing cells, suggesting even low levels of \( \Delta \text{PH}\text{-AKT1} \) or \( \Delta \text{PH}\text{-AKT2} \) over-expression are sufficient to prolong viability independently of BIM after IL-3 withdrawal. Together this data shows that regulation of BAD or BIM by \( \Delta \text{PH}\text{-AKT} \) did not significantly contribute to the function of AKT to suppress apoptosis.
*Puma*/*- cells starved of IL-3, expressing the eGFP construct, had increased viability compared to WT cells. After 24 hours in the absence of IL-3, *Puma*/*- cells had ≈60% viability, decreasing to 50% after 48 hours. This alone supports previous observations that PUMA plays a critical role in IL-3 withdrawal induced apoptosis [149]. Induction of ΔPH-AKT2 over-expression in *Puma*/*- cells also significantly increased viability after IL-3 withdrawal above the baseline viability of PUMA knockout cells, compared to uninduced and eGFP over-expressing cells. *Puma*/*- FDM cells over-expressing ΔPH-AKT2 had >75% viability after 24 hours IL-3 deprivation, and ≈70% viability after 48 hours. This again suggests PUMA independent mechanisms are utilised for ΔPH-AKT2 to maintain viability after IL-3 withdrawal.

Strikingly, induced ΔPH-AKT1 over-expression was not sufficient to increase viability when compared to uninduced or eGFP over-expressing cells at any time-point measured. This observation was consistent across multiple independent clones and in multiple independent experiments. This suggested that ΔPH-AKT1 over-expression requires PUMA to increase viability after IL-3 withdrawal.

### 4.2 Puma is required for AKT1 to maintain clonogenic potential after IL-3 withdrawal

The clonogenic potential of cells is a measure of the capacity of a single cell to form a colony under optimal conditions. In the following experimental paradigm, clonogenicity measures the capability of a cell to survive a period of IL-3 deprivation and then, under conditions in which IL-3 is restored, measures the capacity of the cell to re-enter the cell cycle, re-establish metabolism and proliferate to give rise to a colony of daughter cells. For example, in a population of WT FDMs starved of IL-3, the number of cells at progressive time points that can give rise to a colony rapidly diminished as increasing numbers of cells undergo apoptosis. The cells that do give rise to colonies have remained viable and retained the functional capacity to proliferate under the right conditions. *Apaf1*/*- FDM cells on the other hand do not possess a critical initiating gene for the caspase cascade [203]. Therefore, when starved of IL-3 these cells did not appear to lose plasma membrane integrity in the early stages of IL-3 deprivation (a caspase-dependent event), but due to a loss of mitochondrial activity (from BAX and BAK oligomerisation), these cells had lost the functional capacity to respond when IL-3 was restored. Thus, they had committed to a cell death fate, but
lacked the required genes to manifest the usual morphology associated with apoptosis. Thus, the importance of combining clonogenic assays with the flow cytometry determination of viability is to capture cells which may appear to have survived IL-3 deprivation but which have lost the capacity to divide. Further, as would be the case in Bax^{-/-}/Bak^{-/-} cells, clonogenic assays will establish if ΔPH-AKT over-expression may alter the proportion of cells that can form colonies independently of the intrinsic apoptosis pathway.

The same experimental logic from the previous experiments was applied to the clonogenic assays, that if ΔPH-AKT over-expression were capable of preventing the loss of clonogenicity caused by IL-3 withdrawal, then the deletion of a gene that was critical for ΔPH-AKT to regulate would abrogate the effect of ΔPH-AKT over-expression. Every experiment performed included an eGFP expressing control and matched WT cell lines. Cells from figure 4.2 were removed from culture at the indicated time-points and replated into media containing 0.5 ng/mL IL-3, 20% FCS and 0.3% soft agar. After 10-14 days the number of colonies formed were counted and expressed relative to the number formed at time 0 of each experiment (ie before IL-3 starvation) (figure 4.3).
Figure 4.3: AKT over-expression requires PUMA to prolong clonogenic potential after IL-3 withdrawal

At least three independent cell lines derived from cells of the indicated genotypes were infected with the ΔPH-AKT1 (red lines, left side), ΔPH-AKT2 (red lines, right side) or eGFP (green lines). The cells were cultured in the presence or absence of 4-OHT for 24 hours before being starved of IL-3. At the indicated time-points clonogenicity was determined relative to the number of colonies counted before IL-3 starvation. These data indicate that ΔPH-AKT1 or ΔPH-AKT2 over-expression prolongs clonogenicity after IL-3 withdrawal, but does not prevent clonogenic loss from occurring. This phenotype does not require BIM or BAD, but AKT over-expression requires PUMA to maintain clonogenic potential. Data represents average of at least three independent experiments +/- S.E.M. With thanks to Chris Rifkin for independent confirmation of results.
WT cells, either in the presence or absence of 4-OHT, over-expressing eGFP lost clonogenic potential after IL-3 withdrawal, with the proportion of cells surviving to give rise to colonies falling to \( \approx 10\% \) after 48 hours. The induced over-expression of ΔPH-AKT1 significantly increased that proportion of cells with clonogenic potential after IL-3 withdrawal. However, as was consistently observed, prolonged IL-3 deprivation resulted in most cells dying and being unable to contribute to colony formation, whether or not they over-expressed ΔPH-AKT1.

Induction of ΔPH-AKT2 over-expression was also capable of significantly increasing clonogenic potential of cultures after IL-3 withdrawal, although once again ΔPH-AKT2 over-expression was insufficient to prevent eventual loss of clonogenicity.

Interestingly, \( \text{Bax}^{-/-};\text{Bak}^{-/-} \) cells retained clonogenic potential in response to IL-3 withdrawal. eGFP over-expressing cells maintained above 90% clonogenicity over the five day period of IL-3 starvation. Over-expression of ΔPH-AKT1 or ΔPH-AKT2 did not increase clonogenicity. This suggests that the principal reason why ΔPH-AKT1 or ΔPH-AKT2 over-expression increased the proportion of cells that could go on to form colonies was by blocking cell death. If ΔPH-AKT had a function to increase colony formation independent of cell death pathways, it would have been expected to observe increased colony formation in these cells as a result of ΔPH-AKT expression, independent of IL-3 deprivation. This was not the case.

Previous evidence has established that \( \text{Bad}^{-/-} \) cells undergo a loss of clonogenic potential at a similar rate as WT cells when starved of IL-3 [149]. When over-expressing eGFP, \( \text{Bad}^{-/-} \) cells underwent a significantly faster rate of clonogenic loss compared to WT eGFP over-expressing cells. Although no evidence is at hand to explain why this was so, numerous independent experiments using independent \( \text{Bad}^{-/-} \) FDM cell lines did not replicate these results. Nonetheless, inducing ΔPH-AKT1 over-expression in these cells significantly increased clonogenicity compared to uninduced cells after 24 and 72 hours. When compared to cells over-expressing eGFP, there was a significant increase in clonogenic viability caused by induced ΔPH-AKT1 over-expression. This data strongly indicates that ΔPH-AKT1 over-expression does not require BAD to maintain clonogenicity after IL-3 withdrawal.

A similar effect was observed in ΔPH-AKT2 over-expressing cells. Induced ΔPH-AKT2 over-expression increased viability compared to uninduced cells or eGFP over-
expressing cells, indicating ΔPH-AKT2 over-expression maintained viability via BAD independent mechanisms. The importance of this observation will be discussed, but briefly these data, together with other previously published data [149], show that the model in which Akt phosphorylation of BAD is the main intersection between IL-3 signalling pathways and apoptosis pathways is not correct.

*Bim*−/− cells over-expressing eGFP underwent clonogenic loss with similar kinetics as WT cells. Induced over-expression of either ΔPH-AKT1 or ΔPH-AKT2 did not significantly increase clonogenic potential after IL-3 withdrawal compared to uninduced ΔPH-AKT over-expressing cells. When compared to eGFP over-expressing cells, both ΔPH-AKT1 and ΔPH-AKT2 over-expression subtly, yet significantly increase clonogenicity. These data may be interpreted to indicate that ΔPH-AKT does function to repress BIM and increase clonogenic survival, although deletion of BIM did not prevent ΔPH-AKT promoting enhanced viability when assessed by flow cytometry. Alternatively, the subtle increase in numbers of colonies may indicate that ΔPH-AKT functions independently of BIM.

*Puma*−/− FDMs starved of IL-3, expressing the eGFP construct, had increased clonogenic potential compared to WT cells also expressing GFP over the time course of IL-3 deprivation. This independently confirmed previous observations [149]. The induction of ΔPH-AKT1 over-expression in the absence of Puma resulted in no additional formation of colonies when compared to *Puma*−/− FDM cells expressing eGFP, after IL-3 withdrawal. This is the predicted result expected if ΔPH-AKT were functioning to repress PUMA expression after IL-3 deprivation. This further supports the hypothesis that ΔPH-AKT1 over-expression was regulating PUMA expression to prevent cell death after IL-3 withdrawal.

Interestingly, despite ΔPH-AKT2 over-expression increasing viability in the absence of PUMA, a corresponding increase in clonogenicity was only observed at the 48 hour time point when compared to eGFP expressing cells. This may indicate that ΔPH-AKT2 over-expression only plays a role in short term survival by regulation of the BH3-only proteins, but not regulating long term viability.
4.3 Deregulation of AKT over-expression is not the cause of apoptosis after prolonged IL-3 deprivation

In the experiments shown in figure 4.2, a significant proportion of cells were observed to undergo apoptosis 48 and 72 hours after IL-3 withdrawal, despite being induced to express supra-physiological over-expression of constitutively activated ΔPH-AKT. To ensure this apoptosis was not a result of the inability of the inducible construct to effectively over-express ΔPH-AKT in the absence of IL-3 (which may shut down transcriptional and translational machinery), two independent Bax<sup>−/−</sup>Bak<sup>−/−</sup> clones were cultured in the presence or absence of 4-OHT for 24 hours, then starved of IL-3 for up to 72 hours. Lysates were generated and resolved using SDS-PAGE. Immunoblots were then probed for over-expressed ΔPH-AKT using HA antibodies (figure 4.4).

As expected, HA expression corresponded to cells cultured in the presence of 4-OHT. Even after prolonged IL-3 deprivation, 4-OHT was still sufficient to promote HA expression, indicating that the inducible construct still functions after prolonged IL-3 withdrawal. This was also observed in WT cells starved of IL-3 for 48 hours and using the phosphoAKT antibody (see 2.2).

4.4 Puma expression after IL-3 withdrawal is down-regulated by AKT over-expression.

To investigate the effect of ΔPH-AKT over-expression on Puma protein levels, WT cells expressing the ΔPH-AKT1, ΔPH-AKT2 and eGFP inducible constructs were cultured in the presence or absence of 4-OHT for 24 hours before being starved of IL-3 for the indicated amount of time. Cell lysates were resolved using SDS-PAGE and probed with antibodies against PUMA, AKT, pAKT and actin as a control for loading (figure 4.5).

When probed for pAKT (S473) or HA, cells cultured in the presence of 4-OHT had the highest levels of over-expressed AKT protein, followed by uninduced cells, which again indicates background expression of AKT in the absence of 4-OHT. eGFP over-expressing FDM cells did not express HA. In the presence of IL-3 (time = 0) some endogenous pAKT was detectable in eGFP expressing cells. Prolonged exposure of films to detect endogenous AKT saturated the signals, arising from the over-expressed AKT.
Figure 4.4: Prolonged IL-3 deprivation does not affect induction of AKT over-expression

Two independent $Bax^{-/-};Bak^{-/-}$ clones were starved of IL-3 in the presence or absence of 4-OHT. At the indicated time-points cells were lysed and resolved by SDS-PAGE. These results demonstrate that 72 hours after IL-3 starvation, AKT over-expression is still inducible by 4-OHT.
The role of Akt during IL-3 survival signalling

Figure 4.5: PUMA and Mcl-1 expression is delayed by AKT over-expression

WT cells over-expressing either AKT1 (A) or AKT2 (B) or eGFP were cultured in the presence or absence of 4-OHT, then starved of IL-3. At indicated time-points cells were lysed and resolved by SDS-PAGE. (A) These data suggest that the over-expression of AKT1 delays PUMA expression when compared to cells induced to over-express eGFP. The expression of Mcl-1 also appears to be maintained when AKT over-expression is compared to eGFP induced over-expression. (B) These data suggest that the over-expression of AKT2 delays PUMA expression when compared to cells induced to over-express eGFP. The expression of Mcl-1 also appears to be maintained when AKT over-expression is compared to eGFP induced over-expression. Asterisk indicates non-specific band. With thanks to Dr Jarrod Sandow for independent confirmation (who also provided these figures which were included preferentially for clarity of data).
When cells were probed for PUMA expression, eGFP over-expressing cells were observed to rapidly express PUMA after approximately 4 hours, reaching maximal levels after 8 hours of IL-3 deprivation. By 24 hours, PUMA expression levels fell as more cells undergo apoptosis. In both induced ΔPH-AKT1 and uninduced ΔPH-AKT1 cells, changes in PUMA expression were delayed after IL-3 withdrawal. PUMA levels eventually reached similar levels as seen in eGFP expressing cells after 24 hours. Interestingly, PUMA appeared to also be repressed by ΔPH-AKT2 over-expression. These data suggest that the over-expression of both ΔPH-AKT isoforms was sufficient to repress PUMA expression after IL-3 withdrawal and may account for enhanced clonogenic survival.

As mentioned previously, Mcl-1 plays a major role in preventing apoptosis, particularly in haematopoietic cells [198, 200-202, 225, 226], including FDMs. Therefore, it is reasonable to assume that kinases promoting cell survival are obliged to maintain Mcl-1 expression. However, what was observed so far was a partial effect of AKT on cell survival after IL-3 withdrawal, which suggests either partial or transient maintenance of Mcl-1 by AKT. Therefore, immunoblots were also probed for Mcl-1 expression in the presence of or absence of IL-3 and ΔPH-AKT1 or 2. In eGFP over-expressing Mcl-1 protein levels decreased 24 hours after IL-3 deprivation. Uninduced cells expressing either the ΔPH-AKT1 or ΔPH-AKT2 construct also lost Mcl-1 expression by 24 hours, although the decay rate was somewhat slower. Importantly, cells induced to over-express either ΔPH-AKT isoform maintained Mcl-1 expression 24 hours after IL-3 deprivation. This data could be explained by at least two possible mechanisms:

1. AKT over-expression directly regulated Mcl-1 to promote survival after IL-3 withdrawal. This may be by blocking Mcl-1 degradation pathways [154, 227].
2. Akt over-expression promoted cell survival, and the expression of Mcl-1 is an indirect representation of an increased number of viable cells contributing to cell lysates.
4.5 PUMA, but not Mcl-1, is regulated by ΔPH-AKT1 over-expression in Bax−/−;Bak−/− cells after IL-3 starvation

To distinguish between these two possibilities, Bax−/−;Bak−/− cells were used to follow Mcl-1 protein expression after IL-3 deprivation as these cells do not undergo apoptosis, and thus cell loss to apoptosis cannot contribute to a fall in Mcl-1 expression. Two independent Bax−/−;Bak−/− clones were cultured in the presence or absence of 4-OHT for 24 hours before the cells were starved of IL-3 and cell lysates generated after the indicated amount of time. Lysates were then resolved using SDS-PAGE and probed with the indicated antibodies (figure 4.6).

In this experiment Mcl-1 protein was degraded 24 hours after IL-3 starvation, regardless of ΔPH-AKT1, ΔPH-AKT2 or eGFP over-expression. This is expected in eGFP expressing cells as cytokine deprivation has been shown to target Mcl-1 for proteosomal degradation [201, 202]. In this series of experiments, ΔPH-AKT over-expression was incapable of maintaining Mcl-1 levels after IL-3 withdrawal Bax−/−;Bak−/− cells. The distinction here is that, as Bax−/−;Bak−/− cells did not undergo apoptosis, the reduced Mcl-1 expression is not due to apoptosis causing universal protein degradation. Considering these results, it is likely that in figure 4.5 the reason Mcl-1 expression is correlated to ΔPH-AKT over-expression is more likely due to an increased percentage of the cells remaining viable, rather than direct regulation of Mcl-1 by ΔPH-AKT.

In these same cells, AKT was capable of delaying PUMA upregulation when compared to eGFP expressing cells, although the kinetics of the PUMA expression response to IL-3 removal is somewhat slower in Bax−/−;Bak−/− FDM cells than WT cells. AKT over-expression also resulted in phosphorylation of BAD, although over the course of the experiment BAD expression actually increased, rather than decreasing as might be expected by the model proposed by Datta et al [148, 224].

Another BH3-only protein, NOXA, is also inhibited by Mcl-1. NOXA has a similar set of binding partners as PUMA, and therefore NOXA expression was determined after IL-3 withdrawal. NOXA expression increased subtly in response to four hours of IL-3 deprivation whether eGFP or AKT1 is over-expressed. After prolonged IL-3 deprivation NOXA expression is reduced.
The expression of other anti-apoptotic Bcl-2 family members was also analysed in the \textit{Bax}^{/-}\textit{Bak}^{/-} cells. Bcl-2 expression was stable across all conditions tested; suggesting \(\Delta\text{PH-AKT1}\) over-expression is not regulating Bcl-2. Bcl-\textit{X}_{L} expression decreased after 24 hours IL-3 starvation; however, this does not correlate with \(\Delta\text{PH-AKT1}\) activity.

Maurer et al proposed a model whereby GSK-3 repressed Mcl-1 expression. In this model, GSK is a substrate of AKT and negatively regulated by AKT phosphorylation. Thus, in the presence of IL-3 and AKT activation, GSK is phosphorylated, inhibited, and therefore Mcl-1 levels maintained. In the absence of AKT activity, unphosphorylated, active GSK can accumulate and phosphorylate Mcl-1 to promote Mcl-1 degradation. This model would predict that induced over-expression of active AKT would phosphorylate and inactivate GSK-3, allowing Mcl-1 to maintain cellular viability. Rather, it was observed that induced \(\Delta\text{PH-AKT1}\) expression did result in a substantial increase of phosphorylated but not total GSK. However, this was not associated with maintenance of Mcl-1 levels, suggesting that inhibition of GSK by AKT-dependent phosphorylation was not sufficient to inhibit Mcl-1 degradation. Further, in GFP expressing cells, phosphorylated GSK tended to increase over the course of IL-3 deprivation rather than decline. Thus, these results are not consistent with this model. Further work will be required to establish the definitive role of GSK in Mcl-1 regulation in various cell types. However, this is not pursued further in this thesis. The principal finding is that Mcl-1 levels are not maintained by \(\Delta\text{PH-AKT1}\) over-expression after IL-3 withdrawal, even in cells that cannot undergo apoptosis.
Figure 4.6: PUMA expression is delayed by AKT1 over-expression

Two independent Bax<sup>-/-</sup>;Bak<sup>-/-</sup> clones, (A) & (B), over-expressing either AKT1 or eGFP were cultured in the presence or absence of 4-OHT, then starved of IL-3. At indicated time points cells were lysed and resolved by SDS-PAGE. These results suggest that the Bcl-2 family proteins; NOXA, BAD, Bcl-2, Mcl-1 and Bcl-X are not regulated by induced AKT over-expression (indicated by increased pAKT) Whilst BAD appears to be phosphorylated by AKT, there is no suggestion that this regulates the overall levels of BAD. Strikingly, PUMA expression is delayed by AKT1 over-expression, although is ultimately expressed after prolonged IL-3 deprivation. PUMA regulation was then re-determined in an independent clone suggesting AKT1 over-expression represses PUMA expression. Asterisk indicates non-specific band.
4.6 Transcription of PUMA, but not Mcl-1, is regulated by ΔPH-AKT1 over-expression after IL-3 withdrawal

To determine whether the regulation of PUMA and Mcl-1 occurred at a transcriptional level, WT cells expressing the ΔPH-AKT1, ΔPH-AKT2 or eGFP constructs were cultured in the presence or absence of 4-OHT for 24 hours before IL-3 was withdrawn. After the indicated times, RNA was isolated from cells and PUMA or Mcl-1 expression was determined by qRT-PCR (figure 4.7).

Cells over-expressing eGFP expressed up to four fold more PUMA RNA after eight hours in the absence of IL-3. This increase was not affected by culturing cells in the presence of 4-OHT. Cells not induced to over-express ΔPH-AKT1 or ΔPH-AKT2 have less PUMA transcription compared to eGFP expressing cells, once again likely due to leakiness of the inducible lentiviral system. When cells were induced to over-express ΔPH-AKT1 or ΔPH-AKT2 a significant delay in PUMA expression was observed when compared to uninduced cells. These results indicate that ΔPH-AKT over-expression was sufficient to repress PUMA transcription after IL-3 withdrawal.

When Mcl-1 expression was determined in eGFP over-expressing cells, no significant change in RNA was detected in the presence or absence of 4-OHT, nor after prolonged IL-3 deprivation, consistent with the idea that Mcl-1 protein degradation is the principal mechanism for diminished expression levels after IL-3 withdrawal. ΔPH-AKT over-expressing cells also had no significant change in Mcl-1 transcription.

Taken together, this data suggests that ΔPH-AKT1 or ΔPH-AKT2 over-expression is sufficient to repress PUMA transcription after IL-3 deprivation, but has no significant effect on Mcl-1 mRNA levels. Interpreting further, this suggests that ΔPH-AKT indirectly regulates PUMA by way of repressing PUMA transcription factors.

4.7 Conclusions

It was reasoned that the abrogation of the effect of ΔPH-AKT over-expression in a cell line deleted for a specific BH-3 only protein would indicate a potentially critical substrate of ΔPH-AKT. Using this logic, PUMA was determined to be a substrate of ΔPH-AKT whilst simultaneously dismissing BAD, and BIM from the list of candidates. Interestingly, this PUMA specific role seems to be more-so required for ΔPH-AKT1 dependent short-term survival signalling than ΔPH-AKT2.
Figure 4.7: PUMA transcription is repressed by AKT over-expression

Multiple WT cell lines expressing either the eGFP, ΔPH-AKT1 or ΔPH-AKT2 constructs were cultured in the presence or absence of 4-OHT for 24 hours before the cells were starved of IL-3. At the indicated time-points RNA from cells was isolated and quantified using the Taqman® qRT-PCR method. These results indicate that the over-expression of either AKT1 or AKT2 is sufficient to repress PUMA, but not regulate Mcl-1 after IL-3 withdrawal. Data represents average of two independent experiments +/- S.E.M. With thanks to Drs Anissa Jabbour and Dimitra Masouras for performing analysis of isolated RNA.
Despite some evidence of regulation of Mcl-1 by ΔPH-AKT, this appears to be more likely an artefact caused by increased cell number, as Mcl-1 mRNA or protein levels are not regulated by ΔPH-AKT when investigated independently of apoptosis.

One notable observation was the apparent decrease of PUMA transcript caused by ΔPH-AKT over-expression. Numerous models have suggested that AKT directly regulates Bcl-2 family members by phosphorylation of key residues [148, 202, 213, 228, 229]. The data collected instead suggests an indirect mechanism of regulating PUMA by ΔPH-AKT. This model is not without precedence however [217, 219, 230-233]. Chapter 5 will focus on delineating the signalling pathway from AKT to PUMA, focusing on transcription factors possibly involved in this process.
5. AKT regulates p53, controlling PUMA transcription, to maintain viability after IL-3 withdrawal

Chapters 3 and 4 demonstrated how AKT could regulate the sensitivity of cells to IL-3 survival signalling through PUMA repression. Two competing (or perhaps co-operating) models exist describing how PUMA is transcribed when an apoptotic stimulus is detected.

When first cloned, PUMA was described as a p53 Upregulated Modulator of apoptosis [218]. Thus, the question arises as to whether AKT is able to regulate p53-dependent transcription of Puma in response to cytokine withdrawal [217, 218]. Further, there is no indication from the literature that p53 is a direct substrate of AKT, nor any AKT recognition domains within p53 either. There is however, substantial literature indicating that the E3 ubiquitin ligases that regulate p53 abundance in the nucleus, MDM2 in particular, is an AKT substrate [234-236]. Thus, the first thing to establish is whether there is an association between AKT activity and p53 function.

Another proposed model of AKT dependent PUMA transcription involves the forkhead transcription factor FOXO3a. By this model, AKT phosphorylates FOXO3a S253, preventing translocation of the transcription factor into the nucleus [231, 237]. This then follows that in response to cytokine withdrawal, a loss of AKT activity would allow for PUMA transcription.

Using a similar strategy as used in chapter 4, it was reasoned that if either p53 or FOXO3a were required for ΔPH-AKT dependent survival, then the genes deletion would abolish the survival signal. Furthermore, if the critical substrate of ΔPH-AKT were to be identified as p53, then the precise mechanisms by which p53 is repressed would be explored; as it would be unlikely ΔPH-AKT directly phosphorylates/regulates p53.

5.1 p53 is required for ΔPH-AKT to maintain viability after IL-3 withdrawal

As previously explained, it was reasoned that the deletion of a required substrate of ΔPH-AKT to prolong survival after IL-3 withdrawal would abrogate the effect of ΔPH-AKT over-expression. As such, WT, p53−/− and FoxO3a−/− cells were infected with the eGFP, ΔPH-AKT1 or ΔPH-AKT2 inducible over-expression constructs.
In all clones used, it was first established that they uniformly expressed proteins detected by anti-HA antibody (indicating AKT expression) in an inducible manner (chapter 2.9). Each cell line was stimulated with 4-OHT for 24 hours before IL-3 was withdrawn and viability determined by PI exclusion after the indicated amount of time (figure 5.1).

As previously observed, the over-expression of ΔPH-AKT1 or ΔPH-AKT2 but not eGFP significantly prolonged survival, but did not ultimately prevent apoptosis from occurring. FoxO3a−/− cells over-expressing eGFP underwent apoptosis with similar kinetics to WT cells. FoxO3a−/− cells over-expressing ΔPH-AKT1 or ΔPH-AKT2 had subtly, yet significant, increased viability after IL-3 withdrawal. This suggests that AKT over-expression prolonged survival independently of FOXO3a.

As previously observed [230], p53−/− cells had increased survival compared to WT cells when over-expressing eGFP, indicating p53 is a partial requirement of normal apoptosis stimulated by IL-3 withdrawal. The over-expression of ΔPH-AKT1 in p53−/− cells does not increase cellular survival compared to uninduced cells. Surprisingly, ΔPH-AKT2 over-expression is also incapable of increasing viability of cells compared to uninduced or eGFP over-expressing cells. These results indicate that the survival advantage gained from ΔPH-AKT1 or ΔPH-AKT2 is p53 dependent.

**5.2 p53 is required for AKT to maintain clonogenicity after IL-3 withdrawal**

To determine whether either FOXO3a or p53 are required to increase clonogenicity due to AKT over-expression, cells from figure 5.2 were replated into media containing 0.5 ng/mL IL-3, 20% FCS and 0.3% soft agar. After 10-14 days, the resulting colonies were counted (figure 5.2).
Figure 5.1: Akt over-expression requires p53 to prolong survival after IL-3 withdrawal

Four independent cell lines derived from cells of the indicated genotypes were infected with the AKT1 (red lines, left side), AKT2 (red lines, right side) or eGFP (green lines). The cells were cultured in the presence or absence of 4-OHT for 24 hours before being starved of IL-3. At the indicated time-points cellular viability was determined by PI uptake using flow cytometry. These data indicate that AKT1 and AKT2 do not require FoxO3a, but require p53 to promote survival after IL-3 withdrawal. Data represents average of at least 3 independent experiments +/- S.E.M.
Figure 5.2: AKT over-expression requires p53 and FoxO3a to prolong clonogenic potential after IL-3 withdrawal

Four independent cell lines derived from cells of the indicated genotypes were infected with the AKT1 (red lines, left side), AKT2 (red lines, right side) or eGFP (green lines). The cells were cultured in the presence or absence of 4-OHT for 24 hours before being starved of IL-3. At the indicated time-points clonogenicity was determined by PI uptake using flow cytometry. These data indicate that AKT1 and AKT2 require FoxO3a and p53 to maintain clonogenic viability after IL-3 withdrawal. Data represents average of at least three independent experiments +/- S.E.M
WT cells over-expressing ΔPH-AKT1 or ΔPH-AKT2 produced more colonies when plated in soft agar in the presence of IL-3 after varying durations of IL-3 deprivation. eGFP over-expression did not provide any increased colony formation. FoxO3a<sup>−/−</sup> cells over-expressing either ΔPH-AKT1 or ΔPH-AKT2 did not have a significant increase in clonogenic potential after IL-3 withdrawal, although a subtle trend towards more colonies was observed. This suggested that although there is a delay in apoptosis when ΔPH-AKT is over-expressed in these cells, the additional surviving cells have largely lost the capacity to form colonies when IL-3 is restored. Some of the clonogenic potential may therefore be FOXO3a-dependent. Strikingly, the deletion of p53 results in no increased clonogenicity due to ΔPH-AKT over-expression. This suggests that p53 was critically required for ΔPH-AKT over-expression to increase clonogenic proliferation in cells surviving IL-3 deprivation.

### 5.3 AKT inhibition induced apoptosis requires p53, but not PUMA

As both p53 and PUMA were observed to be required for ΔPH-AKT over-expression to prolong survival after IL-3 withdrawal, it was hypothesised that both proteins would be required for apoptosis stimulated by AKT inhibition. To test this, at least three independent WT, Bad<sup>−/−</sup>, Puma<sup>−/−</sup>, FoxO3a<sup>−/−</sup>, p53<sup>−/−</sup> and Bax<sup>−/−</sup>;Bak<sup>−/−</sup> FDM clones were cultured in the presence or absence of IL-3 with the indicated concentration of AKTi VIII for 24 hours before cellular viability was determined (figure 5.3).

As previously observed, only ≈10% of WT cells cultured in the presence of IL-3 underwent apoptosis due to AKT inhibition. Between 30-50% of WT cells cultured in the absence of IL-3 underwent apoptosis due to AKT inhibition. As expected Bax<sup>−/−</sup>;Bak<sup>−/−</sup> cells succumb to neither IL-3 withdrawal or AKT inhibition induced apoptosis.

A significant proportion of Bad<sup>−/−</sup>, FoxO3a<sup>−/−</sup> and surprisingly, Puma<sup>−/−</sup> cells were apoptotic after IL-3 deprivation combined with AKT inhibition. This indicated that each of these proteins was redundant for AKT inhibition induced apoptosis. Strikingly, cells lacking p53 are protected from apoptosis induced by AKT inhibition, even in the absence of IL-3, suggesting p53 is critical for inducing apoptosis via AKT inhibition. This stands in contrast to earlier results that showed that MEK inhibition induced apoptosis was not blocked in the absence of p53 (figure 3.11).
It is interesting that the deletion of PUMA did not prevent apoptosis induced by the AKT inhibitor. This indicates that there are both PUMA dependent and independent mechanisms that are utilised by the inhibitor to promote apoptosis. One explanation is that these cells are undergoing apoptosis via off target effects. This may also be explained by the observation that ΔPH-AKT2 was still capable of promoting survival in Puma<sup>−/−</sup> cells (figure 4.1); perhaps the inhibition of AKT2 specifically by the AKT inhibitor is responsible for inducing apoptosis in the Puma<sup>−/−</sup> cells. Despite this, it is still apparent however that the AKT1 and AKT2 mechanisms for inducing apoptosis require p53.

Other important conclusions from these results were that IL-3 receptor signalling was sufficient to promote survival despite the apoptotic stimuli promoted by the AKT inhibitor, whilst AKT inhibitor dependent apoptosis is exacerbated by IL-3 deprivation. This reinforces previous results suggesting that AKT signalling becomes progressively more critical to maintain cell viability as IL-3 receptor signalling declines (chapter 3.4).

**5.4 AKT inhibition decreases relative clonogenicity of WT and p53<sup>−/−</sup> cells, albeit to different extents**

To determine whether the AKT inhibitor also decreased the clonogenic potential of p53<sup>−/−</sup> cells in a similar manner as in short term survival, the same WT and p53<sup>−/−</sup> cells from Figure 5.3 were replated into media containing 0.5 ng/mL IL-3, 20% FCS and 0.3% soft agar, having first been washed three times in PBS to remove the AKT inhibitor. After 10-14 days, the number of resultant colonies were counted and expressed relative to cells cultured in the presence of IL-3 and absence of the inhibitor (figure 5.4).
Figure 5.3: *p53*/* cells are resistant to apoptosis stimulated by AKT inhibition

At least three independent clones of the indicated genotypes were grown in the presence (blue) or absence (red) of IL-3. To these cultures, the AKT inhibitor was added at the indicated concentrations. Cells were then stained for PI after 24 hours and analysed by flow cytometry. These data show that PUMA, BAD and FOXO3a cells undergo apoptosis in a similar manner to WT cells, suggesting that the mechanism by which the AKT inhibitor works is independent of these genes. There is a subtle but insignificant decrease in viability due to AKT inhibition in *p53* cells, which advocates a *p53* dependent mechanism of cell death. As expected, *Bax*/*Bak* cells are resistant to both IL-3 withdrawal and AKT inhibition. Data represents average of at least three independent experiments +/- S.E.M.
Figure 5.4: *p53*−/− cells have reduced clonogenicity caused by AKT inhibition

Four cell lines from figure 5.3 were replated into media containing soft agar for 10-14 days, then the resulting colonies counted. Results are expressed relative to cells cultured in the absence of inhibitor, but presence of IL-3. These results suggest that *p53*−/− cells are sensitive to AKT inhibition when measured by colony formation potential, although less so than WT cells. Data represents average of at least three independent experiments +/- S.E.M.
Notably, clonogenic potential decreased by 50% in response to AKT inhibition, even in the presence of IL-3. This suggests that even though IL-3 receptor signalling could maintain cell viability in response to treatment with the inhibitor, there was still a significant effect of AKT inhibition since half the surviving cells lost the capacity to form colonies. The inhibition of AKT therefore must influence other processes other than just cell death pathways independently of IL-3 receptor signalling to reduce clonogenicity. These processes might include pathways relating to proliferation (chapter 1.2.3.2), protein synthesis (chapter 1.2.3.3) or metabolism (chapter 1.2.3.4). When cells are cultured in the absence of IL-3, the AKT inhibitor promoted a sharp reduction in clonogenic potential of cells, presumably as a result of the increased rate of apoptosis.

Interestingly, p53−/− cells also underwent a loss in clonogenic potential in either the presence or absence of IL-3 after AKT inhibition, although this clonogenic loss was significantly less than that experienced by WT cells. This is consistent with AKT inhibition reducing clonal proliferation independently of cell survival. Moreover, the decline in clonal proliferation in p53−/− cells was not significantly different in the absence of IL-3, suggesting that the impact of the AKT inhibitor was primarily on those pathways that regulate clonal proliferation and not cell death.

5.5 AKT inhibition, but not MEK inhibition regulates PUMA via a p53 dependent mechanism

As hinted at in chapters 3.10 and 3.11, some evidence exists for a ‘cross-talk’ to exist between the PI3K/AKT pathway and the MEK/ERK pathway. Therefore, both the AKT and MEK inhibitors were then added to WT or p53−/− cells cultured in the presence of IL-3. After 24 hours, lysates were generated and resolved by SDS-PAGE (figure 5.5). The Western blots were probed with antibodies to detect AKT, phosphoAKT (Ser473), ERK, phosphoERK (Thr202, Tyr204), BIM and PUMA. In these Westerns, the total AKT and total ERK provide indications of equal loading of lanes.
Figure 5.5: Puma expression is regulated by Akt in a p53 dependent fashion. WT or p53⁻/⁻ cells were cultured in the presence of IL-3 and either AKTi VIII or UO126 for the indicated periods. Cells were then lysed and resolved by SDS-PAGE. This data suggests that the AKT pathway robustly increases the expression of PUMA when the p53 pathway is intact, whilst only subtly increasing the expression of BIM independently of p53. The data also illustrates that MEK inhibition only subtly increases the expression of BIM and PUMA, which depends on the presence of p53. Furthermore, this data indicates that the inhibition of the AKT pathway does not alter the phosphorylation state of ERK, nor does the inhibition of the MEK pathway alter the phosphorylation state of AKT providing no evidence of cross-talk between the AKT and MEK/ERK.
BIM expression did not increase significantly above background when either AKT or MEK was inhibited, whilst PUMA expression was significantly increased in response to AKT inhibition. This further supports a PUMA dependent, BIM independent model of AKT survival signalling. Strikingly, the MEK inhibitor does not promote PUMA upregulation, suggesting that MEK dependent survival signalling is not PUMA dependent like AKT survival signalling is. Critically, the p53−/− cells no longer express PUMA in response to the AKT inhibitor, providing further evidence that AKT critically requires p53 to regulate PUMA expression.

As expected and as had been previously observed, the AKT inhibitor reduced AKT phosphorylation in WT and p53−/− cells without significantly changing total AKT levels. UO126 treatment similarly diminished pERK levels without influencing total ERK levels in WT and p53−/− cells. Notably, AKT inhibition did not decrease pERK, suggesting AKT does not regulate the MEK/ERK pathway. Furthermore, the MEK inhibitor did not reduce AKT phosphorylation, indicating MEK did not negatively regulate the PI3K/AKT pathway. In fact, these Western blots showed an increased expression of pERK in the presence of the AKT inhibitor. Thus, no evidence was observed that UO126 could increase or decrease AKT activation, and thus no direct evidence of MEK activity regulating AKT activation. In contrast, treatment of these cells with the AKT inhibitor resulted in increased pERK expression, in WT and p53−/− cells. There are two potential explanations for this observation.

1. The MEK/ERK pathway provides a compensatory pathway for survival if AKT signalling is lost
2. AKT signaling negatively regulates ERK activation.

Using the information at hand, it is difficult to determine which of these theories holds true. Whilst the concept of MEK/ERK / PI3K/AKT crosstalk is fairly novel, there is increasing evidence supporting this idea [90]. Meanwhile, if the MEK/ERK pathway can compensate for the loss of AKT signalling this may explain the partial redundancy of AKT during IL-3 withdrawal induced apoptosis.
5.6 AKT inhibition does not promote expression of the Puma-eGFP reporter construct

To verify whether AKT represses PUMA transcription, a reporter construct was utilised in which the p53 binding elements from the PUMA promoter drove expression of eGFP as a reporter. This construct had previously been used to observe PUMA transcription in response to IL-3 deprivation [217]. It was reasoned that AKT inhibition should increase eGFP expression as PUMA transcription is promoted by p53 activity.

In these experiments, low, yet significant increases in eGFP were observed in \textit{Bax}^{-/-};\textit{Bak}^{-/-} cells when starved of IL-3, as had previously been observed. As WT cells underwent apoptosis however, eGFP expression was muted by apoptosis initiated proteosomal degradation, meaning only low levels of eGFP could be detected under all conditions. Therefore, only \textit{Bax}^{-/-};\textit{Bak}^{-/-} cells were used to observe p53 activity using the reporter construct.

Two independent \textit{Bax}^{-/-};\textit{Bak}^{-/-} clones expressing the reporter construct were cultured in either the presence or absence of IL-3 at the indicated concentrations of the AKT inhibitor. After 24 hours, eGFP expression was determined using flow cytometry (figure 5.6).

Unexpectedly, AKT inhibition reduced eGFP activity as increasing concentrations of the inhibitor was added. However, this was most likely a technical limitation of the experimental setup, as this may have been the result of inhibiting other pathways regulated by AKT; specifically the upregulation of protein synthesis. It was likely that AKT inhibition prevented the formation of the ribosomal complex by inhibiting mTORC. Thus whilst eGFP transcript might be produced by p53, the machinery necessary to translate the mRNA fragments is not present.

One possible way to circumvent this effect was to infect the HoxB8 expressing FDM cells with the reporter construct, as well as the dual inducible over-expression construct. Whilst this was attempted, the number of viral molecules required to be expressed by a single cell proved a technical challenge and further attempts to utilise the PUMA-reporter construct were aborted.

Ultimately, qRT-PCR data from figure 4.6 provides the best evidence that p53-dependent transcription is blunted by ΔPH-AKT over-expression.
Figure 5.6: AKTї VIII does not promote p53 reporter GFP expression

Two independent Bax<sup>-/-</sup>;Bak<sup>-/-</sup> clones expressing the p53 reporter construct were cultured in the presence or absence of IL-3 at the indicated AKTї VIII concentrations. After 24 hours, GFP expression was determined by flow cytometry. These results suggest that AKT inhibition is not capable of promoting p53 reporter expression. Data represents a single experiment +/- S.E.M.
5.7 Deletion of PUMA or p53 provides a similar survival advantage in low IL-3 concentrations as AKT over-expression.

As ΔPH-AKT over-expression had been shown to increase viability to a similar degree as PUMA or p53 deletion after IL-3 withdrawal [149, 217], it was hypothesised that the viability and clonal proliferation of WT, Puma−/− and p53−/− cells in limiting IL-3 concentrations would be similar to ΔPH-AKT over-expression. Multiple independent Puma−/−, p53−/− and WT clones were washed and replated into the indicated concentrations of IL-3. After 72 hours, cellular viability was determined.

WT cells had an IC50 of ≈70 pg/mL IL-3 (figure 5.7) (see chapter 3.4 for how this figure is calculated). Both Puma−/− and p53−/− cells had an IC50 of ≈25 pg/mL IL-3, indicating that approximately three times more IL-3 was required to maintain WT cells viability as PUMA or p53 deficient cells. These results demonstrate that there is an increase in viability at both low IL-3 concentrations, as well as in the complete absence of IL-3. Notably, this level of survival advantage is of the same magnitude seen in WT FDM cells over-expressing ΔPH-AKT.

5.8 Deletion of PUMA or p53 prolongs clonogenic viability in low IL-3 concentrations compared to WT cells

Clonogenic survival was also measured in these same assays as previously described in chapter 4.2 (figure 5.8).

WT cells underwent ≈50% clonogenic loss when cultured at 0.1 ng/mL IL-3, >75% clonogenic loss when cultured at 0.02 ng/mL IL-3 and >80% clonogenic loss when cultured in the absence of IL-3 for 72 hours. Cells lacking Puma or p53 did not undergo significant loss in clonogenic potential when cultured in 0.1 ng/mL IL-3, however only p53−/− cells are protected from apoptosis at 0.02 ng/mL IL-3. Despite this, Puma−/− cells are significantly more clonogenic than WT cells at the same IL-3 concentration. In the absence of IL-3, both Puma−/− and p53−/− cells undergo a small but significant decrease in clonogenic potential; although still remain significantly more protected from clonogenic loss than WT cells. These results reveal that the deletion of PUMA or p53 increased clonogenicity after IL-3 starvation; however, deletion of PUMA or p53 was not sufficient to completely maintain clonogenic survival.
The role of Akt during IL-3 survival signalling

Figure 5.7: The deletion of either p53 or PUMA increases sensitivity to low IL-3 concentrations

Two independent WT, p53⁻/⁻ and Puma⁻/⁻ cell lines were cultured in a titration of IL-3 for 72 hours, after which cells were stained with PI and analysed by flow cytometry. These data indicate cells lacking either p53 or PUMA have increased sensitivity to low IL-3 concentrations for survival signalling when compared to WT cells, similar to as seen in AKT over-expressing cells. Data represents average of two independent experiments +/- S.E.M. The IC₅₀s are; WT ≈35pg/mL, Puma⁻/⁻ ≈25pg/mL, p53⁻/⁻ ≈20pg/mL
The deletion of either p53 or PUMA increases relative clonogenicity of cells at multiple IL-3 concentrations

Two independent WT, p53<sup>−/−</sup> and Puma<sup>−/−</sup> cell lines were cultured in a titration of IL-3 for 72 hours, after which cells were replated into media containing soft agar. After 10-14 days, resultant colonies were counted and expressed relative to colonies grown in the presence of IL-3. This data indicates cells lacking either p53 or PUMA have increased clonogenicity when cultured in high, low and the absence of IL-3 compared to WT cells. Data represents average of two independent experiments +/- S.E.M.
5.9 Conclusions

Using similar reasoning as was used to identify PUMA as a substrate of AKT, p53 was identified as a critical requirement for ΔPH-AKT over-expression to prolong survival after IL-3 withdrawal. Further, p53, but not PUMA, was observed to be required for AKT inhibition mediated apoptosis. This is not to say that $p53^{-/-}$ cells are completely refractory to the AKT inhibitor, as clonogenic potential decreased in response to increasing concentrations of the drug.

Whilst AKT inhibition induced apoptosis has been shown to require p53, chapter 3.11 indicated apoptosis induced by MEK inhibition was p53 independent. Indeed, there was little evidence of change in AKT pathway activity when MEK was inhibited, nor a change in MEK pathway activity when AKT was inhibited, thus, once again no evidence of ‘cross-talk’ was observable using the FDM model. What was observed however was a significant increase in PUMA expression, but not BIM expression caused by the AKT inhibitor, an effect that required p53.

It was also evident that the deletion of either PUMA or p53 has a similar protective effect on of cells titrated in IL-3 as constitutively activated AKT over-expression has. Thus, there is a correlation between PUMA or p53 deletion and ΔPH-AKT over-expression.

It should be noted however that there is little evidence to support direct phosphorylation of p53 by AKT, and it is more likely that this interaction is facilitated by another medium, which will be the focus of Chapter 6.
6. AKT phosphorylates MDM2 in order to prevent p53-dependent transcription

Chapter 5 established p53 as the most likely substrate of AKT to promote PUMA transcription in response to IL-3 withdrawal. Whilst an elegant model, p53 does not possess an AKT recognition domain capable of being directly targeted for AKT dependent phosphorylation. Thus, an intermediate must be utilised by AKT to regulate p53 activity. For this model however, a candidate substrate was identified within the literature, which highlights the protein MDM2 as being both a highly specific repressor of p53, as well as a direct substrate of AKT [235, 238, 239]. These studies not only found evidence of AKT dependent phosphorylation of MDM2 at the S166 and S188 sites, but, through use of in vitro kinase assays, proved a direct interaction between these two proteins.

MDM2 is a critical regulator of p53 abundance by regulating its capacity to translocate into the nucleus [232, 234]. In its unphosphorylated state, MDM2 is rapidly ubiquitinated, then targeted for proteosomal degradation. When phosphorylated at the S186 residue, by AKT for example, MDM2 is no longer degraded and therefore allowed to bind directly to p53 [236]. The p53-MDM2 complex is thus no longer capable of entering the nucleus. As a result, p53 dependent transcription is halted and the MDM2-p53 complex rather targeted for proteosomal degradation.

It was therefore hypothesised that AKT exerted p53-dependent effects on cell survival through MDM2 phosphorylation in response to IL-3 signalling.

6.1 IL-3 restimulation results in AKT activation and MDM2 phosphorylation

To determine whether MDM2 was regulated by IL-3 in FDMs, WT cells were starved of IL-3 for 4 hours, before being briefly restimulated with IL-3 for 20 minutes. Protein lysates were then generated and resolved using SDS-PAGE (figure 6.1). Upon IL-3 restimulation, phosphorylation of AKT was promoted on both the S437 and T308 residues, resulting in AKT activation. AKT activity was also measured by GSK-3 phosphorylation. Strikingly, IL-3 restimulation led to a robust increase in phosphorylation of MDM2, indicating that MDM2 phosphorylation correlates with AKT activation in response to IL-3 stimulation.
Figure 6.1: IL-3 restimulation promotes AKT, GSK3 and MDM2 phosphorylation

WT cells were starved of IL-3 for 4 hours (-), before IL-3 was re-added for 20 minutes (+). Cells were then lysed and resolved by SDS-PAGE. Figure indicates that the re-addition of IL-3 results in the simultaneous phosphorylation of AKT, GSK and MDM2 suggesting IL-3 is sufficient to phosphorylate these proteins.
6.2 ΔPH-AKT1 over-expression is sufficient to phosphorylate and stabilise MDM2

To determine whether ΔPH-AKT1 is sufficient to promote MDM2 phosphorylation, *Bax−/−;Bak−/−* cells expressing the ΔPH-AKT1 over-expression construct were starved of IL-3 for 24 hours, before being restimulated with either IL-3, or 4-OHT alone. At the indicated time points, lysates were generated and resolved using SDS-PAGE (figure 6.2).

The loss of IL-3 receptor signalling prevented AKT, GSK-3 and MDM2 phosphorylation, leading to reduced overall levels of MDM2. Four hours after IL-3 restimulation, AKT, GSK3 and MDM2 were phosphorylated, leading to an increase in total MDM2 levels, as the protein was no longer targeted for proteosomal degradation. Induction of ΔPH-AKT1 over-expression alone was sufficient to increase AKT and GSK3 phosphorylation, indicating AKT activity. Critically, ΔPH-AKT1 over-expression was also sufficient to phosphorylate MDM2 independently of IL-3, which in turn led to an accumulation of total MDM2.

These results indicate that ΔPH-AKT1 activity is sufficient to phosphorylate and stabilise MDM2, leading to the accumulation of MDM2 levels in the cellular environment.

6.3 Inhibition of MDM2-p53 complexing induces apoptosis in WT, but not *p53−/−* cells

Although previous data suggests MDM2 is a substrate of both AKT and IL-3 on a molecular level, evidence was sought to confirm that MDM2-p53 binding is critical for AKT dependent survival. Consequently, Nutlin-3 was utilised, a compound which sterically hinders the binding of MDM2 to p53 [240-242]. It was first established whether Nutlin-3 promoted cell death via a p53 dependent mechanism by treating WT and *p53−/−* cells with 0.5, 0.02 or 0 ng/mL IL-3 and the indicated concentrations of Nutlin-3 Cells were analysed for viability after 24 hours (figure 6.3).

WT cells underwent a significant decrease in viability caused by Nutlin-3 inhibition in all concentrations of IL-3 (figure 6.3a). This indicates that the disruption of MDM2-p53 binding is sufficient stimulus to initiate apoptosis despite upstream IL-3 signalling being maintained.
**Figure 6.2: AKT1 over-expression is sufficient to phosphorylate and stabilise MDM2**

*Bax<sup>−/−</sup>;Bak<sup>−/−</sup> cells expressing the inducible ΔPH-AKT1 construct were starved of IL-3 for 12 hours before either IL-3 or 4-OHT was re-added. At the indicated time points the cells were lysed and resolved by SDS-PAGE. Figure indicates that the induced over-expression of ΔPH-AKT1 independently of IL-3 is sufficient to phosphorylate MDM2. This correlates with increased expression of MDM2, proposed to be due its protection from proteasomal degradation.
The role of Akt during IL-3 survival signalling

Figure 6.3: Inhibition of MDM2 induces apoptosis in the presence of IL-3 in WT but not p53−/− cells

Three independent WT (A) and p53−/− (B) cell lines were cultured in the presence of IL-3 and the indicated concentrations of the MDM2 inhibitor Nutlin-3. After 24 hours cells were evaluated for viability by PI exclusion using flow cytometry. This data shows that Nutlin-3 is sufficient to induce apoptosis in the presence or absence of IL-3 in WT but not in p53−/− cells. Data represents average of one experiments +/- S.E.M.
As cells lacking p53 are resistant to Nutlin-3 induced apoptosis (figure 6.3b) this is likely via p53 dependent mechanisms, indicating that Nutlin-3 stimulated apoptosis is not caused by off target, non-p53 dependent, mechanisms.

**6.4 AKT over-expression is insufficient to promote viability after IL-3 withdrawal in the presence of Nutlin-3**

One would predict that if ΔPH-AKT over-expression signaled survival via p53 dependent mechanisms (figure 5.1), then the chemical inhibition of MDM2 binding to p53 would similarly abrogate prolonged ΔPH-AKT dependent survival. Thus, multiple independent WT cell lines expressing the ΔPH-AKT1, ΔPH-AKT2 or eGFP constructs were cultured in the presence or absence of 4-OHT for 24 hours before the cells were replated in the presence or absence of IL-3. To these cultures, the indicated concentrations of Nutlin-3 were added, and then viability tested after 24 hours (figure 6.4).

eGFP over-expressing cells underwent apoptosis when Nutlin-3 was added to cells in the presence or absence of IL-3 (figure 6.4c). When ΔPH-AKT1 (figure 6.4a) or ΔPH-AKT2 (figure 6.4b) expression was induced in the presence of IL-3, increasing concentrations of Nutlin-3 induced a subtle yet significant amount of apoptosis. The small survival advantage provided by ΔPH-AKT1 or ΔPH-AKT2 over-expression in the absence of IL-3 was lost when cells were treated with Nutlin, indicating Nutlin blocks the survival signal promoted by ΔPH-AKT over-expression. This data further supports a model where-by ΔPH-AKT over-expression requires the complexing of MDM2 and p53 to maintain survival after IL-3 withdrawal.

**6.4.1 AKT1 over-expression is insufficient to promote viability after prolonged IL-3 withdrawal in the presence of Nutlin-3**

An alternate experimental set-up was also performed in which WT cells were starved of IL-3 for 48 hours before ΔPH-AKT1 over-expression was induced simultaneously to the addition of Nutlin-3. Twenty-four hours after the addition of 4-OHT / Nutlin-3, viability was determined (figure 6.5).
Figure 6.4: ΔPH-AKT1 over-expression dependent survival is blunted by Nutlin-3

Four independent WT clones expressing the ΔPH-AKT1 (A), ΔPH-AKT2 (B) or eGFP (C) over-expression constructs were stimulated with 4-OHT for 24 hours before IL-3 was withdrawn and Nutlin-3 was added at the indicated concentrations. After 24 hours, cell viability was measured by PI using flow cytometry. This data shows that cells cultured in the presence of IL-3 are subtly sensitive to Nutlin-3. When IL-3 is withdrawn, AKT prolongs cell viability in the absence of Nutlin-3. When Nutlin-3 is added to these cells, then there is no significant increase (N.S) in viability between induced and uninduced ΔPH-AKT1 over-expressing cells. Data represents average of three independent experiments +/- S.E.M.
Figure 6.5: The protective effect of ΔPH-AKT1 over-expression in the absence of IL-3 is blunted by MDM2 inhibition

Three independent WT clones expressing the ΔPH-AKT1 (A) or eGFP (B) over-expression constructs were stimulated with 4-OHT for 24 hours before IL-3 was withdrawn and Nutlin-3 was added at the indicated concentrations. After 48 hours, cell viability was measured by PI using flow cytometry. This data shows that when IL-3 is withdrawn, AKT prolongs cell viability in the absence of Nutlin-3. When Nutlin-3 is added to these cells, then there is no significant increase in viability between induced and uninduced AKT1 over-expressing cells. Data represents one experiment +/- S.E.M.
As expected, ΔPH-AKT1 over-expression in the absence of Nutlin-3 prolonged maintained increased survival 72 hours post-IL-3 withdrawal. Any survival advantage promoted by ΔPH-AKT1 was lost however when Nutlin was added to the cells at concentrations as low as 0.4 µM. Under such circumstances, cells cultured in the presence or absence of ΔPH-AKT1 over-expression had similar viabilities.

It may be that by 72 hours after IL-3 deprivation, particularly in the presence of Nutlin, that cells are committed to apoptosis and that ΔPH-AKT1 over-expression simply cannot reverse cells passed a certain point of no return. It is possible however, that this data is further indication that Nutlin-3 inhibits the pathway by which ΔPH-AKT1 over-expression promotes survival after IL-3 withdrawal.

6.5 AKT1 is not required for Nutlin-3 induced apoptosis

As MDM2 and p53 are proposed to lie downstream of AKT dependent survival signals, one might expect that the deletion of AKT1 would not affect Nutlin-3 induced apoptosis. To test this, WT and Akt1−/− cells were cultured in the presence or absence of IL-3 at the indicated concentrations of inhibitor. After 24 hours, the cells were tested for viability (figure 6.6).

WT and Akt1−/− cells are both susceptible to Nutlin-3 in the presence of IL-3, WT cells being ≈50% viable, whilst Akt1−/− even less so (≈50% viability). In the absence of IL-3 both WT and Akt1−/− cells succumb to apoptosis to ≈25% viability. This indicates that Nutlin-3 induced apoptosis is not dependent on AKT1.
Figure 6.6: MDM2 inhibition induced apoptosis does not require AKT1
Two independent clones of WT and AKT1/− cells were grown in either the presence or absence of IL-3 with the indicated concentrations of Nutlin-3. After 24 hours, cells were stained with PI and analysed by flow cytometry. Results suggest that cells lacking AKT1 are not protected from Nutlin-3 induced apoptosis compared to WT cells. Data represents average of one experiment +/- S.E.M.
6.6 Conclusions

MDM2 was the most likely facilitator of AKT-p53 interaction, as it has long been described as both a direct substrate of AK [232, 234], as well as a potent inhibitor of p53 [243-245].

MDM2 responds to numerous different cytokines, but it was imperative to ensure MDM2 was phosphorylated in response to IL-3 in the FDM system. Robust phosphorylation of MDM2 was observed to correspond with IL-3 restimulation, as well as AKT activation. Further, ΔPH-AKT over-expression was sufficient to promote MDM2 phosphorylation independently of IL-3 signalling.

When Nutlin-3 was used to disrupt the formation of MDM2-p53 complexing, ΔPH-AKT over-expression could no longer promote survival after IL-3 was withdrawn. This would suggest that the ability for MDM2 to bind p53, and therefore prevent p53 translocation into the nucleus is critical to Akt dependent survival signalling.

Thus, a model of AKT survival signalling emerges, where AKT phosphorylates MDM2, which inhibits p53 translocation, preventing the transcription of PUMA to promote apoptosis.
7. Discussion

7.1 Limiting IL-3 concentrations do not reliably separate survival and proliferative signals.

IL-3 receptor signalling absolutely requires the β-common (βc) chain component of the IL-3 receptor. Receptor activation is characterised by phosphorylation of one or more of the eight tyrosine residues on the cytoplasmic domain of the βc-chain, not to mention the possibility of non-tyrosine phosphorylation sites such as the Serine 585 residue.

An additional feature of receptor activation was identified by Gutheridge and Lopez [79, 84, 222]. They studied the human GM-CSF receptor, which also requires βc for signaling, and showed that at limiting GM-CSF concentrations, GM-CSF could maintain cell viability but was not sufficient to drive proliferation. This was a means of separating the survival and proliferative signals. They identified serine phosphorylation events that were uniquely associated with limiting GM-CSF receptor activation, which suggested serine phosphorylation was specifically associated with survival signaling. As the βc-chain is shared by both the GM-CSF and IL-3 receptors, it was hypothesised that IL-3 receptor signaling would show a similar separation of survival and proliferation signals at limiting IL-3 concentrations.

What was observed was significant heterogeneity in WT FDM cells in response to titrating progressively lower IL-3 concentrations. In some cell lines a slight separation could be distinguished in the IL-3 concentrations required to maintain cell viability compared to proliferation, whilst in others, these signaling outcomes could not be easily distinguished (chapter 3.1). This does not necessarily contradict the conclusion that separate βc phosphorylation events also occur in IL-3 signaling, but only indicates that survival and proliferation are shutdown at similar IL-3 concentrations in FDM cells. It was not possible to directly test whether serine phosphorylation characterised IL-3 dependent cell survival, as the concentrations of IL-3 did not sufficiently separate these survival signals from the proliferative signals, and also because the phospo-specific antibody described by Gutheridge et al is human specific and did not detect the murine receptor.
One explanation as to why these cells behave differently to the cells described by Guthridge and Lopez is that FDM cells, being murine cells, possess two βc-chains. Murine cells possess the βC-chain, but also have a closely related, IL-3 specific β-chain (β_{IL-3}). Mechanistically, it is not clear how β_{IL-3} might alter the response to limiting IL-3 concentrations, since deletion of the β_{IL-3} gene has no influence on IL-3 receptor signaling [246].

### 7.2 AKT1 is required for cell survival in limiting IL-3 signalling concentrations

AKT has long been proposed as a critical regulator of cytokine dependent survival [108, 163, 247]. Therefore, the IL-3 dependent FDM cell lines were utilised as a model to determine which roles AKT is required for during IL-3 signalling. Using this model, it was found that the deletion of each AKT isoform does not prevent the generation of IL-3 dependent cell lines. Thus, each AKT isoform does not have essential roles in proliferation, metabolism, protein synthesis maintaining cytokine dependent survival, or indeed any process required for \textit{in vitro} culture. This would suggest that each AKT isoform is redundant for IL-3 dependent signalling (chapter 3.2). This may be due to each AKT isoform being able to compensate for the deleted isoforms, a possible scenario as \textit{Akt1}\textsuperscript{-/-},\textit{Akt2}\textsuperscript{-/-},\textit{Akt3}\textsuperscript{+/+} mice are viable, whilst \textit{Akt1}\textsuperscript{-/-},\textit{Akt2}\textsuperscript{-/-},\textit{Akt3}\textsuperscript{-/-} are not [134, 136]. It is also possible however, that parallel pathways, such as the MEK/ERK pathway, render AKT signalling redundant in response to IL-3. To determine which of these outcomes is true; FDMs generated from compound knockouts of AKT would need to be generated from mice, which were not available at the time of writing.

Although it remains to be seen whether the combination of AKT isoforms plays critical roles in cytokine signalling, it is evident that the over expression of hyper-activated ΔPH-AKT1 or ΔPH-AKT2 cannot compensate for the effects of IL-3 withdrawal. The over-expression of either ΔPH-AKT isoform could not stimulate proliferation independently of IL-3, nor could it prevent the loss of cell cycling or initiation of apoptosis upon IL-3 withdrawal (chapters 3.5, 3.10 & 3.11).

Using these models however, it was found that AKT, specifically AKT1, was critically required to regulate cell survival in limiting concentrations of IL-3. \textit{Akt1}\textsuperscript{-/-}...
cells, or AKT inhibition consistently reduced cellular viability at lower IL-3 concentrations (chapters 3.4, 3.5 & 3.8). This was further confirmed by experiments where over-expressing ΔPH-AKT1 (or ΔPH-AKT2) was capable of maintaining cell viability in the same low IL-3 concentrations compared to eGFP over-expression (chapter 3.5). Thus, it is clear that whilst no single AKT isoform is required for survival signalling in abundant IL-3, AKT1 specifically is critical for maintaining fine control of the survival signal at limiting IL-3 concentrations. It is likely that the enforced over-expression of ΔPH-AKT2 was sufficient to reduce substrate specificity. This may therefore allow ΔPH-AKT2 over-expression to also maintain survival in limiting cytokine concentrations despite AKT2 deletion not having as significant effect on cellular viability AKT1 deletion did. There have been numerous explanations proposed to describe the combination of observed effects;

1. The IL-3 receptor is capable of detecting different concentrations of IL-3, resulting in different molecular responses activating different survival signals. This is based on the Binary Switch Model proposed by Guthridge et al. Using this model, the collected evidence would suggest that AKT1 specifically is responsible for survival when the receptor is signalling ‘low cytokine’ by Serine 585 phosphorylation, whilst alternative pathways are responsible for maintaining survival in high IL-3 concentrations When Tyrosine 577 is phosphorylated (for further details on this model, read [84]).

2. The IL-3 receptor activates multiple redundant pathways in the presence of abundant IL-3. As the IL-3 concentration is decreased, these pathways are sequentially shut down in order of their activation sensitivity. Eventually, a concentration of cytokine would be reached at which AKT1 is the sole remaining survival pathway activated (figure 7.1).

3. It may remain true that AKT is required for survival signalling in response to IL-3, although this is not apparent in the Akt−/− cell lines. This may be due to cross isoform redundancy, which is capable of compensating for survival when a single AKT isoform is deleted. Under this theory of survival signalling, AKT1 may be the predominantly expressed isoform in FDM cells, and in the Akt1−/− cell lines, total levels of AKT2 and AKT3 combined are only sufficient to maintain viability when very high cytokine concentrations are provided.
Nevertheless, this role of AKT may be the mechanism by which AKT activation, either by direct mutation, or by a loss of upstream regulation (i.e. PI3K mutation or PTEN loss) could lead to oncogenesis. This data would suggest that AKT activation could permit cells to survive under conditions normally stimulating apoptosis. Whilst ΔPH-AKT over-expression could not enforce oncogenesis on its own (chapters 3.5, 3.10 & 3.11), the delay in undergoing apoptosis attributed to AKT activation could allow an accumulation of mutations to promote cytokine independence.

7.3 ΔPH-AKT over-expression requires PUMA to maintain survival in the absence of IL-3

The literature provides a number of examples of AKT regulating specific members of the Bcl-2 family, particularly the BH3-only proteins, to maintain survival in response to cytokine [133, 148, 210, 212, 248]. BH3-only protein members such as BAD, BIM and PUMA have all been described to be either directly or indirectly regulated by AKT [148, 149, 215-218]. It was shown above that ΔPH-AKT over-expression could promote survival of cells in limiting IL-3 concentrations. Thus, it was reasoned that if this survival signal regulated a specific BH3-only protein, then the deletion of that BH3-only protein would abrogate the effects of ΔPH-AKT over-expression. Using this approach, it was demonstrated that PUMA was a critical substrate for ΔPH-AKT1 over-expression to maintain survival after complete IL-3 withdrawal.

When either ΔPH-AKT1 or ΔPH-AKT2 was over-expressed in Bad−/− or Bim−/− FDM cells, increased survival after IL-3 withdrawal was observed, similar to that seen in WT cells. This indicates that neither gene is required for ΔPH-AKT over-expression to maintain viability. Thus, Akt can continue to function to promote viability in the absence of these pro-apoptotic molecules. This is an important finding, particularly with respect to BAD, as it contradicts highly cited literature that states that it is an AKT-BAD interaction that mediates IL-3-dependent survival [148, 224] The model described in the literature revolves around the AKT-dependent phosphorylation of BAD, which promotes dimerization of BAD and the chaperone protein 14-3-3. When bound to 14-3-3, BAD is incapable of initiating apoptosis. Upon loss of the cytokine-dependent survival signal, AKT is inactivated, BAD is no longer phosphorylated, 14-3-3 no longer binds to BAD, and thus apoptosis is initiated. This model was somewhat
questionable, as Bad<sup>−/−</sup> cells were not protected from IL-3 withdrawal as WT cells [149]. The data collected above provides further compelling evidence that this direct phosphorylation of BAD is not required to contribute to IL-3 dependent cell survival. This is not to say that BAD is not an Akt substrate. Indeed, figure 4.6 demonstrates ΔPH-AKT1 phosphorylating BAD independently of IL-3 signalling. However, at least with respect to the regulation of apoptosis, this phosphorylation has no obvious physiological significance.

Concerning BIM regulation by AKT, one critical caveat must be remembered in respect to the nature of FDM cells. HOXB8 expression, which is used to generate FDM cells, is associated with the repression of BIM expression. It was recently described that one mechanism by which this happens is through the expression of the microRNA~17-92 cluster [249]. Thus, it is possible that in cells not expressing HOXB8, AKT may contribute to the regulation of BIM expression. However, what is clear is that the deletion of BIM has no effect on the capacity of cells to undergo apoptosis when IL-3 concentrations are reduced, or the capacity of AKT to maintain viability when overexpressed in the same conditions.

In Puma<sup>−/−</sup> cells ΔPH-AKT2 over-expression, but not ΔPH-AKT1 over-expression, was sufficient to increase viability after IL-3 withdrawal. This demonstrates that ΔPH-AKT1, but not ΔPH-AKT2, in some way regulates PUMA to maintain survival in FDM cells after a loss of IL-3 signalling. This role of PUMA as a target of AKT, required for AKT to repress cell death, is not entirely unexpected. PUMA deletion has been shown to increase cell viability and clonogenic survival in IL-3 dependent cells after the removal of IL-3 from cell cultures [149]. What is novel however, is that PUMA is critical for AKT dependent survival signalling. Furthermore, ΔPH-AKT1 over-expression was sufficient to delay PUMA expression after IL-3 withdrawal (chapter 4.4 & 4.5). It is noteworthy that the enhanced survival that results from repression of PUMA, at most delays cell death, since ultimately it is the fate of most cells over-expressing ΔPH-AKT to undergo apoptosis in the absence of IL-3 signaling.

**7.3.1 AKT is not sufficient to maintain Mcl-1 expression**

It should be mentioned that BH-3-only independent mechanisms of AKT survival signalling have been described. One example is the capacity of AKT to regulate
Mcl-1 [154]. Maurer et al. proposed a model whereby AKT directly phosphorylates GSK3β at the S9 residue. When phosphorylated, GSK3β is inactive. Active GSK3β would otherwise phosphorylate the anti-apoptotic Bcl-2 family protein Mcl-1 at the S159 residue. When phosphorylated, Mcl-1 is targeted for proteosomal degradation, thereby initiating apoptosis. This is a particularly tempting model of cytokine dependent survival pathways, as Mcl-1 is of critical importance to normal haematopoietic cell survival, as well as a frequently expressed feature of haematopoietic malignancies [225, 250-253]. Tellingly, it is notable that Mcl1−/− IL-3 dependent cells have not been possible to generate thus far, such is the importance of Mcl-1 for haematopoietic cell survival. Therefore, it was not possible to test the requirement of Mcl-1 for AKT dependent survival in a similar fashion to the other candidate proteins tested. It was shown however, that ΔPH-AKT1 does not maintain Mcl-1 expression upon IL-3 loss (chapter 4.5). To control for the confounding effect of apoptosis on Mcl-1 levels, these experiments were performed in the Bax−/−/Bak−/− cells. The inability of ΔPH-AKT1 to regulate Mcl-1 may partially explain its redundancy for survival signalling; if Mcl-1 is critical to maintain FDM viability, then proteins regulating Mcl-1 must be potent repressors of apoptosis. This would suggest that AKT-independent mechanisms are important to provide Mcl-1 stabilisation. Taken together, this suggests that the mechanism AKT regulates intrinsic apoptosis pathways in FDM cells is primarily through repression of PUMA.

Although PUMA does have a pseudo AKT-recognition domain, little evidence suggests AKT directly regulates or phosphorylates PUMA [219]. Therefore, an intermediary probably facilitates this interaction.

7.4 AKT regulates p53-dependent PUMA transcription

As PUMA is not directly phosphorylatable by AKT [219], an intermediary must facilitate this interaction. As PUMA regulation by AKT was apparent at the transcriptional level (chapter 4.6), it was hypothesised that AKT indirectly regulates PUMA by repressing key transcription factors. p53 and FOXO3a were known to both be transcription factors of PUMA and regulatable by cytokine signalling [218, 230, 231]. PUMA was first cloned as a gene specifically upregulated by p53 in response to DNA damage, and is a key mediator of p53-dependent apoptosis. FOXO3a was shown
to regulate PUMA (and BIM) expression in thymocytes in response to IL-2 withdrawal [254, 255]. In this situation, FOXO3a is a direct substrate of AKT, and negatively regulated by AKT-dependent phosphorylation. Thus, a similar experimental reasoning was used as previous to determine if either of these transcription factors were required to mediate ΔPH-AKT over-expression dependent survival. If either p53 or FOXO3a were required for to regulate survival, then the deletion of p53 or FOXO3a would abrogate the enhanced survival mediated by ΔPH-AKT over-expression.

As was the case in PUMA-deficient cells, deletion of p53 blocked any additional survival caused by ΔPH-AKT1 over-expression. Unlike Puma\(^+\) cells, survival was not enhanced when ΔPH-AKT2 was over-expressed in p53\(^-\) cells deprived of IL-3. This suggests that ΔPH-AKT1 regulates survival by the p53/Puma signalling axis, whilst ΔPH-AKT2 regulates survival via p53 dependent, PUMA independent pathways.

Although the effect is more subtle than as seen in WT cells, over-expression of either ΔPH-AKT1 or ΔPH-AKT2 is capable of prolonging survival in FoxO3a\(^-\) cells after IL-3 withdrawal, indicating FOXO3a is not absolutely required for ΔPH-AKT over-expression mediated survival. The relatively small effect of ΔPH-AKT over-expression in FOXO3a deficient cells, together with the failure of AKT to promote clonogenic survival in FoxO3a\(^-\) cells, means it is not possible to absolutely exclude a role for FOXO3a in mediating ΔPH-AKT dependent survival. However, it had previously been shown that deletion of FOXO3a has no protective effect against IL-3 withdrawal, whereas deletion of p53 provides a substantial survival advantage [230], which strongly suggests that any role FOXO3a has in mediating IL-3 dependent survival is substantially smaller and less significant than that of p53. FOXO3a dependent regulation was described in lymphocytes and in response to IL-2 stimulation [256]. Thus, it is also plausible that the role of FOXO3a in cytokine-mediated cell survival and the regulation of BH3-only proteins are both cell type and cytokine specific.

To further probe whether p53 or PUMA are required for AKT mediated survival signalling, FDMs deleted for PUMA or p53, as well as BAD or FOXO3a, were treated with the AKT inhibitor, which inhibits both AKT1 and AKT2 (chapter 5.3). When the Bad\(^-\) and FoxO3a\(^-\) cells were treated with the inhibitor they were as susceptible to cell death as WT cells, indicating neither gene is required for AKT inhibitor induced apoptosis. Strikingly, Puma\(^-\) cells are also susceptible to apoptosis after AKT...
inhibition, whilst $p53^{-/-}$ cells did not. Additionally, $p53^{-/-}$ cells still lost clonogenic potential after AKT inhibition. This is a somewhat complicated set of results, from which one may draw the following conclusions.

1. The apoptosis induced by the AKT inhibitor may involve upregulation of PUMA expression, but does not require PUMA. Therefore, there are other p53 dependent, PUMA independent, pathways by which this apoptosis may proceed, which are simultaneously being inhibited alongside the AKT1/PUMA dependent survival pathways. This has been alluded to previously, as over-expression of ΔPH-AKT2 promoted PUMA independent, p53 dependent mechanisms of survival (chapter 4.1). These pathways could include metabolic regulation including a loss of glucose importation into cells through GLUT4 activation [147, 257, 258].

2. Cells lacking p53 are resistant to apoptosis induced by the AKT inhibitor, although this drug still retains some activity to block colony formation in clonogenicity assays. This ability to block colony assays is, in essence, a block on proliferation. Since the data indicates that ΔPH-AKT alone is not sufficient to drive proliferation, the loss of colony formation in $p53^{-/-}$ cells may be an off target effect of this inhibitor. This remains to be formally confirmed or refuted. The best method to do so, the generation of an Akt1$^{-/-}$/p53$^{-/-}$ mouse strain, proved to be a technically difficult cross to achieve.

3. AKT dependent survival pathways are no longer required in cells lacking p53, since inhibition of AKT has no pro-apoptotic effect in these cells. This was independently verified for the manuscript published from this thesis; an experiment where siRNA was used to repress p53 expression showed that this also reduced AKT inhibitor induced apoptosis compared to a scrambled siRNA control (see appendix 1, with regards Dr Anissa Jabbour).

When the physiological effects of the AKT and MEK inhibitors are compared from chapters 3.11 and 5.3, it is evident that AKT inhibition requires p53 to promote apoptosis, whilst MEK inhibition does not. In chapter 5.5, at a molecular level it is evident that the AKT inhibitor stimulates PUMA expression (but not BIM expression),
providing further evidence of the capacity of AKT to regulate PUMA. This regulation of PUMA was not evident during MEK inhibition. Further still, AKT can no longer promote PUMA expression in *p53*/* cells, once again indicating a *p53* dependent mechanism for PUMA regulation by AKT.

It should be mentioned at this point that the AKT inhibitor did not affect MEK/ERK signalling, nor did the MEK inhibitor affect the status of AKT phosphorylation. Thus, chapters 3.10, 3.11 and 5.5 do not provide any evidence of ‘cross-talk’ between the PI3K/AKT pathway and the MEK/ERK pathway. In the opinion of the author however, this does not rule out the possibility of cross-talk. Indeed, it is likely that multiple pathways may compensate or fine tune parallel pathways in a complex network of checks and balances. One justification for the results obtained may be that in the system utilised, this fine-tuning may be swamped by the supra-physiological cytokine concentrations responsible for FDM culturing.

Experiments that used a p53-reporter construct in cells treated with the AKT inhibitor were designed to further define the link between AKT inhibition and AKT activation. It was then hypothesised that AKT inhibition would increase p53 activity, leading to increased eGFP expression. The opposite effect was observed, where increased concentrations of the AKT inhibitor decreased eGFP expression. IL-3 withdrawal was still sufficient to promote eGFP expression however, indicating the construct was functioning as previously reported (chapter 5.6). Does this disprove the link between AKT and p53? One could argue that it does not, as there is a critical caveat to these reporter experiments. AKT has a very important role in ribosomal biogenesis and protein translation [142-144]. Since the reporter assay is dependent on new protein production, and this is repressed by the AKT inhibitor, this type of experiment will not adequately distinguish specific effects of this inhibitor on p53 protein (or a surrogate measure using a reporter) and an effect on all protein translation. It would be more accurate to determine whether reporter transcript was being produced in response to AKT inhibitor, although time did not permit such an experiment. Nonetheless, a similar effect was observed in chapter 4.6, when PUMA transcript levels decreased in response to ΔPH-AKT over-expression.
7.5 AKT phosphorylates and stabilises MDM2 to inhibit p53 dependent transcription

One mechanism by which AKT may regulate p53 is via the known AKT substrate MDM2. MDM2 is an E3 ubiquitin ligase, and is a critical inhibitor of p53 protein stability, directly phosphorylated by AKT [236]. Under conditions in which AKT activity decreases, MDM2 is targeted for proteosomal degradation. This permits p53, which would otherwise be bound to MDM2, to translocate to the nucleus, and drive transcription of genes such as PUMA to cause apoptosis. When phosphorylated by AKT, for example in the presence of cytokines such as IL-3, MDM2 has increased stability and a longer half-life, therefore enabling binding to and inhibition of p53. To test whether this form of regulation may contribute to IL-3 dependent control of apoptosis, it was shown that both AKT and MDM2 phosphorylation was stimulated in response to IL-3 signaling (chapter 6.1). Moreover, it could also be demonstrated that inducible ΔPH-AKT alone was sufficient to phosphorylate MDM2. An important feature of these result is that they were performed in Bax⁻/⁻;Bak⁻/⁻ cells that had been deprived of IL-3. This shows that ΔPH-AKT1, activated by IL-3 or when activate in the absence of IL-3 signaling, can phosphorylate MDM2 and thus provides a potential mechanism that would explain how cytokine signaling can regulate p53 activity. This pathway has been described in other systems, notably in response to DNA damage induced by irradiation [259, 260] where AKT activation can repress p53-dependent transcription by blocking MDM2 degradation. This has been less well recognized in cell death induced by cytokine deprivation.

The treatment of cells with Nutlin, an inhibitor of MDM2, was an experimental attempt to establish how important the regulation of MDM2 is in the maintenance of survival in cytokine dependent cells. One could predict that Nutlin would induce apoptosis in IL-3 dependent cells by initiating p53-dependent pathways. Moreover, this apoptosis should not occur in p53-deleted cells. This was in fact the results observed; indicating that, at least to some degree, repression of p53-dependent cell death is a normal function of the survival pathways in these FDM cells. The addition of Nutlin to cells over-expressing ΔPH-AKT abolished the survival advantage derived from AKT over-expression. This provides evidence to support the contention that AKT regulates the binding of MDM2 and p53 to inhibit the induction of apoptosis. It is worth
recalling that Nutlin does not affect the phosphorylation state of MDM2 but rather the binding interaction between p53 and MDM2. Therefore, quantification of phosphorylated MDM2 is insufficient to determine the effect of Nutlin-3. Further, FDM cells do not express levels of p53 under normal conditions that are detectable using immunoblotting techniques. Thus, it is not possible, using these techniques, to demonstrate that Nutlin-3 treatment increased p53 expression levels. Nevertheless, this evidence provides strong support to the hypothesis that AKT functions, in the context of IL-3 signaling, to repress p53-dependent apoptosis, which may be initiated when cytokine concentrations fall below a critical level. This work further indicates that one of the important targets of p53 transcriptional activity in this paradigm is PUMA.
Figure 7.1: Model of IL-3 dependent Akt signalling

The above figure represents one possible interpretation of the data presented in this thesis. Abundant IL-3 promotes signalling through multiple parallel survival and proliferative pathways. In the absence of IL-3, p53 promotes transcription of Puma. In limiting IL-3, AKT1 plays a non-redundant role in promoting survival.
8. Implications

Since its initial description, AKT has been described as a potent oncogene in numerous cancer types (see chapter 1.2.4). The research within this thesis provides some insights into how AKT plays a part in oncogenesis, as well as strategies to treat neoplasms possessing AKT expressing mutations.

AKT expressing mutations are rare in myeloid leukaemias, but are frequent events in certain solid tumours such as prostate and colorectal cancers. PI3K mutations, particularly activating point mutations such as the E545K or the H1047R mutations, are more frequently associated with leukaemias. A quickly growing trend in malignancies with PTEN loss of function mutations are also being described, as we understand more about the underlying gene. Thus, it might be tempting to target the downstream effectors, including AKT, in such diseases.

The research presented here however, suggests that these malignancies are unlikely to rely on AKT for oncogenic drive. Indeed, research performed by the author, but not included in this thesis indicates that over-expression of ‘hyper’-activated ΔPH-AKT, is insufficient to block PI3K inhibition induced apoptosis [183, 184]. Further, it was shown that the deletion of the primary AKT isoform responsible for survival signalling (AKT1) does not blunt PI3K inhibitor effectiveness. This suggests that, at best, AKT1 is redundant for PI3K survival signalling, and is incapable of reversing PI3K inhibition induced cell death. Therefore, activating mutations of AKT detected in diseased tissue would not preclude the use of PI3K inhibitors as treatment options.

AKT is simply not a particularly potent oncogene. Its role in oncogenesis may be to merely delay apoptosis long enough for an accumulation of mutations to drive transformation. At this point, treatment with an AKT inhibitor would be ineffective. Furthermore, a loss of p53 function, a frequent occurrence in many cancers, renders cells completely refractory to AKT inhibitor treatment. One might argue that the AKT inhibitor at least prevented proliferation, but the author would argue that treatments capable of clearing diseased tissue by inducing apoptosis would be a more effective strategy.
9. Conclusions

Many different models of IL-3 survival signalling exist, most involving the AKT/PI3K pathway regulating Bcl-2 family proteins. The purpose of this research was to determine what the role of AKT is in response to IL-3, and the mechanisms by which these roles are fulfilled.

Aim 1: To elucidate the role of AKT during IL-3 signalling

This research has shown that in FDM cells, over-expression of 'hyper'-activated ΔPH-AKT (possessing both a myristoylation domain and lacking the negative regulatory Pleckstrin Homology domain) is insufficient to promote cell cycling or proliferation independently of IL-3. Indeed, even IL-3 independent survival cannot be maintained by ΔPH-AKT over-expression. Similarly, the deletion of each AKT isoform suggests that each isoform is not required for any IL-3 dependent signalling. A major finding of this work however, was the observation that there is a range of IL-3 concentrations where AKT1 specifically is required to maintain survival.

Aim 2: To determine which Bcl-2 family proteins are required for AKT to maintain survival after IL-3 withdrawal

Many competing models attempt to provide insight into the mechanisms by which AKT regulates cellular viability in response to cytokine signalling. The research performed in this thesis indicates that, at least in the FDM model system, this mechanism does not revolve around BAD, BIM or Mcl-1, but most likely PUMA. This is congruous with research performed by Ekert et al. [149], who observed PUMA as the key regulator of IL-3 dependent cellular survival.

Aim 3: To determine how AKT regulates the Bcl-2 family of proteins to maintain survival after IL-3 withdrawal

PUMA is unlikely to be directly regulated by AKT. Therefore, the intermediary genes responsible for PUMA regulation downstream of AKT were sought. These genes were identified to be p53 and MDM2. p53, a key transcriptional regulator of PUMA, is prevented from translocating to the nucleus when bound to phosphorylated MDM2. Thus, a model has been proposed by the author of this research to describe the survival signal promoted by Akt;
In the presence of low IL-3 concentrations, AKT phosphorylates MDM2 at the S166 residue. Phosphorylated MDM2 binds to p53, preventing p53 translocation to the nucleus, but promoting its proteosomal degradation instead. When IL-3 signalling is lost, MDM2 is no longer phosphorylated and rapidly degraded by the proteasome. This allows p53 to translocate to the nucleus to transcribe, PUMA, thus initiating apoptosis (figure 7.1).
10. References


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The role of Akt during IL-3 survival signalling


The role of Akt during IL-3 survival signalling


11. Appendices

11.1 siRNA

The above figure, taken from the authors paper derived from this thesis [261], provides further evidence that AKT inhibition induced apoptosis requires p53. Knockdown of p53 transcript blunts the apoptosis induced by the AKT inhibitor when compared to cells infected with the scramble control.
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