Genetic basis of congenital myeloid failure syndromes in mutant zebrafish

Thesis submitted for the degree of Doctor of Philosophy

By

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Abstract

Zinc finger and BTB domain containing proteins (BTB-ZF) are transcriptional repressors from a family including members with critical roles in haematopoiesis and oncogenesis. From an N-ethyl-N-nitrosourea (ENU) mutagenesis screen for defects in myeloid development, a zebrafish mutant deficient in cells expressing myeloperoxidase (mpx) designated marsanne (man) was identified. Positional cloning identified that man carried a mutation in zbtb11, a largely unstudied BTB-ZF transcription factor, suggesting that zbtb11 is critical for normal neutrophil development.

The mutant man was found in a gynogenetic haploid ENU screen for defective expression of genes along the developmental pathway from mesoderm to mature neutrophil, undertaken to search for novel genetic regulators of myelopoiesis in an unbiased fashion. Since zebrafish are ectothermic, embryos were screened at 33°C to maximise recovery of temperature dependant alleles; man was the single temperature dependant mutant recovered.

man was a recessive, early embryonic lethal mutant with normal expression of genes involved in early haematopoietic differentiation and specification but markedly reduced expression of mpx, a gene expressed in terminally differentiated neutrophils. Erythropoiesis was unaffected. man mutants also developed brain and spinal cord degeneration with hydrocephalus, with marked apoptosis throughout the central nervous system.

Positional cloning resolved the genetic interval containing the man mutation to 52.5 Kb containing the open reading frame of a single gene, zbtb11. Sequencing identified a putative missense mutation at nucleotide 346 (T>A), causing a C116S substitution. Genetic tests implicated this zbtb11 mutation as the cause of the man phenotype. Antisense morpholino oligonucleotide knockdown of zbtb11
recapitulated the *mpx* deficiency and other features of the *man* phenotype. Injection of WT but not mutant *zbtb11* mRNA into *man* embryos completely rescued expression of *mpx* and all other aspects of the *man* phenotype. Human *ZBTB11* mRNA also rescued *man* mutants, indicating a wide evolutionary conservation of Zbtb11 function.

Utilising the rescue of *man* as an *in vivo* bioassay for Zbtb11 function, truncated and mutated forms of Zbtb11 were tested for functionality. The C terminal zinc fingers were not required for Zbtb11 function in this assay. The function of the N terminal domain of the protein, containing the residue mutated in *man*, was also explored.

These studies demonstrate a function for Zbtb11 in vertebrate neutrophil development. Ongoing study of the *man* mutant will provide further insights into Zbtb11 function and define its exact role in haematopoiesis.
Declaration

This is to certify that:

(i) The thesis comprises only my original work towards the PhD except where stated in the preface;

(ii) Due acknowledgement has been made in the text to all other materials used;

(iii) The thesis is less than 100,000 words in length, exclusive of tables, references, appendices and footnotes.

Dr Duncan Carradice

Dr Graham J. Lieschke
(Supervisor)
Preface

I declare that the mutant *marsanne* was derived from a mutagenesis screen performed by Dr Judith Layton in the laboratory of Dr Graham Lieschke. Prior to my commencement of the project Luke Kapitany collected some mutant embryos for mapping purposes. Dr Judith Layton performed the initial genome scan and Luke Kapitany performed the confirmation of linkage to markers flagged by this scan.

I performed the majority of the positional cloning, with some assistance in the later stages of the project from Sony Varma who performed some bulk screening PCRs and RFLP analysis.

As part of the collaborative nature of the laboratory some molecular biology work e.g. restriction digests, ligations, gel electrophoresis and riboprobe construction were performed by John Hayman, Luke Kapitany, Luke Pase, Felix Ellett and Sony Varma. Common laboratory stocks e.g. competent cells, rhodamine dextran etc. were prepared by all members of the laboratory in rotation.

Similarly, other members of the laboratory, including Dr Judith Layton, Luke Kapitany, Luke Pase, John Hayman and Felix Ellett, performed some WISH assays.

I received some assistance in the cloning of zebrafish and human Zbtb11 from Sony Varma who performed some PCRs, DNA ligations and site directed mutagenesis. A UROP student, Joanne Slater performed site directed mutagenesis to create the Δ172-1146 construct. Loretta Cerruti prepared the K562 cells from which the human Zbtb11 cDNA was cloned. Dr Julien Bertrand,
in the laboratory of Dr David Traver, performed the qPCR for *zbtb11* expression in sorted adult haematopoietic cells.

I performed the majority of the embryo microinjections. Joanne Slater performed embryo microinjection and phenotype scoring for some of the deletion constructs and HHCC motif mutant construct experiments.

Sequencing was outsourced to AGRF (QLD, Aust) and the Gandel Trust Sequencing Centre (VIC, Aust). Embryo sectioning and histology staining was performed at the Histology Department, Walter and Eliza Hall Institute. Anna Friedhuber at the Department of Pathology, Melbourne University, performed electron microscopy.

The staff of the zebrafish aquarium, Ludwig Institute for Cancer Research, provided fish husbandry. Other laboratory members set up some pair wise matings and collected embryos.

Helpful discussions on the mapping of *marsanne* took place during regular meetings between the laboratories of Dr Graham Lieschke and Dr Joan Heath, of the Ludwig Institute for Cancer Research.
Acknowledgments

I would like to thank my supervisors, Dr Graham Lieschke and Dr Judith Layton for their support and guidance throughout this project. I would particularly like to thank Graham for taking me on as a student and for his expert knowledge, mentorship and dedication to this project. I have learnt greatly from your rigorous approach to design and analysis of experiments, assessment of the literature and to scientific writing that I will carry forward into the future. Thank you to Judy for providing expertise and helpful comments during the mapping stage of the project and for performing WISH assays and the initial genome scan on marsanne.

I would like to thank all the past and present members of the Lieschke laboratory for their input into this project. Thank you to Sony Varma who performed molecular biology work, zebrafish husbandry, reagent preparation and provided a source of invaluable advice on molecular biology techniques. I would also like to thank Luke Kapitany who was initially working on the marsanne project prior to my commencement and who collected mutant embryos and advanced the mapping of marsanne prior to my arrival. Luke also performed several WISH assays on marsanne embryos. I would like to thank John Hayman for a great deal of collaborative work including molecular biology, fish husbandry and electron microscopy and for his constant enthusiasm. My thanks also go to Luke Pase and Felix Ellett for many helpful discussions on many aspects of the project, especially on initially teaching genetics to a medical practitioner, and for collaboration in molecular biology work and WISH assays.

Thanks must also go to all past and present members of staff in the zebrafish aquarium at the Ludwig Institute including Andrew Hughes, Kelly Costain, Bronwyn Groves, Mark Greer and Prue Chamberlain who diligently maintained all of the marsanne generations.
Finally I would like to thank my wife, Katie for her unwavering support and loyalty throughout the project. Being the partner of a PhD student for the last 3 years and having 3 children during that time is a challenge of major proportions, which she achieved without complaint or question.
Publications and presentations

Publications generated during my PhD candidature

Pase L, Layton JE, Kloosterman WP, Carradice D, Waterhouse PM, Lieschke GJ
“miR-451 regulates zebrafish erythroid maturation in vivo via its target gata2.”
Blood 2009 Feb 19;113(8):1794-804.

Carradice D, Lieschke GJ
“Zebrafish in Haematology: Sushi or Science?”
Blood 2008 Apr 1;111(7):3331-42.

Lieschke GJ, Carradice D (Inventors)
Provisional patent
Title: “Cell Therapy and Compositions Therefor”
Application No: US 11/195100

Selected presentations

Carradice D, Layton J, Heath J, Lieschke G
“Discovering novel genetic regulators of myelopoiesis using ENU mutagenesis in zebrafish.”
Oral presentation at the 10th Australia and New Zealand Zebrafish Workshop, 2009 (Victor Harbor, SA, Australia).

Carradice D, Layton J, Heath J, Lieschke G
“A Novel Transcription Factor, zbtb11 Is Critical for Neutrophil Development in Zebrafish.”
Blood (ASH Annual Meeting Abstracts), Nov 2008; 112: 284.
Oral presentation at the 50th meeting of the American Society of Haematology, 2008 (San Diego, CA, USA).

Carradice D, Layton J, Heath J, Lieschke G
“The mutant marsanne reveals a novel transcription factor critical for normal neutrophil development in zebrafish.”

Carradice D, Layton J, Heath J, Lieschke G
“The myeloid failure mutant marsanne is a lesion in a novel transcription factor.”
Oral presentation at the Asia Pacific Zebrafish Network Meeting, 2008 (Auckland, New Zealand).

Carradice D, Pase L, Layton J, Lieschke G
“Dissecting myelopoiesis in zebrafish.”
Oral presentation at Haematology Society of Australia and New Zealand Scientific Meeting, 2007 (Geelong, VIC, Australia).

Regular seminar presentations during 2006, 2007 and 2008 in the following forums:

Cancer and Haematology Division, Walter and Eliza Hall Institute of Medical Research, Parkville, Australia

Bone Marrow Research Laboratories, Royal Melbourne Hospital, Parkville, Australia
Abbreviations

°C  Degrees Celsius
A   Adenine
AB  AB zebrafish strain
AGM Aorta-gonad-mesonephros region
ALM Anterior lateral mesoderm
BAC Bacterial artificial chromosome
BSA Bovine serum albumin
C   Cytosine
CAT Chloramphenicol acetyl transferase
cDNA Complementary deoxyribonucleic acid
ChIP Chromatin immunoprecipitation
CHT Caudal haematopoietic tissue
CLP Common lymphoid progenitor
cM  Centimorgan
CMP Common myeloid progenitor
cmyb V-myb myeloblastosis viral oncogene homolog (avian)
cpa5 Carboxypeptidase A5
csf1r Colony stimulating factor 1 receptor
DNA Deoxyribonucleic acid
DNase Deoxyribonuclease
dNTP Deoxynucleotide triphosphate
dpf Days post fertilisation
drl Draculin
DVA Ventral wall of dorsal aorta
EDTA Ethylenediamine tetraacetic acid
EM Electron microscopy
ENU N-ethyl-N-nitrosourea
ES cell Embryonic stem cell
evi5 Ecotropic viral integration site 5
FACS Fluorescence-activated cell sorting
fam69a Family with sequence similarity 69, member A
fli1a Friend leukaemia integration site 1a
foxa1 Forkhead box A1
foxn1 Forkhead box N1
G Guanine
gata1 GATA binding protein 1
gfi1 Growth factor independent 1 transcription repressor
GFP Green fluorescent protein
GMP Granulocyte macrophage progenitor
hbae(x) Haemoglobin alpha embryonic-x
hbbe(x) Haemoglobin beta embryonic-x
hhex Hematopoietically expressed homeobox
hpf Hours post fertilisation
HSC Haematopoietic stem cell
ICM Intermediate cell mass
ighm Immunoglobulin heavy chain constant mu
ikzf1 Ikaros
itga2b Integrin, alpha 2b (CD41)
kb Kilobase (1000 bases)
kDa Kilodalton
lcp Lymphocyte cytosolic plastin
lmo2 LIM domain only 2
lyz Lysozyme
mb Megabase
MEP Megakaryocyte erythroid progenitor
µg Micrograms
MGH Massachusetts General Hospital
µl Microlitres
µM Micromoles
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO</td>
<td>Morpholino antisense oligonucleotide</td>
</tr>
<tr>
<td>mpll</td>
<td>Myeloproliferative leukaemia virus oncogene-like</td>
</tr>
<tr>
<td>mpx</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>nkn2.5</td>
<td>NK2 transcription factor related, locus 5 (Drosophila)</td>
</tr>
<tr>
<td>npsn</td>
<td>Nephrosin</td>
</tr>
<tr>
<td>PBI</td>
<td>Posterior blood island</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with 0.1 % Tween20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PLM</td>
<td>Posterior lateral mesoderm</td>
</tr>
<tr>
<td>PTU</td>
<td>Phenylthiourea</td>
</tr>
<tr>
<td>rag1</td>
<td>Recombination activating gene 1</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>rpl5b</td>
<td>Ribosomal protein L5b</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>runx1</td>
<td>Runt-related transcription factor 1</td>
</tr>
<tr>
<td>SK</td>
<td>St Kilda zebrafish strain</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>spi1</td>
<td>Spleen focus forming virus proviral integration oncogene</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium chloride and sodium citrate solution</td>
</tr>
<tr>
<td>SSCT</td>
<td>Sodium chloride and sodium citrate solution with 0.1% Tween20</td>
</tr>
<tr>
<td>SSLP</td>
<td>Simple sequence length polymorphism</td>
</tr>
<tr>
<td>T</td>
<td>Thymidene</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA buffer</td>
</tr>
<tr>
<td>tal1</td>
<td>T-cell acute lymphocytic leukaemia 1 (synonym-SCL, stem cell leukaemia)</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA buffer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>TILLING</td>
<td>Targeting Induced Local Lesions In Genomes</td>
</tr>
<tr>
<td>TU</td>
<td>Tübingen zebrafish strain</td>
</tr>
<tr>
<td>U</td>
<td>Uracil</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>WISH</td>
<td>Whole mount in-situ hybridisation</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>zbtb11</td>
<td>Zinc finger and BTB domain containing 11</td>
</tr>
<tr>
<td>zfpm1</td>
<td>Zinc finger protein, multitype 1 (FOG1)</td>
</tr>
</tbody>
</table>
Nomenclature

Genes, mRNA, protein and microRNAs
To reduce confusion, the nomenclature used in this study is based on that described at the zebrafish information network (http://www.zfin.org). When referring to human genes, names are capitalized and italicised, while human proteins are capitalised but not italicised. Murine genes have the first letter capitalised and are italicised, while proteins are not italicised. Zebrafish genes are in lower case and italicised, while proteins are not italicised. mRNAs use the same formatting conventions as the gene name.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein name</th>
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<tr>
<td><strong>Human</strong></td>
<td></td>
</tr>
<tr>
<td><em>GATA BINDING PROTEIN 1</em></td>
<td><em>GATA1</em></td>
</tr>
<tr>
<td>GATA BINDING PROTEIN 1</td>
<td>GATA BINDING PROTEIN 1</td>
</tr>
<tr>
<td><strong>Mouse</strong></td>
<td></td>
</tr>
<tr>
<td><em>GATA binding protein 1</em></td>
<td><em>Gata1</em></td>
</tr>
<tr>
<td>GATA binding protein 1</td>
<td>GATA binding protein 1</td>
</tr>
<tr>
<td><strong>Zebrafish</strong></td>
<td></td>
</tr>
<tr>
<td><em>gata binding protein 1</em></td>
<td><em>gata1</em></td>
</tr>
<tr>
<td>gata binding protein 1</td>
<td>gata binding protein 1</td>
</tr>
</tbody>
</table>
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