FUNCTIONAL PROTEOMIC ANALYSIS OF LEUKAEMOGENIC PROTEIN TYROSINE KINASE TARGETS

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LIST OF CONTENTS

a) TITLE ............................................................................................................................. 1 

b) LIST OF CONTENTS ................................................................................................. 2 

c) LIST OF FIGURES ....................................................................................................... 6 

d) LIST OF TABLES ......................................................................................................... 9 

e) LIST OF APPENDICES ............................................................................................. 9 

f) LIST OF ABBREVIATIONS and SYMBOLS ............................................................ 10 

g) ABSTRACT .................................................................................................................. 15 

h) DECLARATION ........................................................................................................... 16 

i) COPYRIGHT STATEMENT ...................................................................................... 16 

j) ACKNOWLEDGEMENTS ......................................................................................... 17 

k) DEDICATION ............................................................................................................. 19 

CHAPTER 1. INTRODUCTION .................................................................................. 20

1.1 General introduction ................................................................................................... 21 

1.2 Haematopoiesis ......................................................................................................... 22 

1.3 Bone marrow microenvironment of HSCs ............................................................ 24 

1.4 HSCs homing and mobilisation .............................................................................. 24 

1.5 CXCL12/CXCR4 interaction induces cell motility ............................................... 25 

1.6 Sphingosine-1-phosphate an agonist that regulates cell motility ......................... 28 

1.7 BM tumor microenvironment contribute to leukaemia ........................................ 30 

1.8 Classification of leukaemia ..................................................................................... 30 

1.9 Myeloproliferative neoplasms and related diseases ............................................. 31 

1.9.1 Chronic myeloid leukaemia- Ph positive MPN ................................................ 33 

1.9.2 MPL W515L- Ph negative MPN ........................................................................ 34 

1.9.3 JAK2 V617F- Ph negative MPN .......................................................................... 36 

1.9.4 JAK2 K539L- Ph negative MPN .......................................................................... 37 

1.10 Current treatment strategies for MPN ..................................................................... 38 

1.11 MYC induces leukaemic transformation ................................................................ 40 

1.12 Transforming growth factor beta (TGFβ) and myelofibrosis ............................... 41 

1.13 THOC5/Fms-interacting protein (FMIP) activation downstream of leukaemogenic PTK .......................................................... 43 

1.14 Approaches to proteomic studies .......................................................................... 46 

1.15 Cell lines models for leukaemogenic protein tyrosine kinase studies .................. 47 

1.16 Aims and Objectives ............................................................................................... 48
CHAPTER 2. MATERIALS AND METHODS

2.1 Cell lines and culture conditions................................................................. 50
2.2 Whole cell lysate preparation......................................................................... 50
2.3 Protein assay.................................................................................................. 51
2.4 Western blotting............................................................................................ 51
  2.4.1 SDS-PAGE gel preparation........................................................................ 51
  2.4.2 Sample preparation................................................................................... 51
  2.4.3 Electrophoretic protein transfer................................................................. 52
  2.4.4 Immunoblotting and protein detection....................................................... 52
2.5 Chemotaxis and motility assay....................................................................... 54
  2.5.1 Motility assay............................................................................................ 54
  2.5.2 Cell treatment prior to motility assays....................................................... 55
2.6 Cloning THOC5 Y225F into MSCVneo............................................................ 55
  2.6.1 Plasmid preparation and restriction enzyme digestion of MSCVneo vector and THOC5 Y225F............................................................... 55
  2.6.2 DNA ligation and transformation into E.coli DH5α™............................... 56
  2.6.3 Confirmation of cloning............................................................................. 57
2.7 Transfection of MYC-tagged mutant THOC Y225F/MSCVneo into the retroviral packaging cell line................................................................. 57
  2.7.1 Preparation of Platinum-E packaging cell line........................................... 57
  2.7.2 Formation of DNA-liposome complex...................................................... 58
  2.7.3 Transduction of Ba/F3 MPL W515L cells................................................... 58
2.8 c-MYC siRNA transient transfection............................................................... 58
2.9 Flow cytometry of CD45 and CXCR4 cell surface staining............................ 59
2.10 TGFβ1 measurement..................................................................................... 59
2.11 Protein quantification using eight-channel isobaric tagging for relative quantification (iTRAQ™).................................................................................. 59
  2.11.1 Cell lysate preparation for iTRAQ™......................................................... 60
  2.11.2 Tryptic digestion and iTRAQ™ eight-channel labelling............................ 60
  2.11.3 High-pH reversed phase chromatography for phosphopeptide fractionation............................................................................................................. 61
  2.11.4 Tandem mass spectrometry-QSTAR® Elite............................................. 61
  2.11.5 Data analysis and software employed for protein identification and quantification................................................................................................. 62
2.12 Sphingosine-1-phosphate analysis on LC/ESI-MS/MS.................................... 63
2.13 RNA extraction and Quantitative real time-polymerase chain reaction (qRT-PCR)........................................................................................................ 63
2.14 Cell proliferation assay................................................................................... 64
CHAPTER 3. MYELOPROLIFERATIVE NEOPLASM ASSOCIATED ONCOGENE MPL W515L AFFECTS MOTILITY VIA THOC5 LEADING TO LEUKAEMIC TRANSFORMATION

3.1 Introduction........................................................................................................ 68
3.2 Results.................................................................................................................. 69
3.3 Effects of MPL W515L on STAT signal transduction and MCL-1....................... 69
3.4 MPL W515L alters motile responses which is linked to THOC5 modulation........ 71
3.5 Construction of THOC5 Y225F into MSCVneo.................................................. 74
3.5.1 Cloning of THOC5 Y225F into MSCVneo......................................................... 75
3.5.2 Orientation analysis of THOC5 Y225F insert in MSCVneo......................... 75
3.5.3 Generation of Ba/F3 MPL W515L expressing THOC5 Y225F....................... 76
3.5.4 Western blot of Ba/F3 MPL W515L THOC5 Y225F expressing cells......... 76
3.5.5 THOC5 Y225F expression reduces the MPL W515L induced chemokinesis................................................................. 77
3.6 Modulation of the CXCL12/CXCR4/CD45 axis in Ba/F3 MPL W515L expressing cells................................................................. 78
3.6.1 CXCL12/CXCR4 affects motility....................................................................... 78
3.6.2 Involvement of CD45 in the motility pathway................................................. 80
3.7 Screening of inhibition of signal transduction proteins to identify pathways linked to increase in chemokinesis................................................................. 82
3.8 TGFβ measurement in Ba/F3 MPL W515L expressing cells............................... 83
3.9 Assessment on TGFβ effects on THOC5 Y225 phosphorylation......................... 84
3.10 Targeting MYC in MPL W515L expressing cells affects motility via THOC5......................................................................................................................... 86
3.10.1 MPL W515L oncogene effects on c-MYC expression................................. 86
3.10.2 Effects of JQ1 bromodomain inhibitor on MYC and THOC5 expression....................... 86
3.10.3 Suppression of MYC with siRNA.................................................................... 89
3.10.3.1 Optimization of esiRNA MYC concentration............................................ 89
3.10.3.2 esiRNA MYC knockdown alters cell motility.............................. 90
3.11 SK regulation in MPL W515L linked to THOC5 Y225 phosphorylation, MYC and motility................................................................. 91
3.12 Characterisation the role of S1P on motile response and expression of THOC5 Y225 phosphorylation and MYC in MPL W515L expressing cells................................. 94
CHAPTER 4. PROTEOMIC EFFECTS ON MOTILITY INDUCED BY THE
MYELOPROLIFERATIVE NEOPLASM ASSOCIATED ONCOGENE
MPL W515L VIA THOC5

4.1 Introduction........................................................................................................... 109
4.2 Results.................................................................................................................. 110
4.3 Characterisation of Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cell lines by eight-channel isobaric tagging.......................... 110
4.4 Data analysis using ProteinPilot™ software......................................................... 112
4.5 Identification of differentially expressed protein................................................ 113
4.6 Validation of iTRAQ changes in protein levels by Western blot analysis............ 119
4.7 Ingenuity Pathway analysis................................................................................ 121
4.8 Protein interaction analysis of differentially expressed proteins identified in
Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cells... 123
4.9 Discussion............................................................................................................. 124

CHAPTER 5. REGULATION OF mRNA EXPORT COMPLEX AND THOC5
FUNCTION IN MYELOPROLIFERATIVE NEOPLASMS

5.1 Introduction........................................................................................................... 128
5.2 Results.................................................................................................................. 134
5.3 Analysis of mRNA transcript level by qRT-PCR.................................................. 134
5.4 Western blot analysis of mRNA export protein in MPN oncogenes............... 135
5.5 Cell proliferation studies for mRNA export factor inhibition in MPN associated
oncogenes expressing Ba/F3 cells........................................................................ 140
5.6 Discussion............................................................................................................. 143

CHAPTER 6. GENERAL DISCUSSION, FUTURE WORK AND
CONCLUSION

6.1 General discussion.............................................................................................. 147
6.2 Future work......................................................................................................... 153
6.3 Conclusion.......................................................................................................... 156

CHAPTER 7. APPENDICES

CHAPTER 8. REFERENCES

Word count: 52, 945
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic representation of haematopoiesis</td>
<td>23</td>
</tr>
<tr>
<td>1.2</td>
<td>Mechanism of HSCs homing and mobilisation in the bone marrow</td>
<td>25</td>
</tr>
<tr>
<td>1.3</td>
<td>A schematic representation of the CXCL12/CXCR4 intracellular signal</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>transduction pathways</td>
<td></td>
</tr>
<tr>
<td>1.4</td>
<td>Sphingolipid metabolic pathway</td>
<td>29</td>
</tr>
<tr>
<td>1.5</td>
<td>p210 BCR/ABL fusion protein in CML</td>
<td>33</td>
</tr>
<tr>
<td>1.6</td>
<td>JAK-STAT signalling pathway induced by thrombopoietin (TPO) receptor and</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>activation in the absence of ligand by MPL W515L and JAK2 mutation</td>
<td></td>
</tr>
<tr>
<td>1.7</td>
<td>The structure of Janus kinase</td>
<td>36</td>
</tr>
<tr>
<td>1.8</td>
<td>JAK2 exon 12 mutations</td>
<td>37</td>
</tr>
<tr>
<td>1.9</td>
<td>TGFβ signalling pathway</td>
<td>42</td>
</tr>
<tr>
<td>1.10</td>
<td>THO complex structure</td>
<td>44</td>
</tr>
<tr>
<td>1.11</td>
<td>Schematic domain structure and phosphorylation sites of THOC5</td>
<td>44</td>
</tr>
<tr>
<td>2.1</td>
<td>Schematic diagram of Boyden chamber assay</td>
<td>54</td>
</tr>
<tr>
<td>2.2</td>
<td>pMSCVneo vector map</td>
<td>56</td>
</tr>
<tr>
<td>2.3</td>
<td>Schematic diagrams of Plat-E packaging constructs</td>
<td>57</td>
</tr>
<tr>
<td>3.1</td>
<td>Western blot assessment of the STAT protein expression</td>
<td>71</td>
</tr>
<tr>
<td>3.2</td>
<td>Assessment of MCL-1 protein expression</td>
<td>71</td>
</tr>
<tr>
<td>3.3</td>
<td>Protein changes identified and categorised as a consequence of Ba/F3</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>MPL W515L expression by biological process</td>
<td></td>
</tr>
<tr>
<td>3.4</td>
<td>Differential effects of CXCL12/SDF-1 on Ba/F3 and Ba/F3 MPL W515L expressing</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>cell migration</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>Western blot assessment of the THOC5 and phospho Y225 THOC5 expression</td>
<td>74</td>
</tr>
<tr>
<td>3.6</td>
<td>BglIII digestion of MSCVneo and MSCV THOC5 Y225F IRES GFP</td>
<td>75</td>
</tr>
<tr>
<td>3.7</td>
<td>Western blot analysis of THOC5 and THOC5 Y225F MYC-tag expression</td>
<td>76</td>
</tr>
<tr>
<td>3.8</td>
<td>THOC5 plays a role in MPL W515L induced motility</td>
<td>77</td>
</tr>
<tr>
<td>3.9</td>
<td>CXCL12 effects on motility in MPL W515L expressing cells</td>
<td>79</td>
</tr>
<tr>
<td>3.10</td>
<td>Cell surface expression of CXCR4</td>
<td>79</td>
</tr>
<tr>
<td>3.11</td>
<td>Western blot analysis of CXCR4 expression</td>
<td>80</td>
</tr>
<tr>
<td>3.12</td>
<td>Cell surface expression of CD45</td>
<td>81</td>
</tr>
<tr>
<td>3.13</td>
<td>Western blot analysis of CD45 expression</td>
<td>81</td>
</tr>
<tr>
<td>3.14</td>
<td>Differential effects of tyrosine kinase inhibitors on Ba/F3 and Ba/F3</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>MPL W515L cell migration</td>
<td></td>
</tr>
<tr>
<td>3.15</td>
<td>MPL W515L expression induces TGFβ secretion</td>
<td>84</td>
</tr>
</tbody>
</table>
Figure 3.16  Assessment of THOC5 Y225 phosphorylation following TGFβ treatment 85
Figure 3.17  Assessment of THOC5 Y225 phosphorylation following TGFβ inhibition 85
Figure 3.18  Western blot analysis on c-MYC expression 86
Figure 3.19  JQ1 inhibitor effects on THOC5 Y225 phosphorylation and c-MYC expression 87
Figure 3.20  MYC plays a role in the MPL W515L induced motility 88
Figure 3.21  Western blot analysis of c-MYC expression on esiRNA MYC treated cells 89
Figure 3.22  Western blot analysis of c-MYC, THOC5 and phospho Y225 THOC5 expression on esiRNA MYC treated cells 90
Figure 3.23  Motility assay on Ba/F3 and Ba/F3 MPL W515L cells treated with esiRNA MYC 91
Figure 3.24  THOC5, THOC5 Y225 phosphorylation and c-MYC expression following sphingosine kinase inhibitor (SKi) treatment 93
Figure 3.25  Effect of SKi on MPL W515L induced chemokinesis 93
Figure 3.26  THOC5, THOC5 Y225 phosphorylation and c-MYC expression following S1P treatment 95
Figure 3.27  Effects of S1P on MPL W515L in motility 95
Figure 3.28  S1P analysis in whole cell lysates of Ba/F3 and Ba/F3 MPL W515L expressing cells 97
Figure 3.29  MPL W515L induced chemokinesis in patient material 98
Figure 3.30  Colony forming ability in patient material for TGFβ and JQ1 inhibitor 99
Figure 3.31  Schematic representation of the MPL W515L and JAK2 mutant mediated pathway of THOC5 induced motility via TGFβ, MYC, and S1P 107
Figure 4.1  Overview of iTRAQ™ reagent eight-channel methodology for Ba/F3, Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cells 111
Figure 4.2  Distribution of protein quantification ratios 112
Figure 4.3  Venn diagram of up-regulated proteins compared to Ba/F3 cells 114
Figure 4.4  Venn diagram of down-regulated proteins compared to Ba/F3 cells 114
Figure 4.5  Western blot analysis of EZH2, TOP1, and TMEM57 expression 120
Figure 4.6  Total EZH2, TOP1 and TMEM57 of western blot analysis normalised to Ba/F3 120
Figure 4.7  Ingenuity Pathway Analysis 122
Figure 4.8  The protein interactions modulated by THOC5 Y225F expression 123
Figure 4.9  Composition of PRC2 complex and domain structure of EZH2 125
Figure 5.1  Overview of mRNA export pathway 129
Figure 5.2  SRAG protein level of Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells 136
Figure 5.3: UIF protein level normalised to actin for Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells

Figure 5.4: eIF4E protein level normalised to actin for Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells

Figure 5.5: ALY/REF protein level normalised to actin for Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells

Figure 5.6: Comparison of SRAG protein change against mRNA change for Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells

Figure 5.7: Comparison of UIF protein change against mRNA change for Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells

Figure 5.8: Comparison of eIF4E protein change against mRNA change for Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells

Figure 5.9: Comparison of ALY/REF protein change against mRNA change for Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells

Figure 5.10: Schematic representation of mRNA export for cell proliferation studies

Figure 5.11: Effects of curaxin on cell growth in MPN associated oncogenes expressing Ba/F3 cells

Figure 5.12: Effects of ribavirin on cell growth in MPN associated oncogenes expressing Ba/F3 cells

Figure 5.13: Effects of AdOx on cell growth in MPN associated oncogenes expressing Ba/F3 cells

Figure 6.1: Schematic representation depicting the MPL W515L and JAK2 mutants potentiate THOC5 induced motility
LIST OF TABLE

Table 1.1 Features of myeloproliferative neoplasms diseases 32
Table 1.2 MYC alterations which induce haematological malignancy 41
Table 2.1 List of antibodies used in this studies 53
Table 2.2 List of oligonucleotides used for TaqMan® RT-PCR assays in this study 65
Table 3.1 Proteins identified and quantitated in MPL W515L expressing cells 69
Table 3.2 Cell viability effects on inhibitors treatment 82
Table 3.3 Cell viability effects on JQ1 inhibition 88
Table 3.4 Cells viability effects on SK inhibition 94
Table 4.2 Proteins up-regulated by the action of MPL W515L oncogene altered by THOC5 Y225F expression 115
Table 4.3 Proteins down-regulated by the action of MPL W515L oncogene altered by THOC5 Y225F expression 117
Table 5.1 Components of transcription export complex 132
Table 5.2 Quantitation of mRNA expression in Ba/F3 MPL W515L as well as JAK2 mutant expressing Ba/F3 cells 134

LIST OF APPENDICES

Appendix 7A SDS-PAGE and WB reagents and solutions 158
Appendix 7B Effects of inhibitors on cell cycle 159
Appendix 7C Effects of inhibitors on cell cycle 160
LIST OF ABBREVIATIONS and SYMBOLS

µg: microgram
µL: microliter
µM: micromolar
µm: micrometer
2D: two-dimensional
ALCLs: anaplastic large cell lymphomas
ALL: acute lymphocytic leukaemia
AML: acute myeloid leukaemia
ATM: ataxia telangiectasia mutated
ATP: adenosine triphosphate
Ba/F3: pro-B cell line derived from BALB/c mouse
BALB/c: albino mouse strain
BCR: breakpoint cluster region
BM: bone marrow
BSA: bovine serum albumin
C/EBPs: CCAAT/enhancer binding protein
c-ABL: c-Abelson leukaemia virus
CD: cluster of differentiation
CD45: receptor-type tyrosine-phosphatase C
CEL: chronic eosinophilic leukaemia
Chtop/SRAG: chromatin target of Prmt1
CID: collision-induced dissociation
CLL: chronic lymphocytic leukaemia
CML: chronic myeloid leukaemia
CMML: chronic myelomonocytic leukaemia
CMP: common myeloid progenitor
CLP: common lymphoid progenitor
c-MPL: myeloproliferative leukaemia virus oncogene
c-MYC: cellular homolog of the retroviral myelocytomatosis oncogene
CNL: chronic neutrophilic leukaemia
CO₂: carbon dioxide
CXCL12: C-X-C motif chemokine ligand 12
CXCR4: C-X-C chemokine receptor type 4
CXCR7: C-X-C chemokine receptor type 7
DH5α: E.coli type of competent cells
DMEM: Dulbecco’s modified eagle medium
DNA: deoxyribonucleic acid
ECL: enhanced chemiluminescence
EDTA: ethylenediaminetetraacetic acid
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
eIF4E: eukaryotic initiating factor 4E
ELISA: enzyme linked immunosorbent assay
EP: erythrocyte progenitor
EPO: erythropoietin
EpoR: erythropoietin receptor
ERK1/2: extracellular signal-regulated kinase
ESI: electrospray ionisation
ET: essential thrombocytemia
EZH2- enhancer zeste homolog 2
FAB: French American British
FACS: fluorescence activated cell sorting
FACT: facilitates chromatin transcription
FCS: fetal calf serum
FLT3/ITD: FLT3 internal tandem duplication
FMIP: Fms-interacting protein
g: gram
G-CSF: granulocyte colony-stimulating factor
GFP: green fluorescence protein
GM-CSF: granulocyte-macrophage colony stimulating factor
GMP: granulocyte-macrophage progenitor
GP: granulocyte progenitor
h: hours
HES: hypereosinophilic syndrome
HGFs: haematopoietic growth factors
HPLC: high performance liquid chromatography
HRP: horse radish peroxidase
HSCs: haematopoietic stem cells
HSPC: haematopoietic stem cells/progenitor cells
ICAT: isotope-coded affinity tag
IL-3: interleukin-3
IMF: idiopathic myelofibrosis
IPG: immobilised pH gradient
IRES: internal ribosome entry site
iTRAQ: isobaric tagging for relative and absolute quantitation
JAK-STAT: Janus kinase/signal transducer and activator of transcription
JAK2 K539L: point mutation at position 539 amino acid from lysine to leucine on Janus kinase 2
JAK2 V617F: point mutation at position 617 amino acid from valine to phenylalanine on Janus kinase 2
JAK2 WT: wild type Janus kinase 2
JAK2: Janus kinase 2
JM: juxtamembrane
JQ1: c-Myc BET bromodomain inhibitor
kDa: kilodalton
L: liter
LC: liquid chromatography
M: Molar
MacP: macrophage progenitor
MALDI: matrix-assisted laser desorption ionization
MAPK: mitogen activated protein kinase
MCL-1: myeloid cell leukaemia 1
MCS: multiple cloning site
M-CSF: macrophage- colony stimulating factor
MEP: megakaryocyte erythrocyte progenitor
MESV: murine embryonic stem cell virus
mg: miligram
mIL-3: murine interleukin-3
min: minutes
mL: milliliter
MLL: mixed lineage leukaemia
mM: milimolar
mmol: milimole
MMTS: methyl methanethiosulfonate
MPD: myeloproliferative disorders
MPL W515L: mutation at position 515 from tryptophan to lysine in thrombopoietin receptor
MPNs: myeloproliferative neoplasms
MRM: multiple reaction monitoring
mRNA: messenger ribonucleic acid
mRNP: messenger ribonucleoprotein particle
MS/MS: tandem mass spectrometry
MS: mass spectrometry
MSCV: murine stem cell virus
MW: molecular weight
NBCS: newborn calf serum
NCP: nitrocellulose paper
ng: nanogram
nmol: nanomole
NK: natural killer cells
NPC: nuclear pore complex
NPM/ALK: nucleophosphmine anaplastic lymphoma kinase
NTA: nitriloacetic acid
O₂: Oxygen
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
PDGFR: platelet derived growth factor receptor
Ph: Philadelphia chromosome
pH: potential hydrogen
pI: isoelectric point
PI3K: phosphatidylinositol 3-kinases
PIP3: phosphatidylinositol 3,4,5 trisphosphate
Plcγ: phospholipase C-γ
pmol: picomole
Puro: puromycin
PTK: protein tyrosine kinase
PTMs: post translational modifications
PV: polycythaemia vera
qRT-PCR: quantitative real time-polymerase chain reaction
RIPA: radio immuno precipitation assay
RNA: ribonucleic acid
ROS: reactive oxygen species
RP: reverse phase chromatography
RPMI: Roswell Park Memorial Institute medium
RTK: receptor tyrosine kinase
s: seconds
SAM: S-adenosyl methionine
S1P: sphingosine-1-phosphate
S1PR: sphingosine-1-phosphate receptor
SCF: stimulating colony factor
SDF-1: stromal derived factor-1
SDS: Sodium Dodecyl Sulfate
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM: standard error of mean
SFK: Src family kinase
shRNA: small hairpin ribosomal nucleic acid
SILAC: stable isotope labelling by amino acids in cell culture
siRNA: small interfering ribosomal nucleic acid
SKI: sphingosine kinase inhibitor
SOX: SRY–related high-mobility group box
Src: proto-oncogene identified in Rous sarcoma virus
STAT: signal transducers and activators of transcription
TEAB: triethylammonium bicarbonate
TEMED: N,N,N,N-tetramethylethylenediamine
TGFβ: transforming growth factor beta
TGFβi: transforming growth factor beta inhibitor
THO: suppressors of the transcriptional defects of Hpr1 by overexpression
THOC1: THO complex 1
THOC2: THO complex 2
THOC3: THO complex 3
THOC4: THO complex 4 or ALY/REF
THOC5: THO complex 5
THOC6: THO complex 6
THOC7: THO complex 7
TKD: tyrosine kinase domain
TKs: tyrosine kinases
TOP1: topoisomerase 1
TPO: thrombopoeitin
TpoR: thrombopoeitin receptor
TREX: transcription export complex
UIF: UAP56 interacting protein
UV: ultraviolet
v/v: volume/volume
V: volts
VEGF: vascular endothelial growth factor
w/v: weight/volume
WBCs: white blood cells
WHO: World Health Organization
WT: wild-type
Y225F: mutation at position 225 from tyrosine to phenylalanine on THOC5/FMIP protein
Functional proteomic analysis of leukaemogenic protein tyrosine kinase targets

Norhaida binti Che Azmi
The University of Manchester
PhD in Medicine
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Myeloproliferative neoplasms (MPNs) are clonal proliferative disorders associated with JAK2 mutation (e.g. JAK2 K539L, JAK2 V617F), MPL mutation (e.g. MPL W515L) or product from reciprocal chromosomal translocations in many cases (e.g. BCR/ABL). The mutated thrombopoietin receptor MPL W515L found in thrombocytosis and myelofibrosis is constitutively activated leading to a downstream signal transduction cascade activation including the JAK-STAT signalling pathway. MPL W515L induced JAK2 mutation is associated with polycythaemia vera. Using quantitative proteomics I have investigated the effects of the MPL W515L oncogene on the proteome. This was performed to delineate specific features of MPL W515L action with a view to identifying new therapeutic targets for MPN patients. Within the proteins identified as being differentially expressed as a consequence of MPL W515L expression I observed an enrichment of proteins involved in motility. This was associated with a MPL W515L induced increase in chemokinesis. Further investigation into this altered chemokinesis elucidated a pathway from CXCL12/CXCR4/CD45 mediated Src activation through to THOC5 Y225 phosphorylation that had been compromised by MPL W515L. The MPL W515L induced THOC5 phosphorylation was linked to elevated MYC expression. Either chemical inhibition of MYC or gene silencing reduced both the level of THOC5 Y225 phosphorylation and also the increased chemokinesis. Of interest, because of its reported role in both myelofibrosis and motility, the MPL W515L expressing cells demonstrated increased release of transforming growth factor beta (TGFβ). I demonstrated that TGFβ stimulates the phosphorylation of THOC5. Via the expression of Y225F mutants of THOC5 and the chemical inhibition of TGFβ I show a role for this elevated TGFβ in the increased chemokinesis of MPL W515L expressing cells. TGFβ has been reported to upregulate sphingosine-1-phosphate (S1P) which contributes to fibrosis. Having previously published on the differential effects of S1P on the motility of HSC populations I investigated the potential role of S1P in the MPL W515L induced chemokinesis. Inhibition of sphingosine kinase reduced the increase in chemokinesis and THOC5 Y225 phosphorylation in MPL W515L expressing cells. Furthermore I demonstrated that MPL W515L expression led to an increase in the intracellular levels of S1P suggesting a role for S1P in MPN. To further understand the role of THOC5 phosphorylation in the increased chemokinesis I undertook a discovery proteomics screen of MPL W515L cells co-expressing either wild type or Y225F mutant THOC5. Enhancer zester homolog 2 (EZH2) was shown to increase in MPL W515L as compared to MPL W515L mutant THOC5 Y225F expressing and control cells and as such may be linked to the increases in chemokinesis observed. Present work is aimed at clarifying the role of EZH2 in chemokinesis. In conclusion I have identified a novel pathway disrupted in MPN and allow me to start to understand the mechanisms by which the phosphorylation of THOC5 may contribute to leukaemogenic transformation through links to TGFβ, MYC, and S1P biology.
DECLARATION

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After all, my dreams would not come true without you all here throughout my endure journey in Manchester, UK.

THANK YOU EVERYONE!
DEDICATION

To my husband
Mohd Azuan Bin Mohamad Paudzi

my daughter
Nurnisa Saffiya Khadeeja Binti Mohd Azuan

for their endless support, patience, and understanding through thick and thin during our time abroad.
Chapter 1

Introduction
Chapter 1

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1.1 General introduction

Haematopoiesis is the highly regulated process of blood formation which occurs mainly within the bone marrow (BM) (medullary) within adults but it can also occur in other tissues such as the liver, thymus and spleen (extramedullary) (Weissmann, 2000). The haematopoietic system offers an ability to react in a coordinated manner to changes in environmental stimuli. Disruption of this response can result in hematological diseases including leukaemia (e.g: acute myeloid leukaemia, myeloproliferative neoplasms, lymphoproliferative neoplasms). Myeloproliferative neoplasms (MPNs) are a group of blood disorders characterised by proliferation of one or more haematopoietic cell lineages (Akpınar et al., 2013). It is frequently associated with activation of oncogenic protein tyrosine kinases (PTK) (e.g JAK2 mutants), also mutation in certain haematopoietic-specific cytokine receptors (e.g MPL W515L) and chromosomal translocations (e.g: the t(9;22) translocation forming BCR/ABL chimeric gene).

Chronic myeloid leukaemia (CML) is a MPN that is characterised by the presence of the Philadelphia (Ph) chromosome and the subsequent expression of this BCR/ABL fusion gene whilst non-CML Ph-negative MPNs are stratified into polycythemia vera (PV), essential thrombocythemia (ET), and primary (idiopathic) myelofibrosis (MF) (Akpınar et al., 2013). Despite recent advances in diagnosis and treatment, there remains a clinical need for therapy improvement in the treatment of MPNs. For instance in CML, imatinib mesylate (Glivec®, Gleevec™) has proved a remarkable success as the first line treatment in chronic phase CML patients, however, imatinib does not eliminate the leukaemia stem cell in the patients and resistance to the drug has been increasingly recognised (Henkes et al., 2008). The use of JAK2 inhibitors such as ruxolitinib in MF dramatically reduces the inflammatory-linked symptoms and splenomegaly but has been argued to offer little improvement over conventional treatments such as hydroxyurea (Antonioli et al., 2010; Ricksten et al., 2008). Given the complexity of MF pathogenesis which involves an interplay between the BM
microenvironment and haematopoietic stem cells (HSCs) trafficking, it appears likely that a single oral agent will not sufficiently reduce the burden experienced in MF patients (Hoffman, 2011). Therefore, further investigation on the molecular mechanisms such as those on HSCs trafficking may offer a better understanding towards the resistance to treatment in MPN. Further studies on single or in combination treatment are of critical importance to improve clinic outcomes in MPN patients.

1.2 Haematopoiesis

Haematopoiesis is the process by which haematopoietic stem cells (HSCs) give rise to all the different mature circulating blood cell types. Haematopoiesis occurs within the bone marrow and is a highly hierarchical process involving differentiation, proliferation and development (Weissmann, 2000). The pluripotent HSCs are required throughout the lifespan of the organism because mature cells such as erythrocytes and neutrophils have a short life span (Seita and Weissmann, 2010). However, with their unique characteristics, haematopoietic stem cells constantly maintain their population through the ability to self-renewal (duplication without losing development potential) and their potential to give rise to multiple cell types (differentiation). These multipotent stem cells were first observed by Till and McCulloch in 1961. They showed that injection of syngeneic BM into lethally irradiated mice led to recovery of normal blood cell formation compromised by irradiation (Till and McCulloch, 1961).

Maintaining the balance between quiescent and active HSCs within the BM is crucial in providing the required number of mature blood cells throughout the life span of the organism. The closest progeny of HSCs are called haematopoietic progenitor cells. HPCs lack in maintained self renewal capacity, but they are capable of further differentiation and proliferation to produce the mature blood cells of all haematopoietic lineages (Figure 1.1). Haematopoiesis occurs mainly within the BM in the adult. The major site of haematopoiesis varies during an individual’s life being different prenatal and post-natal. The first recognised place of prenatal haematopoiesis is in the yolk sac and aorta-gonad-mesonephros (AGM) region (Medvinsky and Dzierzak, 1996). Subsequently, after 3-6 months of foetal life haematopoiesis switches to the liver and spleen. Just before birth, haematopoiesis takes place in the BM and is maintained by the long bones including vertebrae, pelvis, ribs and sternum.
throughout life. This suggests that there is a movement of stem cells from the BM to other sites within the body. Haematopoietic development can be divided into three compartments, haematopoietic stem cell, progenitor cell and mature cells. The haematopoietic stem cells give rise to 2 committed lineages, myeloid and lymphoid (Figure 1.1). The myeloid lineage is responsible for the development of erythrocytes, thrombocytes, granulocytes and monocytes whilst the lymphoid lineage is specialised for T and B lymphocytes which are respectively responsible for cytotoxic immune response and antibody production. Once the cell matures, they leave the BM and enter the blood circulation and tissues to perform their normal function. Red blood cells (erythrocytes) are enucleated cells containing large amounts of haemoglobin which mainly function in oxygen ($O_2$) and carbon dioxide ($CO_2$) gas transportation in the body. Platelets or thrombocytes are important in inflammation and blood coagulation. White blood cells (WBCs), granulocytes are classified into three subtypes neutrophils, eosinophils and basophils. These WBCs are involved in the antimicrobial activity, inflammatory response, parasites and allergy reaction. Abnormalities in haematopoietic development and the bone marrow microenvironment can lead to leukaemic transformation.

Figure 1.1: Schematic representation of haematopoiesis. A multipotent HSC loses it long-term self-renewal ability until the cells reach the stage of committed progenitor – committed myeloid progenitor (CMP) & committed lymphoid progenitor (CLP) status. The differentiation of committed to mature cells represented here as a dashed arrow. MEP, megakaryocyte-erythrocyte progenitor; EP, erythrocyte progenitor; MkP, megakaryocyte progenitor; GMP, granulocyte–macrophage progenitor; GP, granulocyte progenitor; MacP, macrophage progenitor; NK, natural killer. (Taken from: Cedar and Bergman, 2011).
1.3 BM microenvironment of HSCs

The majority of HSCs reside in a specific microenvironment known as BM “niches” which are essential for life-long HSC functions. These BM microenvironments can be defined as two distinct niches which either keep the long term HSCs quiescent (osteoblastic/endosteal niche) or in an active state to promote cell proliferation and differentiation (vascular niche) (Alvarez et al., 2013). Haematopoiesis is tightly regulated by the BM microenvironment comprising of numerous cells of mesenchymal origin such as endothelial cells, fibroblasts, adipocytes, osteoblast as well as macrophages of non-mesenchymal origin (Ho et al., 2015). Within the BM niche, these stromal cells and their associated extracellular matrix (ECM) interactions are crucial in maintaining self-renewal and differentiation by secreting multiple cytokines and chemokines including haematopoietic growth factors (HGFs), interleukins or cytokines (Juarez and Bendall, 2004; Pierce and Whetton, 1997). Numerous HGFs have been described since the mid-1980s, including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), erythropoietin (EPO), interleukin-3 (IL-3), and thrombopoietin (TPO) (Bociek and Armitage, 1996).

1.4 HSCs homing and mobilisation

HSCs are not only crucial for maintaining haematopoiesis and evidence suggest they migrate within the body in response to damage, knowing how the cells migrate between BM, blood and peripheral tissues is of great interest in understanding normal and abnormal blood cell production. Migration of HSCs is based on the mechanism that enable these cells to leave the BM and enter the blood circulation (mobilisation) then relocate to a distant tissue and return back to the BM (homing) (Alvarez et al., 2013) (Figure 1.2). Homing and mobilisation are highly coordinated processes which are orchestrated by various intracellular signalling, cytokines, chemokines, proteases, and adhesion molecules, that play an essential role in numerous physiological process including wound healing, cancer metastasis, inflammation and immune response (Gazitt, 2004; Mizuno, 2013). These cellular interactions occur via a
dynamic interplay between the adhesion molecules, deposition of basement membrane or matrix protein interaction and extracellular molecules acting on specific cell surface receptors (Ananthakrishnan and Ehrlicher, 2007; Wang et al., 1999b). The fine balance between homing, mobilisation and retention of HSCs is important in normal cells, however, any slight deviation or malfunction at any step of the pathway may affect normal functions, resulting in transformation and carcinogenesis such as in leukaemia (van den Berk et al., 2014). Acquiring a motile phenotype is an important characteristic of cancerous cells. It is a critical step towards gaining metastatic competence. Thus, targeting cell motility processes will help in introducing novel therapeutic agents against metastatic and invasive tumors.

![Figure 1.2: Mechanism of HSCs homing and mobilisation in the bone marrow (Gordon, 2005).](image)

The stromal cell-derived chemokine gradients across an endothelial barrier induce stem cells to migrate from one side of the cellular barrier to the other. This stem cells trafficking process involves the homing (transendothelial migration from the bloodstream into the marrow microenvironment), and mobilisation (detachment from the microenvironment and transendothelial migration in the reverse direction). Most stromal-haematopoietic cell interactions are mediated by mutual recognition of adhesive receptors/ligands located at the surface of both HSPC and stromal cells as well as in the surrounding extracellular matrix (ECM) (Taken from: Gordon, 2005; Vermeulen et al., 1998).

1.5 CXCL12/CXCR4 interaction induces cell motility

Chemotactic cytokines or chemokines are low molecular weight proteins (8-10 kDa) that are classified accordingly to the number of the first two cysteine residues (CXC, CC, C, and CX3C) (Balkwill, 2004). Chemokines bind to a large family of seven transmembrane G-protein-coupled receptors (GPCRs) that are classified on the basis of chemokine group to which their
ligands belongs (CXCR, CCR, CX3CR, and XCR1) (Barbieri et al., 2011). Upon chemokine binding, a conformational change of the receptor occurs activating heterotrimeric G proteins: the Ga subunit exchanges GDP for GTP and dissociates from the Gβ and Gγ subunit to modulate multiple signalling transduction pathways (Teicher and Fricker, 2010). In addition, the dissociation of the Ga subunit from Gβγ (Figure 1.3), results in inhibition of adenylyl cyclase which initiates the activation of multiple signalling transduction, including ERK1/2, MAPK, JNK, Rac and AKT effectors resulting in a variety of biological response such as proliferation, survival of HPSC, and gene transcription (Alvarez et al., 2013; Lapidot and Petit, 2002; Teicher and Fricker, 2010; Whetton and Graham, 1999). The chemokine stromal derived factor-1 (SDF-1) also known as CXC chemokine ligand 12 (CXCL12) is constitutively secreted from BM stromal cells (Golan et al., 2013; Sugiyama et al., 2006). CXCL12 is a chemotactic factor for immune cells such as T-cell, monocytes, pre-B cells and haematopoietic progenitor cells and also induces CD34+ cell proliferation (Abe-Suzuki et al., 2014; Lataillade et al., 2000). In addition, numerous studies have shown that CXCL12 is also found in the central nervous system (Balkwill, 2004; Shen et al., 2014; Zhu et al., 2012). CXCL12 exerts its physiological function through binding its receptor, CXCR4 which is highly expressed by monocytes, lymphocytes, megakaryocytes, myeloid cells and CD34+ enabling these cells to migrate across a gradient of SDF-1 concentrations within the microenvironment (Aiuti et al., 1999; Burger and Kipps, 2006; Burger and Kipps, 1999; Gazitt and Akay, 2004).

CXCL12/CXCR4 sparked a substantial interest due to their role in cell trafficking and retention of HSCs within the BM niche by regulating homing and mobilisation of haematopoietic stem/progenitor cell (HSPC) (Vagima et al., 2011; Whetton and Graham, 1999). An interplay between CXCL12/CXCR4 binding is important in cell migration and chemotaxis which are accompanied by cytoskeletal rearrangements, actin polymerisation, polarisation, pseudopodia formation, and integrin-dependent adhesion to endothelial cells and other biological substrates (Sun et al., 2010). Previous research has indicated that a deficiency in the CXCL12/CXCR4 axis affects haematopoietic development (Nagasawa et al., 1996). CXCR4/CXCL12 signalling is active and found to be overexpressed in more than 23 human cancers such as ovarian cancer, breast cancer, and prostate cancer (Balkwill, 2004; Sun et al., 2003) as well as in
haematological malignancy including CML (Dürig et al., 2000; Peled et al., 2002), CLL (Burger et al., 1999), and acute leukaemias (Möhle et al., 1998; Möhle et al., 2000). A recent report suggested that the CXCR4/CXCL12 interaction is not unique as another second high affinity chemokine receptor, CXCR7 has been identified to bind with CXCL12. The CXCR7/CXCL12 interaction, however, does not couple to signalling pathways for migration (Alvarez et al., 2013). A better understanding of the processes involved in haematopoietic stem cell maintenance and trafficking are of critical importance to improve transplant efficiency and bone marrow reconstitution after transplantation. Other work has shown that sphingosine-1-phosphate (S1P) stimulates HPSC trafficking by activation of CXCR4-dependent signalling pathways via interaction with its receptor S1P receptor 3 (S1PR3) (Walter et al., 2007).

Figure 1.3: A schematic representation of the CXCL12/CXCR4 intracellular signal transduction pathways (Teicher and Fricker, 2010). CXCL12 binds to CXCR4 triggers GPCR, then a conformational change of the receptor occurs activating heterotrimeric G proteins: the Gα subunit exchanges GDP for GTP and dissociates from the Gβ and Gγ subunit via inhibition of adenyl cyclase mediated cAMP production. This modulates multiple signalling transduction pathway via PI3K/Akt, PLC/inositol triphosphate, p38 and ERK1/2 pathways thus regulating cell survival & proliferation, transcription gene expression and chemotaxis. Beta-arrestin pathway can be activated through GRK to internalise CXCR4, PLC, phospholipase C; PIP2; phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5 trisphosphate; PI3K, phosphoinositide-3 kinase; ERK1/2, extracellular regulated kinase 1/2; GRK, GPCR kinase (Taken from: Würth et al., 2014)
1.6 Sphingosine-1-Phosphate is an agonist that regulates cell motility

Bioactive sphingolipid metabolites consist of sphingosine-1-phosphate (S1P), sphingosine and ceramide and are important regulators in a number of cellular processes including cell survival, cell differentiation, proliferation, apoptosis, motility and cytoskeleton organization (Donati and Bruni, 2006; Sarkar et al., 2005; Spiegel and Milstien, 2011; Taha et al., 2006; Wang et al., 2008). S1P is predominantly generated by stimulated platelets and leukocytes, resulting in up to micromolar concentrations in normal plasma and other extracellular fluids (Graeler and Goetzl, 2002). Numerous studies have clearly shown that S1P secreted into the extracellular milieu ligate to cell surface G protein-coupled receptors subtypes known as S1P receptor 1-5 (S1PR1-S1PR5), formerly known as EDG1, 3, 5, 6, 8 and activate diverse downstream signalling pathways in both an autocrine and paracrine manner (Pitson, 2011; Spiegel and Milstien, 2011). S1P interactions have also been shown to act intracellularly as second messengers whereby their function has been reported to be independent of S1P receptors (Lee et al., 1998; Taha et al., 2006). The formation of sphingolipid mediator is a process with several potential points of regulation and modulation involving the de novo and salvage pathway (Figure 1.4) (Hannun et al., 2001; Hannun and Obeid, 2008; Pitson, 2011; Taha et al., 2006).

S1P is formed primarily by the action of sphingosine kinase (SK) that phosphorylates sphingosine to form S1P (Schnute et al., 2012). SK is a key enzyme that serves as checkpoint that regulates the levels of S1P, sphingosine and ceramide in the sphingolipid metabolic pathway (Selvam and Ogretmen, 2013; Tani et al., 2007). There are two isoforms of SK, SK1 has a mainly cytoplasmic location whilst SK2 is found in the endoplasmic reticulum (ER) or nucleus. SK1 and SK2 play a role in mediating EGF and TGFβ pathway enhanced-migration and invasion in breast cancer cells as well as in esophageal cancer cells (Hait et al., 2007; Hait et al., 2005; Miller et al., 2008). SK1 is commonly up-regulated in tumours and its inhibition has been shown to slow tumour growth which offers a novel therapeutic intervention for cancer cells (Pitman and Pitson, 2010). Interestingly, S1P’s production and S1P receptor activation are linked in an autocrine and paracrine signalling paradigm (Takabe et al., 2008).
S1P produced intracellularly can be released and then elicit autocrine and paracrine effects through activation of S1P receptors on the cell surface. For example, platelet derived growth factor (PDGF) stimulated fibroblast migration (Hobson et al., 2001; Rosenfeldt et al., 2001) and mast cell chemotaxis (Jolly et al., 2004) rely on activation of SK, production of S1P, and activation of S1P receptors. It was shown to be necessary to both make S1P and activate extracellular S1P receptors to induce the cellular responses. Indeed, studies on the balance between sphingolipid and the SK is of particular interest as disruption in this pathway may contribute to the development and progression of malignancy and lead to resistance in chemotherapy (Pitman and Pitson, 2010; Pitson et al., 2011).

![Figure 1.4: Sphingolipid metabolic pathway](image)

Figure 1.4: Sphingolipid metabolic pathway. The pathways shown are the de novo and salvage pathway entry route with formation of ceramide as a key point and bioactive sphingosine via ceramidase. (Taken from: Pitson, 2011).
1.7 The BM tumor microenvironment contributes to leukaemia

Leukaemia is defined as cancer of the blood and BM which is characterised by uncontrolled growth of leukaemic haematopoietic stem cells (LSCs) in the BM. Within the BM, LSCs interfere with the normal HSC-microenvironment ultimately giving rise to a tumor microenvironment creating a leukaemic BM niche (Colmone et al., 2008). LSCs can infiltrate these niches leading to their enhanced self-renewal and proliferation, enforced quiescence, and also resistance to chemotherapeutic agents (Lane et al., 2009). This is associated with a competitive advantage over the normal white blood cells which leads to predominance of malignant cells in the BM (Vardiman et al., 2002).

1.8 Classification of leukaemia

In 2012, approximately 350,000 people were diagnosed with leukaemia worldwide and 265,000 deaths were caused by this disease (World Cancer Report 2014). Leukaemia is the twelfth most common cancer accounting for around 2.5% of all cancers throughout UK (Cancer Research UK, 2012). In the classification of leukaemia, these diseases were traditionally divided into two major groups which is either acute or chronic. Secondly, further subclassification was through the origin of the blood cells being either of myeloid or lymphoid lineage. Leukaemia from the myeloid lineage is called myeloid leukaemia while lymphoid malignancies are known as lymphoid leukaemia. Thus, chronic and acute leukaemia were then classified as chronic myeloid leukaemia (CML) and chronic lymphocytic leukaemia (CLL), acute myeloid leukaemia (AML) and acute lymphocytic leukaemia (ALL). CLL and ALL were further divided into either bursal equivalent B or thymus derived T-cells origin (Ruccione, 1983). CML is one of the chronic myeloproliferative neoplasms (MPNs) which is the most common form of chronic leukaemia. Besides CML, there are other groups of MPNs such as polycythaemia Vera (PV), chronic idiopathic myelofibrosis (MF), essential thrombocythemia (ET), chronic neutrophilic leukaemia (CNL), chronic eosinophilic leukaemia (CEL), chronic myelomonocytic leukaemia (CMML) and juvenile chronic myeloid leukaemia (JCML) (Harris et al., 1999, 2000; Tefferi et al., 2009).
1.9 Myeloproliferative neoplasms and related diseases

MPNs are clonal haematological that are characterised by overproduction of mature blood cells due to haematopoietic stem cell transformation (Akpinar et al., 2013). According to WHO, 2008, MPNs are classified into classic (Philadelphia positive MPN) and non-classic (Philadelphia negative MPN) disease as summarise in Table 1.1 (Azzato and Bagg, 2015; Tefferi et al., 2009). CML is a MPN that is characterised by the presence of the Philadelphia (Ph) chromosome (BCR/ABL fusion gene) whilst Ph-negative MPNs are polycythaemia vera (PV) (characterised by an increase in the erythrocyte mass in PV), essential thrombocythaemia (ET) (an increase in the platelet number), and primary (idiopathic) myelofibrosis (MF) (advanced fibrosis in the bone marrow) (Pikman et al., 2006; Tefferi, 2010).

Recent studies have demonstrated that calreticulin (CALR) mutations are observed in JAK2 and MPL unmutated primary myelofibrosis (MF) and essential thrombocythaemia (Cazzola and Kralovics, 2014; Lavi, 2014). CALR is a highly conserved protein with pleiotropic roles related to its distribution in the endoplasmic reticulum and cytosol. The CALR gene is located on chromosome 19p13.2 and contains 9 exons and spans 4.2 kb (Tefferi et al., 2014). All CALR mutations seen in MPN involve exon 9 frameshift mutations and are found in 50–80% of JAK2/MPL unmutated ET and MF. These mutations are either somatic insertions or deletions (Ha and Kim, 2015; Klampfl et al., 2013; Nangalia et al., 2013; Tefferi et al., 2014). More than 80% of the CALR mutations are categorised as type 1 (L367fs*46) due to a 52 bp deletion which is the most frequent in MF whilst type 2 (K385fs*47) are the result of a 5-bp TTGTC insertion (Klampfl et al., 2013; Nangalia et al., 2013; Tefferi et al., 2014). These mutations lead to an alteration of the C-terminal of the protein resulting in the loss of an endoplasmic reticulum retention motif, which appears to activate the STAT5 signalling pathway (Klampfl et al., 2013; Malcovati et al., 2014). In addition, the abnormal expression of the CALR gene has also been found in various cancers such as pancreatic and ovarian cancer (Sheng et al., 2014; Vaksman et al., 2013).
Table 1.1: Features of myeloproliferative neoplasms diseases

<table>
<thead>
<tr>
<th>Entity</th>
<th>Clinical features</th>
<th>Histopathological features</th>
<th>Common molecular aberration(s)</th>
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<tbody>
<tr>
<td>Chronic myelogenous leukaemia (CML)</td>
<td>Peripheral blood leukocytosis (12–1000 × 10⁹/L) consisting of neutrophils in various stages of development, often with a myelocyte bulge; platelet count may be normal or increased (chronic phase)</td>
<td>Increased bone marrow cellularity due to granulocytic proliferation, prominent eosinophils, dwarf megakaryocytes, and occasional moderate to marked reticulin fibrosis (chronic phase)</td>
<td>BCR/ABL1 fusion</td>
</tr>
<tr>
<td>Polycythemia vera</td>
<td>Haemoglobin &gt;18.5 g/dL (men) &gt;16.5 g/dL (women), subnormal serum erythropoietin level</td>
<td>Bone marrow myeloproliferation JAK2 exon 12 mutation</td>
<td>JAK2 V617F JAK2 K539L</td>
</tr>
<tr>
<td>Essential thrombocythemia</td>
<td>Platelet count ≥ 450 × 10⁹/L</td>
<td>Bone marrow megakaryocyte proliferation with large and mature morphology JAK2 V617F MPL exon 10 mutations (MPL W515L/K) CALR exon 9 indels</td>
<td></td>
</tr>
<tr>
<td>Primary myelofibrosis</td>
<td>Anemia, leukoerythroblastosis, increased serum LDH</td>
<td>Bone marrow megakaryocyte proliferation and atypia with either reticulin and/or collagen fibrosis JAK2 V617F MPL W515K/L CALR exon 9 indels</td>
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(Taken from: Azzato and Bagg, 2015).
1.9.1 Chronic myeloid leukaemia- Ph positive MPN

CML is a neoplastic haematopoietic stem cell disease, the cytogenetic hallmark of which is the Philadelphia (Ph) chromosome. This Ph abnormality was first described by Nowell and Hungerford in 1960 as a shortened chromosome 22. Then, Janet Rowley in 1973 identified this as reciprocal translocation between the long arm of chromosome 9 containing the c-Abelson leukaemia virus (c-ABL) protein tyrosine kinase (PTK) and the breakpoint cluster region (BCR) on chromosome 22. This translocation results in the additional of a 3' portion of c-ABL gene from chromosome 9q34 to the 5' of BCR gene region of 22q11 (Rowley, 1973).

The ABL gene encodes a non-receptor PTK with a molecular mass 145kd (p145<sub>ABL</sub>) while BCR gene encodes a protein of 160 kd (p160<sub>BCR</sub>) (Kurzrock and Talpaz, 1991). The reciprocal translocation t(9:22)(q34;q11) of CML produces several hybrid fusion proteins ranging from 190 to 230 kd dependant upon the exact site of the breakpoint in the BCR region (Kurzrock and Talpaz, 1991). Numerous studies have shown that more than 90% of CML patients express the p210 BCR/ABL fusion protein (Figure 1.5) and 5-10% in cases of acute myeloid leukaemia (Cortes et al., 1996; Kurzrock and Talpaz, 1991).

![Figure 1.5: p210 BCR/ABL fusion protein in CML.](image)

Meanwhile the p190/p185<sub>BCR/ABL</sub> fusion protein, containing BCR sequence from exon 1 fused to exon 2-11 of c-ABL, is seen in 10% cases of acute lymphoid leukaemia (Kurzrock and Talpaz, 1991). The largest 230-kd (p230<sub>BCR/ABL</sub>) is associated with Ph-positive chronic neutrophilic leukaemia variant and thrombocytosis. These chimeric fusion proteins are
constitutively activated cytoplasmic tyrosine kinases with an activity several fold higher than normal c-ABL (Kurzrock and Talpaz, 1991). This BCR/ABL fusion protein results in activation of multiple signal-transduction cascades such as RAF, RAS, PI3K, MYC and STAT, causing abnormalities in growth and differentiation of cells observed in CML (Al-Achkar et al., 2013; Passegué et al., 2003; Sawyers, 1999). Following understanding in CML Philadelphia positive event, there was another oncogenic tyrosine kinase discovered that could be involved in molecular pathogenesis of Philadelphia negative myeloproliferative neoplasm (Akpinar et al., 2013; Pardanani et al., 2006).

1.9.2 MPL W515L-Ph negative MPN

The thrombopoietin receptor (TpoR) and its ligand thrombopoietin plays a role in the maintenance of haematopoietic stem cells and is a major regulator of platelet production, the critical cell in blood coagulation. Sequence analysis of the MPL gene coding for TpoR led to discovery of a new molecular abnormality of JAK2 mutation-Ph negative MPN (Akpinar et al., 2013; Pardanani et al., 2006). The myeloproliferative leukaemia oncogene (c-MPL) is expressed on haematopoietic stem cells, immature haematopoietic progenitor cells, megakaryocytes and platelets (Debili et al., 1995). c-MPL encodes a 635 amino acid protein made up of two extracellular cytokine receptor domains and an intracellular domain. The extracellular domains contains a 200 amino acid motif displays 4 conserved cysteines and a WSXWS or Trp-Ser-X-trp-Ser near its carboxyl end (Ihle et al., 1998; Souyri et al., 1990). The major cytoplasmic domain is MPL-P encodes for 122 amino acids while MPL-K is a minor 66 amino acid in length (Cosman et al., 1990; Mignotte et al., 1994). The intra-cytoplasmic domain is composed of 121 amino acids, including two short, membrane-proximal motifs (box1 and box2), but displays no consensus regions for either protein kinase activity or enzymatic motif. The Box 1 located between residues 17 and 20 are spatially conserved with proline (PXXP) among most members of the haematopoietic cytokine receptor superfamily whereas box 2 at residues 46 and 64 which contain rich-serine and glutamic acid (Drachman and Kaushansky, 1997). TpoR contains an amphipathic (molecule that possesses both hydrophobic and hydrophilic elements) RWQFP motif that separate the transmembrane (TM) and cytoplasmic domains which functions in maintaining the receptor in inactive conformation
in the absence of ligand. However, mutations in the TpoR can cause JAK-STAT activation in the absence of ligand. The most common mutations concerned the W515 residue which was substituted either tryptophan for a leucine W515L, a lysine W515K or an asparagine W515A (Staerk et al., 2006) in MF patients. MPL W515L is a somatic mutation (a G to T transition at nucleotide 1544) resulting in substitution of tryptophan to leucine at codon 515 of the transmembrane region. MPL, like JAK2 also displays a docking site for SH2 proteins allowing STAT binding. The MPL W515L mutation results in constitutive activation of JAK-STAT signalling via homodimerization of type I cytokine receptor of thrombopoietin receptor (MPL) increased transcription activity and ultimately MPN (Pikman et al., 2006) (Figure 1.6). This MPL W515L is found in a significant proportion of JAK2 V617F-negative patients with essential thrombocytosis and myelofibrosis with myeloid metaplasia (MMM) due to increase in production of megakaryocytic lineage cells (Campbell and Green, 2006; Pardanani et al., 2006).

Figure 1.6: JAK-STAT signalling pathway induced by thrombopoietin (TPO) receptor and activation in the absence of ligand by MPL W515L and JAK2 mutation. Upon binding of TPO to the MPL receptor JAK2 undergoes phosphorylation which leads to the creation of docking sites for SH2 receptor containing proteins. This enables the activation of STAT. The activated STATs then translocate to the nucleus and activate gene transcription. However, in MPL W515L, JAK2 V617F and JAK2 K539L, the JAK-STAT pathway is constitutively activated in the absence of ligand leading to increased transcription activity and ultimately MPN. Normally, JAK2 signalling is negatively regulated by SOCS proteins, most notably SOCS1 and SOCS3. LNK also serves as a negative regulator by inhibiting JAK-STAT signalling. Cbl proteins function as multifunctional adaptor proteins and ubiquitin ligases that are involved in trafficking and degradation of tyrosine kinases. SOCS; suppressor of cytokine signalling, LNK; lymphocyte adaptor protein. (Taken from: Schmidt et al., 2012).
1.9.3 JAK2 V617F-Ph negative MPN

JAK2 belongs to the Janus kinase (JAKs) family of non receptor tyrosine kinase, which play an important role in haematopoiesis and immune responses. Mutations in JAK2 consequently have an effects on haematopoiesis, resulting in alteration in proliferation of haematopoietic cell lineages. The JAK family members consists of seven conserved regions denoted JAK homolog (JH) domains 1-7: JH1 and JH2 at the C terminal constitute the kinase and pseudokinase domains respectively. Next, the JH3 and JH4 domains show homology to SH2 domains but their function remains unclear. The amino terminal JH5-JH7 domains have a FERM-like motif are important for the interaction of JAK2 receptors-binding module (Figure 1.7) (Dusa et al., 2010; Yamaoka et al., 2004).

![Figure 1.7: The structure of Janus kinase.](image)

The JAK2 V617F mutation was first described in 2005 as the major underlying molecular event of BCR/ABL negative MPN (James et al., 2005; Kralovics et al., 2005). The JAK2 V617F acquired somatic mutation involves exon 14, which encodes part of the JH2 auto-inhibitory pseudokinase located on chromosome 9p24. This mutation presents as a substitution of valine (V) for phenylalanine (F) at amino acid position 617 (Levine et al., 2005) which consequently disrupts the autoinhibitory function of JAK2. This leads to constitutive activation of JAK-STAT downstream signalling pathway causing an increase in transcription factor activity and cellular growth (Baxter et al., 2005; James et al., 2005; Lu et al., 2005). The JAK2 V617F mutation is observed in approximately 95% of patients with PV, 50% in ET and MF respectively and none of those with CML (Baxter et al., 2005; Dameshek, 1951). This suggests that JAK2 V617F mutant play a role in the pathogenesis of PV (Haan et al., 2009; James et al., 2005).
1.9.4  JAK2 K539L-Ph negative MPN

A gain of function mutation of JAK2 was identified in patients with myeloproliferative neoplasms. However, there were some cases of PV or idiopathic erythrocytosis identified that were negative for the JAK2 V617F mutation. So, to understand this phenomenon, Scott et al carried out a study on samples not mutated at V617 in patients diagnosed with PV or idiopathic erythrocytosis (Scott et al., 2007). Their work identified a somatic JAK2 K539L mutation of JAK2 in exon 12 in JAK2 (V617F)-negative patients (Scott et al., 2007). This mutation is due to substitution of mutation at position 539 from lysine to leucine in exon 12 of the JAK2 gene located between the pseudokinase and SH2-like domain (Pardanani et al., 2007). Then, Pietra et al. observed that JAK2 exon 12 mutations involved tandem point mutation, missense and in frame deletions in the hotspot residues between 537-547 (Pietra et al., 2008). The most frequent exon 12 mutations are del/N542-E543, delF537-K539ins/L, K539L and H538QK539L (Figure 1.8).

![Diagram of JAK2 exon 12 mutations](image)

**Figure 1.8: JAK2 exon 12 mutations.** The exon 12 mutations involved tandem point mutation, missense and in frame deletions in the hotspots residues between 537-547 (Taken from: Pietra et al., 2008).
In fact, murine retroviral transplantation model studies demonstrate that mutations in exon 12 results in a PV like phenotype with stimulation of erythroid proliferation. Schnittger et al. suggests that there is a 9% mutation rate of exon 12 in patients with erythrocytosis similar to Williams et al. 2007 (Schnittger et al., 2009). Most frequent were the N542-E543del, H538QK539L, and F537-K539delinsL subtypes with previously described in cases carrying the hotspots exon 12 mutation (Scott et al., 2007 and Pietra D et al.,2008).

1.10 Current treatment strategies for MPN diseases

Activation of the JAK-STAT pathway by driver mutations in BCR/ABL, JAK2 or MPL are the pathogenesis of MPN. CML is a clonal neoplastic disorder of haematopoietic stem cells characterised by Ph positive MPN. Expression of BCR/ABL fusion gene is sufficient to cause chronic phase (CP) CML, followed by more or less rapid progression through an accelerated phase (AP) to blast crisis (BC). Imatinib mesylate, IM (Glivec), was the first available BCR/ABL-targeted therapy that produced complete cytogenetic responses in 70–85% of patients with CML in the early chronic phase (CP) (Druker et al., 2006). IM inhibits the BCR/ABL tyrosine kinase through competitive inhibiton at the adenosine triphosphate binding site of the enzyme, leading to the inhibition of tyrosine phosphorylation of downstream targets ultimately blocking the tumour growth by inducing apoptosis (Azzato and Bagg, 2015; Vlahovic and Crawford, 2003). However, despite the remarkable efficacy of this agent, IM does not completely eradicate residual leukaemic stem cells and progenitors (Graham SM et al.,2002).

At present intensive research is aimed to further understand the molecular pathogenesis of CML and the resistance to IM in CML patients. Currently, a variety of 2nd and 3rd generation TKIs (such as dasatinib) is available for treatment of CML which reduces the leukaemic disease burden in CML (Azzato and Bagg, 2015; Henkes et al., 2008).

The current treatment strategies for Ph negative MPNs are associated with relieving of the symptom burdens and pathology of the disease. Numerous clinical studies testing various JAK2-inhibitors in PV, ET as well as in primary and secondary myelofibrosis (MF) are under way (Stein et al., 2015; Geyer & Messa, 2014; Wolf et al., 2011). However among PV, ET and
MF, MF carries the most adverse prognosis impart due to their molecular complexity (Stein et al., 2014). Therapies for MF have included cytoreductive agents such as hydroxyurea, however such agents only treat the symptoms of MF and splenomegaly that result in transient response and myelosuppression. The process of leukaemogenesis involves the activation of PTKs in a variety of different diseases including the MPN. PTK inhibitors have offered advances in managing PTK-associated leukaemias but there is still a need for new insights to develop new treatment and curative strategies. JAK2 inhibitors alone such as ruxolitinib or in combination with other MF-related therapies have entered clinical trial. However there are limitations associated with the use of ruxolitinib as well as other JAK2 inhibitors (for instance; momelotinib), which are now in clinical development, as they may not sufficiently improve the cytopenias or give resolution of bone marow fibrosis (Mascarenhas et al., 2014; Odenike, 2013; Stein et al., 2015).

Besides JAK2 and MPL mutations calreticulin (CARL) mutations have been identified in MF patients and are sensitive to JAK inhibition (Klampfl et al., 2013; Lavi, 2014; Malcovati et al., 2014). The discovery of CALR in MF patients offers new therapeutic target in MPL W515L. In adddition, studies have shown that chromatin modifying agents or epigenetic modulators in MF are associated with epigenetic deregulation of EZH2, SOCS, IDH1/IDH2 could offer therapeutic value in MPN (Stein et al., 2015; Stein et al., 2014; Vannucchi et al., 2013). Despite the recent advances in diagnosis and clinical treatment in MF, management of MPN patients also requires an individual approach that incorporates prognosis and disease burden (Odenike, 2013) since current treatment is not sufficient to reduce disease burden in MF (Wolf et al., 2011). Further study is needed to improve the understanding of the pathogenetic mechanisms driving this MPN to ultimately address remaining unmet clinical needs. Combinations of such targeted therapies with new drug and specifically by targeting pathways of potential importance in the pathogenesis of MPN may hold effective therapeutic value in patients. It is hoped that MPN patients especially myelofibrosis MPL W515L will soon achieve a better control of their disease.
MYC was first discovered as an oncogene in leukaemia and sarcoma of chickens (Sheiness and Bishop, 1979). c-MYC is located at chromosome 8q24.21, a gene that was discovered by translocation of t(8;14) in Burkitt's lymphoma (BL) (Delgado and León, 2010; Nowell and Croce, 1990; Nowell et al., 1984; Wilda et al., 2004). MYC protein (c-MYC, MYCN and L-MYC) has an ability to regulate activities such as cell cycle, growth and metabolism, differentiation, apoptosis transformation and genomic instability (Albihn et al., 2010). c-MYC is an immediate early response gene for many ligands and their activity is regulated at multiple levels through transcriptional, posttranscriptional, translational, and posttranslational mechanisms. MYC is downstream of various signal transduction pathways such as Sonic hedgehog, receptor tyrosine kinase, and transforming growth factor beta (TGFβ) that are activated both during normal development and in cancer. In addition, c-MYC also has been implicated in the pathogenesis of multiple myeloma, colon (Yang et al., 1996), prostate (Nupponen et al., 1998) and breast cancer (Pertschuk et al., 1993) either by elevated levels of MYC expression or genetic alterations such as translocation, gene amplifications and mutations which directly affects MYC expression (Hermeking, 2003; Vita and Henriksson, 2006; Wang et al., 2007; Wilda et al., 2004) (Table 1.2). Accumulating evidence shows that c-MYC is one of the transcription factors playing a major role in haematopoiesis. The study of genetically modified mice with either overexpression or deletion of MYC has shown that c-MYC is required for the correct balance between self-renewal and differentiation of haematopoietic stem cells (HSCs). For instance studies on c-MYC knockout mice results in pancytopenia and rapid lethality (Laurenti et al., 2008). In addition, JAK2 mediates the increase in c-MYC mRNA expression induced by BCR/ABL and it also interferes with proteasome-dependent degradation of c-MYC protein (Xie et al., 2002). Studies have also shown that JAK2 activated following transcriptional activation of Htert (telomerase catalytic subunit) induced MYC mRNA overexpression in myeloproliferative neoplasm from essential thrombocythemia (Theophiile et al., 2008).
Table 1.2: MYC alterations which induce haematological malignancy

<table>
<thead>
<tr>
<th>Cancer</th>
<th>MYC involvement</th>
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</thead>
<tbody>
<tr>
<td><strong>Myeloid neoplasms</strong></td>
<td></td>
</tr>
<tr>
<td>Acute myeloid leukaemia (AML)</td>
<td>MYC mRNA overexpression (20-24%)</td>
</tr>
<tr>
<td>Essential thrombocythemia</td>
<td>MYC mRNA overexpression</td>
</tr>
<tr>
<td>Chronic myeloid leukaemia</td>
<td>MYC mRNA overexpression</td>
</tr>
<tr>
<td><strong>Lymphoid neoplasms</strong></td>
<td></td>
</tr>
<tr>
<td>Burkitt Lymphoma</td>
<td>MYC translocation and overexpression (&gt;90%)</td>
</tr>
<tr>
<td>Diffuse Large B-cell lymphoma (DLBCL)</td>
<td>MYC translocation (6-16%)</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>MYC translocation (15-50%)</td>
</tr>
<tr>
<td>Chronic lymphocytic leukaemia</td>
<td>Low c-MYC expression</td>
</tr>
<tr>
<td>Plasma cell leukaemia</td>
<td>MYC translocation (13%)</td>
</tr>
<tr>
<td>B-ALL</td>
<td>MYC rearrangement (47-52%)</td>
</tr>
<tr>
<td><strong>Breast cancer</strong></td>
<td>MYC overexpression (56%)</td>
</tr>
<tr>
<td><strong>Colon cancer</strong></td>
<td>MYC overexpression</td>
</tr>
<tr>
<td><strong>Prostate cancer</strong></td>
<td>MYC overexpression (29%)</td>
</tr>
</tbody>
</table>

(Taken from: Delgado and León, 2010).

1.12 Transforming growth factor beta (TGFβ) and myelofibrosis

TGFβ is secreted from a large inactive precursor molecule known as latent TGFβ (inactive form), which is activated by proteolytic activity (such as plasmin), to exhibit its biological effects (Jean-Jacques Lebrun, 2012; Koli et al., 2001; Saharinen et al., 1999). The TGFβ families consists of TGFβ, bone morphogenetic protein (BMP), activins and other related proteins (Katz et al., 2013; Mishra et al., 2005). The activation of TGFβ signalling pathways begin with the binding of TGFβ ligand to a TβR receptor I and II (serine/threonine kinase) which allows receptor II to phosphorylate the receptor I kinase domain, which then propagates the signal through phosphorylation of the Smad proteins (Figure 1.9) (Derynck and Zhang, 2003; Shi and Massagué, 2003). Then, activated R-Smads 2/3 binds Smad4 assembles to form heteromeric complexes leading to transcriptional activation and/or repression complexes to control the targeted gene expression. The inhibitory Smads; Smad6 and Smad7, negatively regulate TGFβ signalling by competing with R-Smads for receptor or Co-Smad interaction and by targeting the receptors for degradation (Derynck and Feng, 1997; Derynck and Zhang, 2003).
Figure 1.9: TGFβ signalling pathway. Schematic diagram depicting the canonical TGFβ SMAD-dependent signalling pathway and SMAD-independent; non-canonical signalling pathway of p38/JNK, mitogen-activated protein kinase (MAPK), extracellular-signal-regulated kinases (ERK), phosphoinositide 3-kinase (PI3K/Akt), and RHO/GTPase. Upon activation of the TGFβ ligand binds to the TGFβ receptor which induces the phosphorylation of Smad2/3 Smad4 complex results in various biological responses within the targeted cells. The inhibitory Smads; Smad6 and Smad7, negatively regulate TGFβ signalling by competing with R-Smads for receptor or Co-Smad interaction for degradation. TF; transcription factor. EMT; epithelial-mesenchymal transition. (Taken from: Derynck and Zhang, 2003; Shi and Massagué, 2003).

Although the Smads are responsible for most of the TGFβ response, the activated TGFβ receptor complex also transduces a signal through other non-Smad pathway via p38/JNK (Bakin et al., 2002), mitogen-activated protein kinase (MAPK) (Lee et al., 2007), PI3K/Akt, and RHO/GTPase. This activation leading to various biological responses such as proliferation, differentiation, and migration (Figure 1.9) (Massagué, 2008, 2012; Massagué and Xi, 2012 and Edlund et al., 2002).) TGFβ has been shown to induce migration of different cell types such as epithelial cells (Boland et al., 1996), and mast cells (Gruber et al., 1994; Olsson et al., 2000) which may contribute to tumor invasiveness and metastasis. TGFβ and megakaryocytes are involved in the pathogenesis of myelofibrosis via increased TGFβ secretion and abnormal megakaryocyte expression (Schmitt et al., 2000; Terui et al., 1990). This myelofibrosis is associated with the interaction between megakaryocytes, monocytes, fibroblasts, endothelial cells and bone marrow stromal cells (Ciurea et al., 2007; Mascarenhas et al., 2014). Myelofibrosis was reported and characterised by abnormal CD34+ cell trafficking resulting from
constitutive mobilisation of CD34+ cells (Barosi G et al., 2001). Furthermore, this constitutive mobilisation was also observed due to BM-repopulating cells that belongs to a malignant clone (Xu et al., 2005a). Further studies on the interaction between MPN disease targeting TGFβ via small molecular inhibitors offers a potentially better understanding of the complexity and possibly offer a therapeutic strategy in myelofibrosis.

1.13 THOC5/Fms-interacting protein (FMIP) activation downstream of leukaemogenic PTK

Our group has previously published that THOC5 is a phospho target of many leukaemogenic oncogenes such as in NPM/ALK and TEL/PDGFR (Pierce et al., 2008). THOC5 phosphorylation is increased in cells from CML patients (Griaud et al., 2013). The THO complex (suppressors of the transcriptional defects of hpr1 delta by overexpression complex, THOC), is a multi-protein complex that is important in that it promotes coupling between transcription and mRNA processing (Rondón et al., 2010). The THOC is part of the TRanscription EXport (TREX) that is conserved from yeast Saccharomyces cerevisiae to human as a five protein complex (Tho2p, HPR1p, TEX, Mft1P and Thp2p) (Figure 1.10) which plays role in transcription elongation, nuclear RNA export and genome stability (Katahira et al., 2009; Reed and Cheng, 2005). In higher eukaryotes such as humans and Drosophila melanogaster, three extra proteins and an additional unique protein were identified. THOC4/ALY REF, THOC5/FMIP, THOC6, and THOC7 as new members of THO complex (Masuda et al., 2005; Tamura et al., 1999). THOC5 was first discovered in yeast 2 hybrid assays by Tamura et al, (1999) as a substrate and binding partner for the Macrophage Colony Stimulating Factor receptor (M-CSF) tyrosine kinase (FMS) and named FMS interacting protein (FMIP) (Tamura et al., 1999). THOC5 is phosphorylated by extracellular stimuli. For instance, THOC5 is phosphorylated by protein kinase C which results in THOC5 nuclear/cytoplasmic shuttling (Mancini et al., 2004). Furthermore, THOC5 is also phosphorylated at threonine 328 following insulin stimulation during adipocyte differentiation (Gridley et al., 2005) and DNA damage leads to THOC5 phosphorylation by ATM kinase at serine 307/312/314 (Matsuoka et al., 2007). All the above studies are suggest that
extracellular stimulation regulates the function of THOC5 via its phosphorylation (Figure 1.11) (Guria et al., 2011). THOC5 has been reported to have a role in granulocyte/macrophage as well as adipocyte differentiation (Jimeno and Aguilera, 2010; Mancini et al., 2010).

Figure 1.10: THO complex structure. a) Saccharomyces cerevisiae consists of five protein complex (Tho2p, HPR1p, TEX, Mft1p and Thp2p) and b) higher eukaryotes human and Drosophila melanogaster with the addition of THOC5/FMIP, THOC4, THOC6 and THOC7 subunit.

MOUSE/HUMAN THOC5 (683 AA)

<table>
<thead>
<tr>
<th>NLS (7-10)</th>
<th>WWB (265-269)</th>
<th>PEST (303-319)</th>
<th>LZ (620-636)</th>
<th>683</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61</td>
<td></td>
<td></td>
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pS5, pS6  
pY225  
pS307/312/314  
pT328

mRNA complex formation  
THOC7 binding site  
THOC1 complex formation

Figure 1.11: Schematic domain structure and phosphorylation sites of THOC5. Arrangement of potential functional domains in THOC5. NLS: nuclear localisation signal; WWB: potential binding site for proteins with WW-domains; PEST: potential PEST domain; LZ: potential leucine zipper; p: phosphorylation sites. Numbers represent amino acid residue numbers of mouse THOC5. (Taken from: Tran et al., 2014a).
THOC5 is also involved in transcription factor expression regulation such as CCAAT/enhancer binding protein (C/EBPs), MYC and Hoxb3 (Carney et al., 2009; Tamura et al., 1999). Further work has indicated that THOC5 is required for development and maintenance of normal haematopoiesis in mammals (Mancini et al., 2007; Mancini et al., 2004). In addition, studies in a murine conditional THOC5 knockout model showed that THOC5 deletion led to rapid haematopoietic failure (Mancini et al., 2010). Tamura et al, (1999) have shown that THOC5 shuttle between the cytosol and nucleus raising the possibility that THOC5 could be critical in linking external cell signalling input to mRNA metabolism. Further to this, our group found that THOC5 phosphorylation is a cytosolic event (Griaud et al., 2013; Pierce et al., 2008b) suggesting that THOC5 involved in cancer development. In addition, THOC5 Y225 is elevated in CD34+ cell population from patients with chronic myeloid leukaemia (CML) (Griaud et al., 2013). Thus, there is possibility these oncogenes modulate mRNA processing contributes to the pathogenesis of leukaemia. Recently, THOC5 has been reported to play an important roles in the response to intrinsic and extrinsic signals of immediate early genes (IEGs) such as MYC, and SOX9 involved in cell proliferation and differentiation in mouse embryonic fibroblast (MEF) systems (Saran et al., 2013; Tran et al., 2014a; Tran et al., 2013). In addition, THOC5 has been found to interact with polyadenylation specific factor 100 (CPSF100) of the target genes, however, how this mechanism works still needs further studies (Tran et al., 2013; Tran et al., 2014b).
1.14 Approaches to proteomics studies

Proteomics is an experimental approach which can be used for the large-scale study of expressed proteins (proteome) in a cell or tissue of organisms. The proteome is the entire complement of proteins encoded by the genomes and mRNA in which the level of expression, state of modification, sub-cellular localization and protein abundance depends on the physiological state of the cell or tissue (Aebersold and Goodlett, 2001; Cristea et al., 2004). Proteomics is complementary to other functional genomic approaches including microarray-based studies, systematic genetics etc. The integration of these datasets and availability of genomic DNA databases provide a source of references for identifying proteins expressed which now makes proteomics a realistic proposition for studies in biology (Larance and Lamond, 2015; Tyers and Mann, 2003). The proteome is more difficult to study than the genome or transcriptome (the study of mRNA transcripts instead of proteins) perhaps due to limited sensitivity of protein expression analysis, since unlike DNA or RNA, amplification of protein, cannot be achieved. Proteomics allows not just study of the proteins in the given cells but also the protein isoforms, protein interaction and protein modifications (Tyers and Mann, 2003). Thus proteomics research has advantages over transcriptomic analysis by defining the cells functional entities and their modifications as well as their sub-cellular localization (Unwin et al., 2006a; Unwin et al., 2005a; Unwin and Whetton, 2006). Traditionally, protein identification was performed by de novo peptide sequencing through Edman degradation method (Steen and Mann, 2004).

Tremendous progress has been made over the years in generating large scale datasets for protein-protein interactions, protein profiles and protein activity in cancer patients (Tyers and Mann, 2003). For instance, post translational modifications (PTMs) are reported in regulated biological processes in a cell (Witze et al., 2007; Eyrich et al., 2011). There are many forms of PTM modulated differences. This includes leukaemias where aberrant protein phosphorylation events are catalysed by oncogenic PTK (Pierce et al., 2008a; Pierce et al., 2008b). Relative quantification proteomics using higher sensitivity mass spectrometry techniques now offers the potential to compare and contrast the effects of leukemogenic PTKs.
in cancer studies (Pierce et al., 2008b; Pierce et al., 2008c). In this study, I used an isobaric tagging fo relative amino acid quantification (iTRAQ) method to identify the global proteomic effects that could contribute to motility in MPN associated oncogenes MPL W515L.

1.15 Cell line models for leukaemogenic protein tyrosine kinases studies

The oncogenic potential of each leukaemogenic PTK described above was assessed by the ability to transform growth factor-dependent cells such as Ba/F3 cell lines (Levine et al., 2005; Pikman et al., 2006). Ba/F3 cells are haemopoietic suspension cells that were purified from bone marrow of BALB/c mice which possess the ability for self-renewal and proliferation in-vitro in response to murine interleukin-3 (Palacios and Steinmetz, 1985). Ba/F3 cell lines used in this study were retrovirally transfected with the oncogenes such as MPL W515L, whilst JAK2 wild type (WT) has been transfected with JAK2 V617F and JAK2 K539L to allow comparison of oncogenes effects (Pierce et al., 2008a; Pierce et al., 2008b).
1.16 Aims and Objectives

As previously outlined, activation of the JAK-STAT pathway by driver mutations in BCR/ABL, JAK2 or MPL are key in the pathogenesis of MPN. MPL W515L is a somatic mutation (a G to T transition at nucleotide 1544) resulting in substitution of tryptophan to leucine at codon 515 of the transmembrane region. The MPL W515L mutation results in essential thrombocytosis and myelofibrosis in patients (Campbell and Green, 2006; Pardanani et al., 2006). Current treatment strategies for myelofibrosis are underway using JAK2 inhibitors alone such as ruxolitinib or in combination with other myelofibrosis related therapies. However there are limitations associated with the use of ruxolitinib as well as other JAK2 inhibitors (such as momelotinib), as they may not sufficiently improve the cytopenias or bone marow fibrosis. Here I plan to study the effects of the MPL W515L oncogene to understand its role in leukaemogenesis and myelofibrosis. This will be achieved by using discovery methods such as proteomics and also genomics to understand which of the genes or gene products are disrupted in leukaemic transformation in MPL W515L expressing cells via THOC5. Combinations of such target identification with targeted therapies with new drugs and specifically by targeting pathways of importance in the pathogenesis of MPN may hold effective therapeutic value in patients.

Therefore, the objectives of this study were:

- To identify and gain insight into which pathways disrupted in the myeloproliferative neoplasms (MPNs) via THOC5 in an attempt to develop novel therapeutic strategies.

- To understand and gain insight into the function of THOC5 Y225 phosphorylation in myeloproliferative neoplasms (MPNs) via its role in motility.
Chapter 2

Materials and Methods
Chapter 2

Materials and Methods

2.1 Cell lines and culture conditions

Ba/F3 cells expressing leukaemic oncogenes (MPL W515L, JAK2 K539L, JAK2 V617F) were cultured in the absence of interleukin-3 (IL-3) in RPMI media containing 10% (v/v) horse serum (Invitrogen, Paisley, UK) and 2% (v/v) L-Glutamine (Invitrogen, Paisley, UK). Ba/F3-MSCV control cells were grown in the above medium with the addition of 5% (v/v) murine-IL-3 condition medium while Ba/F3 JAK2-wild type expressing cells were grown in the presence of erythropoietin (1 µL/mL). The routine maintenance of the cell lines was performed every two days by subculture to 1x10^5 cells/mL when they reached a cell density of approximately 1x10^6 cells/mL. The cells were then incubated at 37°C in a humidified 5% (v/v) CO₂ incubator. Cells used for experiments were in mid-log growth phase. Murine IL-3 was produced by X63-Ag-653 cells (Karasuyama & Melchers, 1988) grown in Gibco® McCoy’s 5A medium (Invitrogen, Paisley, UK).

2.2 Whole cell lysate preparation

Cells were pelleted by centrifuge at 800 RCF for 5 min at 4°C and washed twice using ice-cold phosphate buffered saline (PBS) prior to snap freezing in liquid nitrogen. Cell pellets were stored at -80°C. Pellets were lysed by resuspending (50 µL per 10^6 cells) in ice-cold radio immune precipitation assay (RIPA) buffer [Tris-Hcl 50 mM pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1mM EDTA] supplemented with freshly added cocktail of phosphatase inhibitor II & III, protease inhibitor (10 µL/mL) and sodium orthovanadate (0.2 mM) (Sigma-Aldrich Chemie GmBH, Steinheim, Germany). The mixture was vortexed and incubated on ice for 30 min. Finally, the lysates were vortexed for 30s and insoluble material removed by centrifugation at 4°C in a microcentrifuge at 14,000 RCF for 10 min. Supernatants were transferred into new eppendorf tubes and kept in -80°C prior to protein concentration determination using the Bradford protein assay.
2.3 Protein assay

The protein concentration of cell lysates was quantified using a Bradford Protein Assay (Bio-Rad laboratories Ltd, Herts,UK) in a 96-well plate format. Bovine serum albumin (BSA) (Sigma–Aldrich, Dorset, UK) was used to produce a standard curve ranging from 0 to 3.5 µg of proteins per well (samples in triplicate in a volume of 80 µL). For sample concentration determination, cell lysates were diluted ten and twenty fold to ensure values were on the standard curve. Sample concentration was determined in triplicate by adding 5 µL diluted sample to 75 µL of distilled water in a 96 well plate. 20 µL of Bradford reagent were then added to each well and samples mixed evenly avoiding generation of bubbles. Absorbance was measured on a spectrophotometer at a wavelength of 620nm and reference at 450nm (Lab systems Affinity Sensors, Cambridge, UK). The protein concentration was determined with reference to the standard curve.

2.4 Western blotting

2.4.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel preparation

Both stacking and separating solutions were prepared using Protogel™ which contains 0.8% (w/v) Bis/30% (w/v) Acrylamide (Flowgen Bioscience, Nottingham, UK). Polymerisation of the gels was initiated by addition of N,’N,’N, N- Tetraethylenediamine (TEMED) and Ammonium persulphate (APS) (Appendix 7A).

2.4.2 Sample Preparation

Equal amounts of protein between 30 µg and 50 µg were used to qualitatively compare samples by western blot. Samples for western blotting were prepared by adding 4x Laemmli buffer (Appendix 7A) to the appropriate amount of cell lysate. The samples were heated at 98°C for 5 min prior to loading and separation on 6%-10% SDS-PAGE gel. Molecular weight markers (Fermentas Spectra™ Multicolour Broad Range Protein Ladder, USA) were used to allow estimation of molecular weight. The gel was run at 25mA in running buffer (3.02% w/v Tris-base, 14.4% w/v glycine, 0.1% SDS).
2.4.3 Electrophoretic protein transfer

Once the protein samples were separated through the polyacrylamide gel, the protein was transferred onto nitrocellulose paper (NCP) to allow detection of proteins according to the method of Towbin et al. (Towbin et al., 1979). A sheet of NCP paper (0.45µm pore size) (Amersham™ Hybond™ ECL, GE Healthcare, UK) was pre-wetted with transfer buffer (Appendix 7A) and laid on blotting paper (GE Healthcare, UK) which was supported by a stiff plastic grid. The gel to be blotted was put on the nitrocellulose sheet and further blotting paper added to form sandwich like structure. Care was taken to avoid the introduction of air bubbles between the gel and the NCP in order to avoid incomplete transfer of proteins. The second plastic grid was applied against the pad. The assembly was then submerged into a chamber containing transfer buffer and proteins were transferred at 100V for 45 min in a Transphor electrophoresis unit (Hoefer Scientific, UK) such that the negatively-charged proteins travel towards the positively-charged electrode and bind to the membrane.

2.4.4 Immunoblotting and protein detection

Following protein transfer, the NCP membranes were blocked by incubation in blocking solution [5% (w/v) dried milk in PBS-Tween 20 (PBS-T) [0.05%,(v/v)] for 1 ½ hours to prevent non-specific background binding of the primary and/or secondary antibodies to the membrane. The primary antibodies (Table 2.1) were diluted as per manufacturer’s instructions in 1% (w/v) milk/PBS-T. The membranes were incubated with the primary antibodies overnight on a rocker at room temperature. The blot was then washed three times for 10 min with PBS-T [0.05%,(v/v)]. The secondary antibodies used are shown (Table 2.1). The secondary antibodies were diluted in 1% (w/v) milk/PBS-T and incubated with the membrane for 1 hour at room temperature and then washed three times for 10 min with PBS-T [0.05%,(v/v)]. The protein detection was performed using Supersignal® West Pico Chemiluminescent Substrate (Thermo Scientific, USA) and visualised using a digital camera and Quantity One-1D software (Bio Rad, UK).
Table 2.1: List of antibodies used in this studies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Source</th>
<th>Dilution factor*</th>
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</thead>
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<tr>
<td>CD45</td>
<td>BD Pharmingen (610226)</td>
<td>Mouse</td>
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<tr>
<td>c-MYC</td>
<td>Cell Signalling Technology (#5065)</td>
<td>Rabbit</td>
<td>1/500</td>
</tr>
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<td>Goat</td>
<td>1/200</td>
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<tr>
<td>donkey anti-goat IgG-HRP</td>
<td>Santa Cruz Biotechnology, Inc. (sc-2020)</td>
<td>Donkey</td>
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<td>Amersham™ GE Healthcare (Lot No. 9505538)</td>
<td>Sheep</td>
<td>1/10000</td>
</tr>
<tr>
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<td>Sigma-Aldrich (A5060)</td>
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</tr>
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* All dilution factor used are based upon optimization and recommendation by manufacturers. The catalog no. are shown in brackets.
2.5 Chemotaxis and motility assay

2.5.1 Motility assay

Motility assays were carried out using Boyden chamber assays (Whetton et al., 2003). Cell migration was assayed by using a 6.5-mm polycarbonate membrane (5.0-μm pore size; Corning Inc.) in a 24 well plate format (Figure 2.1). 100 μL (1 x 10⁵) of cells were placed in the upper chambers whilst 600 μL of RPMI medium with 10% (v/v) Horse serum; 5% (v/v) m-IL3 placed in the lower chamber with presence or absence of the chemoattractant, CXCL12 at 200 ng/mL. Water was added into the spare well to avoid evaporation. The chambers were incubated in a humidified 37°C incubator in 5% (v/v) CO₂ for 6 hours. Each sample was done in triplicate and the number of cells migrating into the lower chamber assayed. The 600 μL of media was removed from the lower chamber and any cells migrating pelleted by centrifugation at 10,000 RCF for 3 min. The supernatant was discarded and the pellet containing cells was then resuspended in 10 μL of medium and 10 μL of trypan blue. Cells were counted and viability assessed using a hemocytometer under the light microscope. Non-migratory cells in the upper chamber (treated/non-treated cells) were also counted to assess their viability at 6 hours after initiate of the motility assay.

Figure 2.1: Schematic diagram of Boyden chamber assay (Whetton et al., 2003).
2.5.2 Cell treatment prior to motility assays

To investigate the involvement of particular pathways in motility, $1 \times 10^6$ cells/mL were pretreated with inhibitors for 2 hours at 37°C in a humidified 5% (v/v) CO$_2$ incubator prior to undertaking Boyden chamber assays. The inhibitors used were 10 µM of MEK 1/2 inhibitor, U0126 (MEKi) (Cell Signalling™, UK), 20 µM of Src inhibitor, SU6656 (SRCi) (Selleckbio, USA), 100 µM of Rac inhibitor, NSC23766 (Raci) (Calbiochem, USA), 10 µM of PI3K inhibitor, LY294002 (PI3Ki) (Calbiochem, USA), 5 µM of TGFβ RI kinase inhibitor, (TGFβi) (Calbiochem, USA), 50 µM of JAK2 inhibitor, (ruxolitinib), INCB018424 (Selleckbio, USA), 500 nM MYC inhibitor, (JQ1) (Calbiochem, USA), 5 µg/mL of D-erythro sphingosine-1-phosphate, (S1P) (Acros Organic), and 10 µM of sphingosine kinase inhibitor, (SKi) (Calbiochem, USA).

2.6 Cloning THOC5 Y225F into MSCVneo

2.6.1 Plasmid preparation and restriction enzyme digestion of MSCVneo vector and THOC5 Y225F

Plasmid DNA for MSCVneo and Myc-tagged mutant THOC5 Y225F MSCV-GFP was prepared using the Qiaprep Spin miniprep kit (Qiagen, USA) as per manufacturers instructions. THOC5 Y225F (2300bp) was excised and cloned into the BglII site of the retroviral vector MSCVneo (6500bp) (Figure 2.2). Three µg of the vector (MSCV-GFP) and 1 µg of THOC5 Y225F MSCV-neo were digested with 0.5 µL of BglII enzyme (10U/µL) (Promega, USA) at 37°C for 2 ½ hours in a final volume of 50 µL. The vector was treated with 2 µL of 5-10U/µL calf intestine alkaline phosphatase, CIP (Promega, USA) for 15 min prior to completion. Addition of CIP is useful to dephosphorylates the 5’ termini phosphate group of digested vector, which should prevent self-ligation and recircularisation. The digested plasmids were subject to agarose gel electrophoresis on a 0.6% (w/v) agarose gel to separate the digested fragments. The band(s) of interest were removed under short wavelength UV illumination (Bio Rad, UK). The DNA was recovered from the gel fragment before proceeding with ligation and transformation.
Figure 2.2: pMSCVneo vector map. These vectors contain the long terminal repeat (LTR) that allows the constitutive expression of target gene in mammalian cells. The vector contains the retroviral packaging signal $\Psi^+$ which promotes virus production. The phosphoglycerate kinase (PGK) promoter functions in control the expression of neomycin resistance gene (Neo$^+$) for an antibiotic selection in eukaryotic cells. The vector also includes the pUC origin of replication and *E.coli* Amp$^+$ gene for propagation and antibiotic selection in bacteria.

2.6.2 DNA ligation and transformation into *E.coli* DH5α™

DNA was recovered from the agarose gel using a QIAquick gel extraction kit (Qiagen, USA) according to the manufacturer’s instructions. The gel purified vector and DNA insert was quantified on an agarose gel prior to undertaking ligation. The vector and insert were mixed in a molar ration of 1:3 and the ligation performed in 10 µL, by the addition of 1 µL of T4 DNA ligase (5U/µL) and 1 µL of 10x Buffer (Promega, USA) at 16°C overnight. A control was prepared with no insert in the reaction. Ligation products were transformed into chemically DH5α™ competent cells (Invitrogen, USA) and colonies selected using ampicillin. DNA was prepared from the clones selected and positive colonies identified by restriction digest analysis. Orientation and faithful replication was ensured by DNA sequencing.
2.6.3 Confirmation of cloning

Successful cloning of the THOC5 Y225F insert was assessed via restriction digestion analysis before confirmation via DNA sequencing at Molecular Biology Core Facility (MBCF) at Manchester Institute of Cancer Research. The MSCVneo forward primer 5'-CCCTTGAACCTCCTCGTACC-3' was used for sequencing and the sequencing results were analyzed by Chromas Lite 2.0 Software.

2.7 Transfection of MYC-tagged THOC5 Y225F/MSCVneo into the retroviral packaging cell line

2.7.1 Preparation of Platinum-E packaging cell line

The Platinum-E (Plat-E), a potent ecotropic retrovirus packaging cell line based on the 293T cell line, consists of novel packaging constructs with an EF1α promoter and the Kozak’s sequence upstream of the initiation codon resulting in high expression of virus structural proteins (gag, pol, env) in Plat-E cells (Figure 2.3) (Morita et al., 2000). For the transfection process, the Plat-E packaging cell line was seeded the day before transfection at 3.5 x 10⁵ cells/mL in 6-well plates to reach 75-80% confluent at the time of transfection. The Dulbecco’s Modified Eagle Medium (DMEM) for the packaging cell lines was made supplemented with 10% (v/v) fetal bovine serum and 2% (w/v) L-Glutamine.

Figure 2.3: Schematic diagrams of Plat-E packaging constructs. The fragment carrying the selectable marker, the blastcidin resistant gene (bs') or the puromycin resistant gene (puro'). Packaging construct consists of 3 primary genes: gag, pol, and env. Gag (group specific antigen) codes for proteins coating the capsid, pol (polymerase) codes for reverse transcriptase and other enzymes (protease and integrase), and env (envelope) codes for proteins making up the envelope (Morita et al., 2000). Internal ribosome entry site; IRES sequence is to ensure the stable expression of viral structural genes.
2.7.2 Formation of DNA-liposome complex

Lipofection was performed using Lipofectamine™ 2000 reagent (Invitrogen, USA). 10 µL of lipofectamine 2000 was mixed gently with 250 µL of Optimem. In a separate tube, 4 µg of THOC5 Y225F/MSCVneo DNA was added into 250 µL of Optimem and mixed gently. Both reactions were incubated at room temperature for 5 min before combining and incubating for a further 20 min at room temperature to form lipid-DNA complexes. The Plat-E packaging cells were washed with 2 mL of Optimem. 500 µL of Optimem was added to the lipid-DNA complexes mixed gently and overlaid onto the washed Plat-E packaging cells. The 6-well plates were rocked gently to ensure mixing and an even distribution. Then the plates were incubated for 6 hours at 37°C. After 6 hours incubation, 1 mL of DMEM plus 20% newborn calf serum (NBCS) was added into the plate without removing the transfection mixture. The cell were cultured at 37°C in 5% (v/v) CO₂ incubator for 24 hours.

2.7.3 Transduction of Ba/F3 MPL W515L expressing cells

The Ba/F3 MPLW515L target cells were prepared to be in logarithmic growth phase $5 \times 10^5$ cells/mL prior to transduction. The cell supernatant from Plat-E cells was collected 24 hours post transfection and overlaid onto Ba/F3 MPL W515L expressing cells. At 72 hours post-transduction, the Ba/F3 MPL W515L expressing cells were passaged into the selection medium containing geneticin (500 ng/mL) (Geneticin® G418-Life Technologies, UK). Ba/F3 control cell lines were also generated by infection with the MSCVneo empty vector.

2.8 c-MYC siRNA transient transfection

The Ba/F3 and Ba/F3 MPL W515L expressing cells from logarithmic growth phase were prepared at $2 \times 10^6$ cell/ mL prior to transfection. Cells were transiently transfected with the MISSION® esiRNA targeting mouse MYC (EMU075291, Sigma Aldrich, UK) or siRNA sequences Ambion® Silencer® Select Negative Control #2 siRNA (Ambion, UK) at a final concentration of 260nM (in a total of 2 ml medium). Transient transfection was achieved using the Amaxa cell line nucleofector II device (Lonza, Verviers, Belgium) following the
manufacturer’s protocols optimized for Ba/F3 cells (Kit V and Program X-001, Lonza). The cells were incubated at 37°C in 5% (v/v) CO₂ humidified incubator. Post transfection (48 hours) cell migration was assessed by Boyden chamber assays and cell lysates produced to assess the effect on protein expression by western blotting using the appropriate antibodies.

2.9 **Flow cytometry of CD45 and CXCR4 cell surface staining**

Cells (2 x 10⁵) were pelleted by centrifugation for 2 min at 300 RCF and resuspended in 100 μL of FACS wash (10% (v/v) fetal calf serum/0.01% (w/v) sodium azide/ PBS). APC rat-anti mouse CD184/CXCR4 (1/200), or PE Rat anti-mouse CD45 (1/1000) (BD Biosciences, Oxford, UK) were added and incubated on ice for 10 min. Samples were also stained with isotype matched antibody as a control which was coupled to the same fluorochrome. Following labelling, 500 μL of FACS wash was added and cells pelleted by centrifugation. Finally, cells were resuspended in 300 μL of FACS wash and analysed by flow cytometry using the FACS CALIBUR™ 3 color cell analyzer (Becton Dickinson, Oxford, UK). Data acquisition was performed with FACSDiva (Becton Dickinson) and the output data were analysed using FlowJo 7.6 (Tree Star, USA) software. All steps were performed using ice-cold buffers and cells were kept on ice during the staining process and before data acquisition.

2.10 **TGFβ1 measurement**

TGFβ1 concentrations in the cell culture supernatants were measure by ELISA using the “Quantikine” kit from R&D systems (Abingdon, UK) as per manufacturer’s instructions.

2.11 **Protein quantification using eight-channel isobaric tagging for relative quantification (iTRAQ™)**

The relative protein quantification experiments were carried out using the iTRAQ™ technique to compare the relative abundance of proteins from different samples. The method is based on the covalent labeling of the N-terminus and side-chain amines of peptides from protein digestions with tags of varying mass. These samples are then pooled and usually
fractionated by nano liquid chromatography, followed by analysis using tandem mass spectrometry (MS/MS). A database search is then performed using the fragmentation data to identify the labeled peptides and hence the corresponding proteins. The fragmentation of the attached tag generates a low molecular mass reporter ion that can be used to relatively quantify the peptides and the proteins from which they originated. Mass spectrometry data were processed using ProteinPilot™ software (ABSciex version 3).

2.11.1 Cell lysate preparation for iTRAQ™

1–2x10^7 of Ba/F3, Ba/F3 MPL W515L, and Ba/F3 MPL W515L THOC5 Y225F cells pellet were lysed in 200 µL of 0.5 M triethylammonium bicarbonate, TEAB, 0.05% w/v SDS, Protease and Phosphatase inhibitor cocktail (10 µL/mL) and Benzonase (0.2 mM) (Sigma-Aldrich, St. Louis, MO) on ice for 20 min with regular vortexing. Lysates were centrifuged at 10,000 RCF at 4 °C for 20 min, supernatant removed, and protein was quantified using the modified Bradford protein assay (Bio-Rad Laboratories).

2.11.2 Tryptic digestion and iTRAQ™ eight-channel labelling

Samples of Ba/F3, Ba/F3 MPL W515L, and Ba/F3 MPL W515L THOC5 Y225F was prepared in 0.5 M TEAB/ 0.05% (w/v) SDS (final volume 20 µL) and disulphide bonds reduced by adding 0.1 volumes of 50 mM Tris-(2-carboxylethyl) phosphine hydrochloride (TCEP) (Sigma Aldrich, Poole, UK), with vortexing followed by 1 h incubation at 60 °C. Reduced cysteine residues were then alkylated using cysteine blocking reagent by addition of 0.05 volumes of 200mM methyl methane thiosulphate (MMTS) in isopropanol, vortexed and incubated at room temperature for 10 min. Proteins were digested by the addition of 5 µL of trypsin reconstituted at 2 g/L (Promega, Madison, WI, USA) and incubated overnight at 37°C. After reduction, alkylation and digestion, the samples were labeled with the different iTRAQ™ reagents (Applied Biosystems, Warrington, UK). One unit of each tag (defined as the amount required for labelling of 100 µg of protein) was thawed on the bench for 2-3 min and then pulse centrifuged to ensure all reagents are at the bottom of the tube. Each reagent
was reconstituted in 70 µL isopropanol (Sigma Aldrich, Poole, UK), and the reagent solution added to the digested samples as follows: Ba/F3, iTRAQ 113 (control-replicate 1); Ba/F3 MPL W515L, iTRAQ 114 (replicate 1); Ba/F3 MPL W515L THOC5 Y225F, iTRAQ 115 (replicate 1); Ba/F3, iTRAQ 116 (control-replicate 2); Ba/F3 MPL W515L, iTRAQ 117 (replicate 2); Ba/F3 MPL W515L THOC5 Y225F, iTRAQ 118 (replicate 2); Ba/F3 MPL W515L, iTRAQ 119 (replicate 3); Ba/F3 MPL W515L THOC5 Y225F, iTRAQ 121 (replicate 3). The samples were then vortexed and incubated for 2 hours at room temperature. All post labeling samples were pooled and dried down using a SpeedVac vacuum concentrator until about 15 µL was left in each tube before storage at -20°C (Unwin et al., 2005b).

2.11.3 High-pH reversed phase chromatography for peptide fractionation

Peptide fractionation was performed off line using a reversed phase chromatography system. Peptides were resuspended in 1 mL high pH Buffer A (0.1% ammonium hydroxide, adjusted to pH 10.5 with formic acid) and loaded onto reversed phase chromatography column (Fortis technologies, C18 (3 µm, 100 x 4.6 mm) by using LC Packing Ultimate LC system. The gradient was run at 700 µL/min using initially 0.5% high pH buffer B (1% ammonium hydroxide, 99.9% acetonitrile), then, after 30 min the high pH buffer B was increased to 50%. Buffer B percentage was increased to 75% in 4 min then remained for 4 min at this concentration before being reduced back to 0.5%. Fifteen second fractions were collected during gradient duration. The volume of each fraction were then concentrated with a SpeedVac and stored at -80°C for online MS/MS.

2.11.4 Tandem mass spectrometry-QSTAR® Elite

Peptides were identified by RP-LC-MS/MS on a QSTAR® Elite mass spectrometer (AB Sciex, UK). Dried peptide fractions were re-suspended in 15 µL of 3% (v/v) acetonitrile, 0.1% (v/v) formic acid and 20 mM citric acid. For each analysis, 5 µL of the peptide sample was loaded onto a nanoACQUITY UPLC Symmetry C18 Trap, 5 µm, 180 µm×20 mm and flow was set to 15 µL/min of 3% (v/v) acetonitrile, 0.1% (v/v) formic acid and 20 mM citric acid for 5 min. Analytical separation of the peptides was performed using nanoACQUITY UPLC BEH
C18 Column, 1.7 µm, 75 µm × 250 mm. Briefly, peptides were separated over a 91 min solvent gradient from 3% (v/v) acetonitrile, 0.1% (v/v) formic acid to 40% (v/v) acetonitrile, 0.1% (v/v) formic acid, on-line to a QSTAR® Elite mass spectrometer (AB Sciex, UK). Data was acquired using an information dependent acquisition (IDA) protocol where, for each cycle, the 4 most abundant precursors multiply charged peptides (2+ to 4+) above a 150 count threshold in the MS scan with m/z between 400 and 2000. Each peptide was dynamically excluded (±50 mmu) for 90 seconds.

2.11.5 Data analysis and software employed for protein identification and quantification

Data was processed by search against the UniProt/SwissProt mouse database containing 532146 sequence entries using ProteinPilot™ Software 4.1, revision number 460, using the Paragon™ Algorithm 4.0.0.0, 459 as search engine. The search parameters allowed for one missed or nonspecific cleavage (AB SCIEX, Framingham, USA), methyl methanethiosulfonate (MMTS) and 8-plex iTRAQ fixed modifications as default setting. The calculation by ProteinPilot™ was used to derive an average ratio where multiple spectra were found for a given distinct phosphoentity (that is, a distinct sequence and set of modified amino acids). As the number of identifications for an entity is usually very low the p-value was calculated where possible but not used to determine a change. All proteins identified must have ≥ 95% confidence, and the protein confidence threshold cut-off was set to 1.3 (unused) with at least more than one peptide above the 95% confidence level. The true value for the average ratio was expressed as an error factor \( \text{EF} = 10 \text{ (95% confidence interval)} \) and calculated according to the reports. An EF < 2 was set for the quantification quality to be satisfied. In addition, a p-value < 0.05 was significant for protein quantification. To designate significant changes in protein expression, fold-changes > 1.34 or < 0.74 were set as cut-off values. Furthermore, in order to decrease the artificial error, the bias correction option was executed (Lu et al., 2012).
2.12 Sphingosine-1-phosphate analysis on LC/ESI-MS/MS

Sphingosine-1-phosphate was extracted and analysed by liquid chromatography coupled to electrospary ionisation tandem mass spectrometry (LC/ESI-MS/MS) (Bielawski et al., 2006; Kelly et al., 2011). Briefly, cell pellets were homogenised in ice-cold isopropanol:water:ethyl acetate (30:10:60; v/v/v; 4 mL per sample). Internal standard (C17-sphingosine-1-phosphate; Avanti Lipids, Alabaster, Alabama) was added (50 pmol per sample) and samples were incubated on ice for 30 min. Protein precipitates were removed by centrifugation (1500xg, 4°C, 10 min), the resulting clear supernatant evaporated under nitrogen, and the lipid residue redissolved in methanol with 0.1% formic acid. LC/ESI-MS/MS was performed on an Acquity UPLC system coupled to an electrospray triple quadrupole TQ-S mass spectrometer (Waters, Elstree). Sphingosine-1-phosphate was separated on a C8 column (Acquity UPLC BEH, 1.7 µm, 2.1 x 100 mm; Waters, Elstree) using a gradient of solvent A (water:formic acid; 100:0.1; v/v) and solvent B (methanol:formic acid; 100:0.1; v/v) as follows: 60% B (0-6 min), 60-96% B (6-9 min), 96-99% B (9-18 min), 99% B (18-20 min), 99-60% B (20-23 min) and 60% B (23-26 min). Sphingosine-1-phosphate was monitored in the positive ion mode using the transition m/z 380>264. Results are expressed as pmol/10^7 cells. This study was performed by Prof. Anna Nicolaou and Dr. Alexandra C Kendall from Manchester Pharmacy School, The University of Manchester, UK.

2.13 RNA extraction and quantitative real time-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from fresh cell pellets (kept on ice) immediately, with Qiagen RNeasy Plus Mini kit (cat. No. 74134). RNA was dissolved in RNase free water and quantified on a nanodrop 2000C spectrophotometer (Thermo Scientific, UK). RNA samples were stored in -80°C freezer if not required immediately. First strand cDNA was synthesised from 2 µg RNA with RT-PCR kit (Promega, M-MLV reverse transcriptase, cat. No. M170A) following the instruction of the manufacturer. The synthesised cDNA was then diluted to 2 µg/mL for loading on Q-RT-PCR plate. Primers and probes were designed using the Universal Probe Library Assay Design Centre from Roche Applied Sciences (Table 2.2). Q-
RT-PCR assays were performed in triplicate on a 7900HT Fast Real-Time PCR System. \(\Delta\Delta C_t\) values were calculated for each sample against the average of the two housekeeping genes that were used to calculate fold change using the \(2^{-\Delta\Delta C_t}\) method.

### 2.14 Cell proliferation assay

To determine the cell proliferation assay in response to curaxin, ribavirin and Adox, the WST-1 cell proliferation kit (Roche Diagnostic, Manheim, Germany) was used in this study. 100 µL (2 \times 10^4/mL) of Ba/F3, Ba/F3 MPL W515L, Ba/F3 JAK2 K539L, and Ba/F3 JAK2 V617F expressing cells were seeded triplicate in 96-well plate format. Then, curaxin (0 nM-1 µM) (CBL 0137; Sigma Aldrich, UK), ribavirin (0-200 µM) (R9644; Sigma Aldrich, UK), and Adox (0-200 µM) (A7154; Sigma Aldrich, UK) were added to the plates and incubate at 37 ºC in 5% (v/v) humidified CO\(_2\) incubator for 24 hours. After 24 hours, 10 µL of WST-1 reagent was added to each well and incubate for another 1 hour. The cell proliferation was measured at a wavelength 450/620nm using the plate reader (Lab systems Affinity Sensors, Cambridge, UK).
Table 2.2: List of oligonucleotides used for TaqMan® RT-PCR assays in this study. Sequences are listed in 5’-3’ order from left to right for forward and reverse primer with the location of primers are shown in the bracket. The ideal of amplicon size also shown in the table for optimal PCR efficiency.

<table>
<thead>
<tr>
<th>gene name</th>
<th>NM_ number</th>
<th>Size</th>
<th>Cds</th>
<th>forward primer</th>
<th>reverse primer</th>
<th>amplicon</th>
<th>probe#</th>
</tr>
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<td>(1506)gcttccccggtgcactgttcagtc(1525)</td>
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<td>(192)cataattgtgctcctgatac(215)</td>
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</table>
2.15 Statistical methods

2.15.1 One-way ANOVA

Statistical analysis of all data was performed with Prism 6.0 software (GraphPad Software, USA). The one way analysis of variance (ANOVA) parametric test was used with Turkey multiple comparison post test since the number of comparison more than two groups in most of experiments assuming the equal variance and normal distribution in cell migration. The result with a p-value less than or equal to 0.05 were considered significant (*), p≤0.01 is highly significant (**), and p≤0.001 very highly significant (***) and extremely significant p<0.0001 (****).

2.15.2 Standard error of Mean (SEM)

The error bars shown in graphic (histograms or scatter plots) represent the standard error of the mean (SEM) calculated from replicate observations. It is defined by:

\[ SEM = \frac{s}{\sqrt{n}} \]

Where:
s = sample standard deviation (see formula below)
n = size (number of observations) of the sample

\[ s = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (x_i - \bar{x})^2} \]

Where:
s = sample standard deviation
x₁, ..., xₙ = the sample data set
\( \bar{x} \) = mean value of the sample data set
N = size of the sample data set

(Adapted from: http://www.miniwebtool.com/standard-error-calculator/)
Chapter 3

Myeloproliferative neoplasm associated oncogene MPL W515L affects motility via THOC5 leading to leukaemic transformation
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Myeloproliferative neoplasm associated oncogene MPL W515L affects motility via THOC5 leading to leukaemic transformation

3.1 Introduction

Our group have systematically analysed the global proteomic effects of BCR/ABL and other leukaemia oncogenes using advanced mass spectrometry techniques (Pierce et al., 2008). This identified some common effector mechanisms for the oncogenes amongst a large amount of disparate effects. However in further work we elucidated a novel pathway of Src-mediated CD45 signalling induced THOC5 phosphorylation downstream of leukaemogenic PTKs (Griaud et al., 2013). THOC5 was found to be phosphorylated at tyrosine 225 (Y225) by the action of numerous leukaemogenic PTKs including BCR/ABL, NPM/ALK and TEL/PDGFR (Pierce et al., 2008). In addition, THOC5 is phosphorylated on Y225 following CXCL12 stimulation and found to be involved in the motility response of haemopoietic cells (Griaud et al., 2013). Based on the data previously obtained, THOC5, a member of the mRNA splicing/export THO complex, was chosen for further investigation to enhance our knowledge of leukaemogenic transformation in the context of MPL W515L action. MPL W515L is a somatic mutation when tryptophan is substituted for leucine at codon 515 of the transmembrane region of the thrombopoietin receptor (TpoR). The MPL W515L mutation results in the constitutive activation of JAK-STAT pathway leading to essential thrombocytosis and myelofibrosis (MF) (Campbell and Green, 2006; Pardanani et al., 2006). Current treatment strategies for MF using JAK2 inhibitors alone, such as ruxolitinib, or in combination with other MF related therapies, have entered clinical trial. However current treatment is not sufficient to reduce disease burden in MF (Wolf et al., 2011). Further study is needed to improve the understanding of the mechanisms driving this MPN as targeting pathways of importance in the pathogenesis of MPN may hold effective therapeutic value for patients. To achieve this, a proteomic study was performed on nuclear and
cytoplasmic enriched fractions of MPL W515L expressing cells to determine it’s mode of action. This data linked MPL W515L action to motility and then in turn to the THOC5 protein.

3.2 Results

3.3 Effects of MPL W515L on STAT signal transduction and MCL-1

The mechanistic detail underlying the effects of MPL W515L were systematically investigated using proteomics. The consequences of MPL W515L expression on over 3300 nuclear and 3500 cytoplasmic proteins were assessed using relative quantification isobaric tag mass spectrometry (iTRAQ). Our proteomic dataset indicated that the MPL W515L oncogene induced an increase in the expression of MCL-1, STAT5, MYC and Src family kinase member, Lyn (unpublished observation). MPL W515L and JAK2 mutants identified as somatic mutations have previously been shown to cause constitutive activation of the JAK-STAT signalling pathway (Campbell and Green, 2006; Pardanani et al., 2006). In addition to the JAK-STAT pathway, the MPL W515L mutations has been shown to constitutively activate the SHC-Ras-Raf-MAPK/JNK and PI3K-Akt-Bad pathways (Abe et al., 2002). Lyn tyrosine kinase is involved in the survival signalling pathway of macrophage/monocyte since the antisense oligonucleotide prevented the antiapoptotic activity of GM-CSF (Epling-Burnette et al., 2001). Also MPL W515L stimulation results in the activation of Lyn kinase through MAPK regulation (Lannutti and Drachman, 2004).

Table 3.1: Proteins identified and quantitated in MPL W515L expressing cells. STAT3 is not changing. STAT5 and MCL-1 identified as changing in the nuclear fraction whose expression is altered by the expression of Ba/F3 MPL W515L expressing cells. We defined a protein change as any protein having an iTRAQ reporter ion-based relative quantification ratio outside the range in which 95% of protein ratios for the internal replicate are found and a p-value of 0.05 or less in comparison to Ba/F3 control cells.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ba/F3 MPL W515L expressing cells to Ba/F3 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclear fraction</td>
</tr>
<tr>
<td>STAT3</td>
<td>1.1</td>
</tr>
<tr>
<td>STAT5</td>
<td>2.0</td>
</tr>
<tr>
<td>MCL-1</td>
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</tr>
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</table>

These data support our approach but to further validate our results, western blots were performed on proteins identified as changing according to the iTRAQ data. STAT3, STAT5 and
MCL-1 proteins were identified and quantitated in both the nuclear and cytoplasmic fractions of Ba/F3 MPL W515L expressing cell line (Table 3.1). Ratios shown in Table 3.1 were established against the Ba/F3 control cell lines enabling identification of any expression change in the Ba/F3 MPL W515L oncogenic cell. A ratio demonstrating greater than a 1.5 fold increase was significant and regarded as a change in expression. The ratios for STAT3 is less than 1.5, and therefore representative of not changing either in the cytoplasmic or nuclear fraction. STAT5 results, however clearly demonstrate a change in expression in the nuclear fraction of MPL W515L expressing cell line, with the ratio greater than 1.5. This change is not present in the cytoplasmic fraction with ratio lower than 1.5. For MCL-1, the ratio shows changes greater than 1.5 in nuclear fraction of MPL W515L expressing Ba/F3 cells. In order to validate this proteomic dataset, we chose a panel of proteins to be assessed by western blot. In this western experiment, STAT5 (90 kDa) and phospho-STAT5 (Tyr694)(90 kDa) expression was assessed in Ba/F3 MPL W515L expressing cells and control cells (Figure 3.1). STAT3 protein expression shows equal expression in either Ba/F3 control or Ba/F3 MPL W515L expressing cells, however the phospho-STAT3 (Tyr705) (79 kDa) (Cell Signalling, UK) increased in the MPL W515L expressing cells as compared to Ba/F3 control cells. In addition, MCL-1 expression (40kDa) (Figure 3.2) also display an increase in Ba/F3 MPL W515L expressing cells as compared to Ba/F3 control cells. MPL is known to activate JAK (Pikman et al., 2006). Here, the western blot analysis observed does validate isobaric tagging proteomic results seen in MPL W515L expressing Ba/F3 cells.
**Figure 3.1: Western blot assessment of the STAT protein expression.** The expression level of the proteins indicated were assessed by western blot analysis in whole cell lysates from Ba/F3 and Ba/F3 MPL W515L. Actin was used as a loading control. Representative blots from three independent biological replicates.

**Figure 3.2: Assessment of MCL-1 protein expression.** The expression level of MCL-1 was assessed by western blot analysis in whole cell lysates from Ba/F3 and Ba/F3 MPL W515L. Actin was used as a loading control. Representative blots from three independent biological replicates.

### 3.4 MPL W515L alters motile responses which is linked to THOC5 modulation

The effects of Ba/F3 MPL W515L expressing cells on over 3300 nuclear and 3500 cytoplasmic proteins were relatively quantified using advances isobaric tag mass spectrometry. Within the proteins identified as changing, we saw an enrichment of proteins
Figure 3.3: Protein changes identified and categorised as a consequence of Ba/F3 MPL W515L expression by biological process. Pie charts of all discovered (left) and changing (right) cytoplasmic proteins categorised by biological process.
involved in motility which is double that compared to control (Figure 3.3) (p<0.05). From the protein dataset generated, gelsolin was down regulated as a consequence of MPL W515L expression. Our group have previously reported that BCR/ABL expression, the causative agent for chronic myeloid leukaemia, leads to the down regulation of gelsolin and to a reduced response to chemotactic and chemokinetic stimuli (Unwin et al., 2005b). Given the pathology of myelofibrosis that accompanied by the constitutive mobilisation and abnormal trafficking of haematopoietic/progenitor stem cells into the peripheral blood and also to the extramedullary site of liver and spleen (Xu et al., 2005b), I therefore asked the question whether in the MPL W515L oncogenes led to a disruption in motility response. To investigate the motility of Ba/F3 MPL W515L expressing cells, the migration assay was performed using a Boyden chamber assay as previously described (Whetton et al., 2003). 1x10^5 cells were added to the top well and 200 ng/mL of CXCL12 to the bottom well as indicated. The number of cells migrating to the bottom well were counted after 6 hours. Interestingly, in the absence of chemoattractant, the Ba/F3 MPL W515L showed a greater than 3 fold (p<0.0001) significant increase in chemokinesis compared to control cells (Figure 3.4).

![Figure 3.4: Differential effects of CXCL12/SDF-1 on Ba/F3 and Ba/F3 MPL W515L expressing cell migration.](image)

The CXCL12 (200 ng/mL) induced chemotactic response on Ba/F3 control and Ba/F3 MPL W515L expressing cell was assessed in Boyden chamber assays. 1x10^5 cells were added to the top well and the number of cells migrating into the bottom well counted after 6 hours. Cell viability in the top well was also assessed after 6 hours. Data shown are the mean ± SEM from three experiments done in triplicate. Significance differences from controls were determined by One-way ANOVA with multiple comparison Turkey post test whereby p<0.05 was considered significant. *p<0.05, **p<0.001, ****p<0.0001.
MPNs are often characterised by a large increase in the number of circulating CD34+ cells indicating that adhesion within or egress from the stem cell niche is disrupted. THOC5 was found to be phosphorylated at tyrosine 225 (Y225) by CXCL12 action which governs motility (Griaud et al., 2013). Therefore given the above proteomic data on the effects of MPL W515L on motility proteins (Figure 3.3) and chemokinesis, we assessed the involvement of THOC5 Y225 phosphorylation in MPL W515L action. As shown in Figure 3.5, the phosphorylation of THOC5 on Y225 was seen in leukaemogenic Ba/F3 MPL W515L expressing cells to a greater extent than in control cells. Having identified a pathway potentially disrupted by MPL W515L, the subsequent objective was to assess the role of THOC5 Y225 phosphorylation in MPL W515L mediated transformation by targeting the THOC5 pathway constituents.

![Figure 3.5: Western blot assessment of the THOC5 and phospho Y225 THOC5 expression.](image)

**Figure 3.5: Western blot assessment of the THOC5 and phospho Y225 THOC5 expression.** The expression level of the proteins indicated were assessed by western blot analysis in whole cell lysates from Ba/F3 and Ba/F3 MPL W515L. Actin was used as a loading control. Representative blots from three independent biological replicates.

### 3.5 Construction of THOC5 Y225F into MSCVneo

Having published that addition of CXCL12 induced THOC5 Y225 phosphorylation and modulates motile response, further screening on Ba/F3 MPL W515L expressing cells transfected with a mutant THOC5 Y225F was performed in an attempt to identify the role of THOC5 and Y225 phosphorylation effects on motility in MPN associated oncogene. To achieve this, THOC5 Y225F mutant was cloned into the retroviral vector MSCVneo and transfected into Ba/F3 MPL W515L expressing cells.
3.5.1 Cloning of THOC5 Y225F into MSCVneo

The THOC5 Y225F-MYC tagged construct was excised from MSCV-GFP by BglII digestion (lane 2, 2300bp) and cloned into the BglIII site of MSCVneo (lane 1, 6500bp) (Figure 3.6). The purified insert and vector were then subject to ligation, overnight at 4°C using T4 DNA ligase. The ligation product was then transformed into DH5α E.coli competent cells. Four colonies were selected for plasmid isolation and verification of successful cloning.

![Image of gel with bands](image)

**Figure 3.6: BglII digestion of MSCVneo and MSCV THOC5 Y225F IRES GFP.** The products of a BglII restriction enzyme digest of MSCVneo (lane 1) and MSCV THOC5 Y225F IRES GFP (lane 2) were separated on 0.6% agarose gel. Lane M- DNA molecular weight Marker II (Boehringer Manheim, Roche USA); Lane 3- uncut MSCVneo plasmid (control) and lane 4- uncut THOC5 Y225F plasmid (control)

3.5.2 Orientation analysis of THOC5 Y225F insert in MSCVneo

Successful cloning of the insert was confirmed by restriction digest analysis and the correct orientation by DNA sequencing using MSCV forward and reverse primer. DNA sequencing was performed in the Molecular Biology Core Facility (Paterson Institute of Cancer Research, UK). A suitable clone was chosen for transduction into Ba/F3 MPL W515L cells.
3.5.3 Generation of Ba/F3 MPL W515L cells expressing THOC5 Y225F

Once the Plat-E packaging cells are transfected with THOC5 Y225F MSCVneo expression vector, the viral genomic transcript containing THOC5 Y225F target gene and selectable marker are packaged into infectious virus within 24-48 hours. These particles were then used to infect Ba/F3 MPL W515L expressing cells. A stable transfected cell line of Ba/F3 MPL W515L cells expressing THOC5 Y225F were produced by selection in 50 µg/mL geneticin, G418 for 2 weeks using the method that we generated. Successful transfection and expression of THOC5 Y225F was verified by western blot analysis.

3.5.4 Western blot of Ba/F3 MPL W515L THOC5 Y225F expressing cells

The successful transfection and expression of THOC5 Y225F in the Ba/F3 MPL W515L cells was verified by western blot using both MYC-tag and THOC5 antibodies. Ba/F3 MPL W515L THOC5 Y225F clearly express a MYC-tag and display increased THOC5 expression as compared to endogenous levels in Ba/F3 MPL W515L cells (Figure 3.7).

![Western blot analysis of THOC5 and THOC5 Y225F MYC-tag expression](image)

Expression levels of endogenous and transfected THOC5 Y225F was assessed by western blot analysis on whole cell lysates using antibodies to THOC5 and MYC-tag. Actin was used as a loading control.
3.5.5 THOC5 Y225F expression reduces the MPL W515L induced chemokinesis

The effects of THOC5 Y225F expression on cell motility in the Ba/F3 MPL W515L expressing cells was assessed using a Boyden chamber assay. Interestingly, the Ba/F3 MPL W515L THOC5 Y225F expressing cells displayed significantly decreased chemokinesis and chemotaxis in the presence of CXCL12 (p<0.001) (Figure 3.8) when compared to Ba/F3 MPL W515L cells. So, to further understand the role of THOC5 phosphorylation in Ba/F3 MPL W515L induced changes in motility, I investigated the signalling pathways that our group have previously linked to THOC5 and motility (Griaud et al., 2013). In addition, a quantitative proteomic screen of Ba/F3, Ba/F3 MPL W515L, and Ba/F3 MPL W515L THOC5 Y225F expressing cells was performed to gain insight into the pathways which may contribute to these motility changes (discussed further in Chapter 4). Given I observed such a large difference in chemokinesis, I investigated the CXCL12/CXCR4/CD45/THOC5 pathway in an attempt to understand the mechanism of motility stimulus in MPL W515L expressing cells.

![Figure 3.8: THOC5 plays a role in MPL W515L induced motility.](image)

The CXCL12 induced chemotactic response of control MPL W515L and MPL W515L THOC5 Y225F expressing cell was assessed in Boyden chamber assays. 1x10^5 cells were added to the top well and 200ng/mL CXCL12 to bottom well were indicated (-/+)The number of cells in the bottom well was counted after 6 hours incubation. Cell viability in the top well was also assessed after 6 hours. Data shown are the mean ± SEM from three experiments done in triplicate. Significance differences from controls were determined by One-way ANOVA with multiple comparison Turkey post test whereby p<0.05 was considered significant. **p<0.01, ***p<0.001, ****p<0.0001, ns-not significant.
3.6 Modulation of the CXCL12/CXCR4/CD45 axis in Ba/F3 MPL W515L expressing cells

3.6.1 CXCL12/CXCR4 affects motility

CXCL12 and its receptor CXCR4 have recently sparked substantial interest because of their role in tumorigenesis inducing multiple signal transduction pathways including tumor growth, invasion, and metastasis (Teicher and Fricker, 2010). This chemokine and its receptor mediate a variety of cellular functions such as cell migration, proliferation, and survival. Indeed, there is supporting evidence for the essential role of CXCR4 in enhance invasion in several types of cancer (Furusato et al., 2010). Our initial results had displayed that Ba/F3 MPL W515L showed a significant increase in chemokinesis (in the absence of CXCL12) compared to control cells (Figure 3.4). To further understand this mechanism, the migration assays was performed to study the role of CXCL12 in the increase in spontaneous movement (chemokinesis) in Ba/F3 MPL W515L expressing cells. CXCL12 (200 ng/mL) was added to either the top (+/-) or bottom well (-/+)) in a Boyden chamber (Figure 3.9). The Ba/F3 MPL W515L cells displayed an increase in chemokinesis (more than 70%) as compared to Ba/F3 cells in the absence of CXCL12 (p<0.0001). However, addition of CXCL12 to the top well significantly decreased the cell migration (40%) (p<0.01) in Ba/F3 MPL W515L cells as compared to the absence of CXCL12 or its addition to the bottom well. This results indicates that the CXCL12 response to CXCR4 receptors is not lost in Ba/F3 MPL W515L expressing cells. To further elucidated the role of CXCR4/CXCL12 effects on motility, I next measured the CXCR4 expression in both Ba/F3 and Ba/F3 MPL W515L expressing cells. The number of cells expressing CXCR4 on the cell surface was reduced in Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cells (p<0.0001) (Figure 3.10). This result was also reflected with a reduction in overall level of CXCR4 expression (40-47kDa) assessed on whole cell lysates by western blot (Figure 3.11).
Figure 3.9: CXCL12 effects on motility in MPL W515L expressing cells. The CXCL12 (200ng/mL) induced chemotactic response of Ba/F3 and Ba/F3 MPL W515L expressing cells was assessed in Boyden chamber assays by adding CXCL12 to either the top (+/-) or bottom (-/+). 1x10^5 cells were added to the top well and CXCL12 as indicated. The number of cells in the bottom well was counted after 6 hours incubation. Data shown are the mean ± SEM from three experiments done in triplicate. Significance differences from controls were determined by One-way ANOVA with multiple comparison Turkey post test whereby p<0.05 was considered significant. **p<0.01, ***p<0.001, ****p<0.0001.

Figure 3.10: Cell surface expression of CXCR4. The cell surface CXCR4 expression was assessed using FACS calibur 3 color flow cytometer on Ba/F3, Ba/F3 THOC5 Y225F, Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cells. Results were analysed using FlowJO software and expressed as the number of positive staining cells +/-SEM of three experiments. Significance differences from controls were determined by One-way ANOVA with multiple comparison Turkey post test whereby p<0.05 was considered significant. ***p<0.001, ****p<0.0001.
3.6.2 Involvement of CD45 in the motility pathway

Since our group (Griaud et al., 2013) indicated a role for the CXCR4/CXCL12/CD45 axis in changes to motility induced by oncogenic PTKs via THOC5, I also investigated the effects of MPL W515L on CD45 and THOC5 Y225 as well as in the THOC5 Y225F mutant transfected cells. Using CD45-null/wild-type mast and Lin− cells, our laboratory have shown that CD45 phosphatase is required for Src activation (as determined by its autophosphorylation on tyrosine 416) and THOC5 phosphorylation on tyrosine 225 (Griaud et al., 2013). Our group also identified a novel phosphorylation event on CD45 that is required for CXCL12-induced chemotaxis in primitive haematopoietic cells (Williamson et al., 2013). Thus, these data have consolidated a physiological pathway between CD45 mediated Src activation potentiating THOC5 phosphorylation and leukaemogenic PTKs. The number of cells expressing cell surface CD45 was measured by flow cytometry. Ba/F3 MPL W515L cells display a reduced level of CD45 as compared to Ba/F3 control cells, p < 0.0001 (Figure 3.12) and this also reflected by western blot analysis of whole cell lysates showing reduced overall level of CD45 expression (180-220 kDa) (Figure 3.13). Given that haemopoietic progenitors from CD45null mice display a decreased response to CXCL12 but no change in chemokinesis, it can be concluded that the effects of MPL W515L on chemokinesis are perhaps independent of these changes in CD45 expression (Whetton A.D, Cadeco, S; personal communication).
**Figure 3.12: Cell surface expression of CD45.** The cell surface CD45 expression was assessed using FACS calibur 3 color flow cytometer on Ba/F3, Ba/F3 THOC5 Y225F, Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cells. Results were analysed using FLOWJO software and expressed as the mean fluorescence intensity +/-SEM (n=3). Significance differences from controls were determined by One-way ANOVA with multiple comparison Turkey post test whereby p<0.05 was considered significant. *p<0.05, ***p<0.001, ****p<0.0001.

**Figure 3.13: Western blot analysis of CD45 expression.** Expression levels of CD45 were assessed by western blot analysis on whole cell lysates of Ba/F3, Ba/F3 THOC5 Y225F, Ba/F3 MPL W515L, and Ba/F3 MPL W515L THOC5 Y225F expressing cells. Actin was used as a loading control. Representative blots from three independent biological replicates.
3.7 Screening of inhibition of signal transduction proteins to identify pathways linked to the increase in chemokinesis

Given the down modulation of CXCR4 and CD45 expression which important in motility, I undertook a screen of inhibition of signal transduction proteins to gain insight into which pathways may be contributing to the increased motility seen in the Ba/F3 MPL W515L expressing cells. Since MPL W515L leads to the constitutive activation of the PI3K and ERK pathway (Pulikkan et al., 2012), Src is involved in motility signalling via the CXCR4/CD45 axis (Griaud et al., 2013) and TGFβ involved in motility and myelofibrosis (Martyré, 1995), I chose inhibitors to these pathways. The effect of these inhibitors on chemokinesis in Ba/F3 MPL W515L expressing cells was measured in Boyden chamber assays as described by pre-incubation of the cells in the presence of the inhibitors for 2 hours. The number of cells migrating across the membrane in 6 hours was then assessed. The cell viability of Ba/F3 and Ba/F3 MPL W515L, post experiment was greater than 97% (Table 3.2). The results shown are the mean ± SEM of three experiments. JAK2 inhibition by ruxolitinib can be seen to totally abolish the chemokinetic behaviour in Ba/F3 MPL W515L expressing cells. Of the inhibitors studied, only MEK and TGFβ inhibition had a significant effect (p<0.001) (Figure 3.14). The JAK2 activation was suggested to be downstream effector of MPL W515L oncogene as treatment with ruxolitinib strongly inhibited THOC5 Y225 phosphorylation (unpublished observation).

Table 3.2: Cell viability effects on inhibitors treatment. The number of cells in the top well were counted at 6 hours post motility assay. Data shown are the mean ± SD from three independent experiments (each in triplicates).

<table>
<thead>
<tr>
<th>Cell treatment with/out inhibitors</th>
<th>Cells viability (mean)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba/F3 no addition</td>
<td>98.7%</td>
<td>0.3%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L no addition</td>
<td>98.1%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L + PI3Ki</td>
<td>97.9%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L + SRCi</td>
<td>97.8%</td>
<td>0.9%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L + RACi</td>
<td>97.6%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L + MEKi</td>
<td>97.8%</td>
<td>0.9%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L + Ruxolitinib</td>
<td>98.5%</td>
<td>0.6%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L + TGFβi</td>
<td>97.7%</td>
<td>0.2%</td>
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Figure 3.14: Differential effects of tyrosine kinase inhibitors on Ba/F3 and Ba/F3 MPL W515L cell migration. 1x10^5 cells pre-treated for 2 hours with the inhibitors shown were used in Boyden chamber assay. The inhibitors used were 10 µM PI3Ki (LY294002), 20 µM Src inhibitor, SRCi (SU6656), 10 µM Mek 1/2 inhibitor (U0126), 5 µM TGFβ inhibitor, TGFβi (LY364947) and 50 µM of JAK2 inhibitor ruxolitinib (INCB018424). The number of cells that had migrated into the bottom well were counted after 6 hours. Data shown are the mean ± SEM from three experiments done in triplicate. Significance differences from controls were determined by One-way ANOVA with multiple comparison Turkey post test whereby p<0.05 was considered significant. **p<0.01, ***p<0.001, ****p<0.0001.

3.8 TGFβ measurement in Ba/F3 MPL W515L expressing cells

Our preliminary proteomic studies found that TGFβ is up-regulated by leukaemogenic oncogenes (Pierce et al., 2012). Of note, there was also an indication in one LC/MSMS iTRAQ experiment that this cytokine was elevated by MPL W515L expression. Given the effect of TGFβ inhibitor in motility (Figure 3.14) and its role in MPN myelofibrosis (Martyré, 1995), I sought to investigate the levels of TGFβ in the extracellular media of MPL W515L expressing cells. This experiment was performed by using ELISA assays on the level of TGFβ in cell culture supernatant of Ba/F3 MPL W515L expressing cells. Interestingly, the ELISA assessment showed a marked increase in the secretion of TGFβ (Figure 3.15) which leads to an increase in chemokinesis observed in MPL W515L expressing cells.
Figure 3.15: MPL W515L expression induces TGFβ secretion. The TGFβ levels in the cell culture supernatants of Ba/F3 and Ba/F3 MPL W515L expressing cells were measured using the Quantikine ELISA from R&D systems (Abingdon, UK). Results are displayed as pg/ml of cell culture supernatant. Data shown are the mean ± SEM from three experiments done in triplicate.

3.9 Assessment on TGFβ effects on THOC5 Y225 phosphorylation

Having observed that THOC5 Y225 phosphorylation and TGFβ were involved in the MPL W515L induced motility, the next experiment undertaken was to determine the effect of TGFβ on THOC5 Y225 phosphorylation. The Ba/F3 cells were treated with 5 ng/mL of TGFβ and the whole cell lysates prepared and assessed by western blot. The result shows that THOC5 Y225 phosphorylation displayed a time dependent response to the addition of TGFβ reaching maximum at 20 min of induction and reduced again at 60 min treatment (Figure 3.16). To gain insight into TGFβ regulation in MPL W515L oncogenes, I then treated the Ba/F3 MPL W515L with 5 µM TGFβi to confirm their activities in MPN oncogenes. The result clearly indicate that TGFβ inhibition reduced the level of THOC5 Y225 phosphorylation (Figure 3.17).
**Figure 3.16: Assessment of THOC5 Y225 phosphorylation following TGFβ treatment.**
Ba/F3 cells were treated with TGFβ1 (5 ng/mL) for the times shown and the effects on THOC5 phosphorylation at Y225 assessed by western blot analysis of whole cell lysates. Actin was used as a loading control. Representative blots from three independent biological replicates.

**Figure 3.17: Assessment of THOC5 Y225 phosphorylation following TGFβ inhibition.**
Cells were treated with 5 µM TGFβi for the 6 hours and the effects on THOC5 phosphorylation at Y225 assessed by western blot analysis of whole cell lysates. Actin was used as a loading control. Representative blots from three independent biological replicates. N/A; DMSO (carrier control).
3.10 Targeting MYC in MPL W515L expressing cells affects motility via THOC5

3.10.1 MPL W515L oncogenes effects on MYC expression

Having demonstrated the effects of Ba/F3 MPL W515L on TGFβ levels and THOC5 phosphorylation, and given the ability of THOC5 to modulate MYC expression (Griaud et al., 2013; Tamura et al., 1999; Tran et al., 2014b), I sought to investigate whether the Ba/F3 MPL W515L expressing cells had any effect on MYC expression. Based on western blot analysis, the level of MYC was increased in the Ba/F3 MPL W515L expressing cells and reduced in the Ba/F3 MPL W515L THOC5 Y225F cells (Figure 3.18). This result perhaps indicate that THOC5 Y225 phosphorylation modulates MYC expression affects motility in MPN associated oncogenes. This observation led to further studies on the THOC5 Y225 phosphorylation and motility effects on MYC expression using the MYC inhibitor, JQ1.

![Figure 3.18: Western blot analysis on c-MYC expression](image)

**Figure 3.18: Western blot analysis on c-MYC expression.** The whole cell lysates of Ba/F3, Ba/F3 MPL W515L & Ba/F3 MPL W515L THOC5 Y225F expressing cells were prepared for western assessment on c-MYC antibodies. Actin was used as a loading control (n=3).

3.10.2 Effects of JQ1 bromodomain inhibitor on MYC and THOC5 expression

JQ1, also known as BET inhibitor targeting the bromodomain (BRD) and extra-C terminal domain, (BET) targets c-MYC and offers an effective therapeutic-benefit in diseases involving up-regulation of MYC (Filippakopoulos et al., 2010; Zuber et al., 2011). Many studies conducted by different groups show that JQ1 treatment successfully represses MYC expression in various types of cancer (Delmore et al., 2011; Filippakopoulos et al., 2010; Mertz et al., 2011; Ott et al., 2012; Zuber et al., 2011). Therefore, the JQ1 BET bromodomain
inhibitor was chosen in this study to further evaluate the involvement of MYC regulation in MPN associated changes to motility and THOC5 phosphorylation. I demonstrated that treatment of Ba/F3 MPL W515L expressing cells for 6 hours with JQ1 (500 nM) result in reduction of the elevated MYC levels and THOC5 phosphorylation at Y225 (Figure 3.19).

![Figure 3.19: JQ1 inhibitor effects on THOC5 Y225 phosphorylation and c-MYC expression.](image)

Given the effect of JQ1 on reducing the level of MYC and THOC5 phosphorylation in MPL W515L expressing cells, I performed a motility assay in the presence of JQ1 to observe any effects on chemokinesis in Ba/F3 and Ba/F3 MPL W515L expressing cells. The Boyden chamber assay was performed as described earlier. The cell viability of Ba/F3 and Ba/F3 MPL W515L, post 6 hours treatment with JQ1 inhibitors was greater than 97% (Table 3.3). Interestingly, the number of cells migrating substantially decreased in Ba/F3 MPL W515L expressing cells upon addition of the MYC inhibitor JQ1 as compared to non-treated Ba/F3 MPL W515L cells (40%, p <0.001) (Figure 3.20). The results obtained provide information on the disrupted pathways in MPL W515L expressing cells and provide further data to identify therapeutic targets in MF.
Table 3.3: Cell viability effects on JQ1 inhibition. The number of cells in the top well were counted at 6 hours post motility assay. Data shown are the mean ± SD from three independent experiments (each in triplicates).

<table>
<thead>
<tr>
<th>Cell treatment with/out inhibitors</th>
<th>Cells viability (mean)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba/F3 no addition (-/-)</td>
<td>98.7%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Ba/F3 no addition (-/+ CXCL12)</td>
<td>98.0%</td>
<td>0.3%</td>
</tr>
<tr>
<td>Ba/F3 + JQ1 (+/-)</td>
<td>97.9%</td>
<td>1.1%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L no addition (-/-)</td>
<td>98.3%</td>
<td>0.4%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L no addition (-/+ CXCL12)</td>
<td>97.8%</td>
<td>0.5%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L + JQ1 (+/-)</td>
<td>98.0%</td>
<td>1.0%</td>
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Figure 3.20: MYC plays a role in the MPL W515L induced motility. Cells were treated with 500 nM of the BET bromodomain inhibitor JQ1 for two hours prior to undertaking a Boyden chamber assay. 1x10^5 were added to the top well. 200 ng/mL of CXCL12 was added to the bottom well where indicated (-/+). The number of cells migrating into the bottom well was counted after 6 hours incubation. Data shown are the mean ± SEM from three experiments done in triplicate. Significance differences from controls were determined by One-way ANOVA with multiple comparison Turkey post test whereby p<0.05 was considered significant. ***p<0.001, ****p<0.0001.
3.10.3 Suppression of MYC expression with siRNA

3.10.3.1 Optimization of esiRNA MYC concentration

Since JQ1 is not a MYC specific inhibitor, I sought to underline these data on the role of MYC using a siRNA, gene silencing approach. This was achieved by using esiRNA MYC transfection prior to undertaking a Boyden chamber assay. Initial experiments were undertaken to optimise esiRNA concentration and identify the time point for optimum suppression of MYC. The esiRNA MYC concentrations tested were 130 nM, 260 nM, and 380 nM based on the calculation recommended by the manufacturer. Suppression of c-MYC at the protein level was confirmed by western blot (Figure 3.21). There is no MYC inhibition in MPL W515L at 24 hours post siRNA transfection, however the MYC expression at 48 hours of post transfection was decreased (Figure 3.21). From these experiments an esiRNA MYC concentration of 260 nM and a time point of 48 hours were chosen for further experiments. As with the studies using JQ1, siRNA supression of MYC in the MPL W515L expressing cells led to a decrease in the elevated levels of THOC5 phosphorylation (Figure 3.22). No such effects were observed in non-treated and negative control cells within 48 hours treatment.

![Western blot analysis of c-MYC expression on esiRNA MYC treated cells.](image)

The transient transfected cells using Amaxa Nucleofactor® technology were subjected to no treatment (no addition; N/A), negative control (NC) treated with siRNA sequences Ambion® Silencer® Select (#2 Ambion) and esiRNA MYC 130 nM, 260 nM, and 380 nM for 24 hours and 48 hours. Actin was used as a loading control.
Figure 3.22: Western blot analysis of c-MYC, THOC5 and phospho Y225 THOC5 expression on esiRNA MYC treated cells. Cells were transfected using Amaxa Nucleofactor® technology with no treatment (no addition; N/A), negative control (NC) esiRNA sequences Ambion® Silencer® Select (#2 Ambion) and 260 nM esiRNA MYC. Cell lysates were produced 48 hours post transfection are subject to western blot analysis. Actin was used as a loading control. Representative blot from three independent biological replicates.

### 3.10.3.2 eSIRNA MYC knockdown alters cell motility

Given the effects of JQ1 MYC inhibitor and MYC gene silencing leads to decrease in the elevated levels of THOC5 phosphorylation in MPL W515L expressing cells and the ability of Ba/F3 MPL W515L in modulating c-MYC expression and chemokinesis, I decided to investigate the knockdown effects of MYC in Ba/F3 MPL W515L expressing cells on motility. Therefore, by using the cells at 48 hours esiRNA MYC post transfection, the motility assay was performed using a Boyden chamber assay. As previously, the Ba/F3 MPL W515L expressing cells showed an increase in chemokinesis (89%, p<0.001) as compared to Ba/F3 (-/-), however, with esiRNA MYC knockdown the migration ability of Ba/F3 MPL W515L expressing cells was restricted to 40% without any CXCL12 stimuli (+/-) whilst 49% with presence of CXCL12 (+/+), (p<0.01) respectively when compared to non-treated Ba/F3 MPL W515L (-/-) (Figure 3.23). In summary, all the above observations have consolidated the previous novel
pathway depicted that MPL W515L oncogene modulate THOC5 Y225 phosphorylation possibly through MYC activation which induces motility.

Figure 3.23: Motility assay on Ba/F3 and Ba/F3 MPL W515L cells treated with esiRNA MYC. 1x10⁵ cells 48 hours post transfection with esiRNA MYC were added to the top well of a Boyden chamber assay. 200 ng/mL of CXCL12 was added to bottom well were indicated (-/+) The number of cells migrating into the bottom well was counted after 6 hours incubation. All data were obtained from three independent experiments. Error bars represent means ± SEM. Significance differences from controls were determined by One-way ANOVA with multiple comparison Turkey post test whereby p<0.05 was considered significant. *p<0.01, **p<0.001, ****p<0.0001.

3.11 SK regulation in MPL W515L linked to THOC5 Y225 phosphorylation, MYC and motility

Our studies have demonstrated that inhibition of TGFβ led to a partial reduction in chemokinesis in the MPL W515L expressing cells (Figure 3.14). I have also shown that Ba/F3 MPL W515L expressing cells display increased TGFβ secretion and TGFβ effects have been linked to chemokinesis. Several studies have shown a connection between TGFβ and S1P signalling pathways. For instance, studies by Miller et al. (2008) showed crosstalk between TGFβ1 and S1P signalling promotes migration and invasion of esophageal cancer via activation of ERK signalling pathway (Miller et al., 2008). Milara et al. (2012) concluded that TGFβ may activate and upregulate S1P contributing to lung fibrosis and myofibroblast progression (Milara et al., 2012). In addition, our group have previously published on the differential effects of S1P on the motility of primitive haematopoietic cells. There is possibility
that TGFβ activates a secondary pathway in the MPL W515L expressing cells. To further understand the motility behavior modulation by MPL W515L oncogene, I were keen to explore the role of SK and the bioactive sphingolipid; S1P since they have been implicated in the regulation of cell migration and cytoskeleton organization (Calise et al., 2012; Donati and Bruni, 2006; Spiegel and Milstien, 2000).

The results in Figure 3.24 again demonstrate that Ba/F3 MPL W515L expressing cells display increased THOC5 phosphorylation at Y225 and MYC expression. In addition, treatment with sphingosine kinase inhibitor (SKi) at 10 µM both reduced the THOC5 phosphorylation and c-MYC expression (no effects are seen on cells treated with the SKi carrier DMSO). These results led us to further investigate the role of sphingolipid in MPL W515L effects on motility. To look at effects of sphingosine kinase inhibition on the increased chemokinesis in Ba/F3 MPL W515L expressing cells, I undertook the migration assay as previously described via Boyden chamber assay (Whetton et al., 2003). The Ba/F3 and Ba/F3 MPL W515L expressing cells were treated with SKi (10 µM) for 2 hours prior to undertaking the migration assay. Interestingly, in the presence of SKi, the motility response in Ba/F3 MPL W515L expressing cells was reduced drastically either in the absence (+/-) or presence of CXCL12 (+/+), p <0.0001, n=3 (Figure 3.25). The cell viability of Ba/F3 and Ba/F3 MPL W515L, post 6 hours treatment with SKi was greater than 98% (Table 3.3). It could be hypothesised that sphingosine pathway mediated MYC elevation and THOC5 Y225 phosphorylation played a role in Ba/F3 MPL W515L increased chemokinesis.
Figure 3.24: THOC5, THOC5 Y225 phosphorylation and c-MYC expression following sphingosine kinase inhibitor (SKI) treatment. Ba/F3 and Ba/F3 MPL W515L expressing cells were incubated with SKi at 10 µM for 6 hours. The level of THOC5 and phospho Y225 THOC5 and c-MYC was determined in whole cell lysate by western blot analysis. Actin was used as a loading control. Representative blot from three independent biological replicates. N/A: no addition of treatment (control).

Figure 3.25: Effect of SKi on MPL W515L induced chemokinesis. Cells were treated with Sphingosine kinase inhibitor (10 µM) for 2 h prior to undertaking the Boyden chamber assays. 1x10^5 were added to the top well. 200 ng/mL of CXCL12 was added to bottom well were indicated (-/+). The number of cells migrating into the bottom well were counted after 6 hours. Data shown are the mean ± SEM from three experiments done in triplicate. Significance differences from controls were determined by One-way ANOVA with multiple comparison Turkey post test whereby p<0.05 was considered significant. ****p<0.0001.
Table 3.4: Cells viability effects on SK inhibition. The number of cells in the top well were counted at 6 hours post motility assay. Data shown are the mean ± SD from three independent experiments (each in triplicates).

<table>
<thead>
<tr>
<th>Cell treatment with/out inhibitors</th>
<th>Cells viability (mean)</th>
<th>SD</th>
</tr>
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<tbody>
<tr>
<td>Ba/F3 no addition (-/-)</td>
<td>99.2%</td>
<td>0.3%</td>
</tr>
<tr>
<td>Ba/F3 no addition, CXCL12 (-/+</td>
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<td>0.7%</td>
</tr>
<tr>
<td>Ba/F3 + SKi (+/-)</td>
<td>98.2%</td>
<td>1.2%</td>
</tr>
<tr>
<td>Ba/F3 + SKi, plus CXCL12 (+/+</td>
<td>98.0%</td>
<td>1.2%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L no addition (-/-)</td>
<td>97.8%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L no addition, CXCL12 (-/+</td>
<td>98.6%</td>
<td>0.6%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L + SKi (+/-)</td>
<td>97.8%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Ba/F3 MPL W515L + SKi, plus CXCL12 (+/+</td>
<td>97.6%</td>
<td>0.7%</td>
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3.12 Characterisation of the role of S1P on motile response and expression of THOC5 Y225 phosphorylation and MYC in MPL W515L expressing cells

Since sphingosine kinase is responsible for the production of S1P by phosphorylation of sphingosine to form S1P, and THOC5 phosphorylation was down-regulated following SK inhibitor treatment, I am interested to know the effects of S1P on THOC5. THOC and MYC expression was down-regulated following 6 hours treatment with different S1P concentrations; 1 µg/mL, 2.5 µg/mL and 5.0 µg/mL (Figure 3.26). To understand the S1P effects on motility I undertook the migration studies using Boyden chamber assay as previously described (Whetton et al., 2003). The addition of a S1P gradient did not enhance motility in Ba/F3 cells, yet reduced the elevated chemokinesis to about 50-70% when compared with no addition (S1P) in Ba/F3 MPL W515L cells (p<0.0001) (Figure 3.27). These results suggests that Ba/F3 MPL W515L cells increased chemokinesis without the influence of extracellular action of S1P gradient. As S1P is known to have dual functions signalling inside and out of the cells and sphingosine kinase have implicated in the formation of S1P, we measured the intracellular levels of S1P in MPL W515L oncogenes.
Figure 3.26: THOC5, THOC5 Y225 phosphorylation and c-MYC expression following S1P treatment. Ba/F3 and Ba/F3 MPL W515L expressing cells were incubated with S1P at 1 µg/mL, 2.5 µg/mL and 5.0 µg/mL for 6 hours. The level of THOC5, phospho Y225 THOC5 and c-MYC expression was determined in whole cell lysate by western blot analysis. Representative blot from three independent biological replicates Actin was used as a loading control.

Figure 3.27: Effects of S1P on MPL W515L in motility. Cells were treated with sphingosine-1-phosphate (S1P) (5 µg/mL) for 2 h prior to undertaking a Boyden chamber assays. 1x10^5 cells were added to the top well. The number of cells migrating into the bottom well was counted after 6 hours incubation. Data shown are the mean ± SEM from three experiments done in triplicate. Significance differences from controls were determined by One-way ANOVA with multiple comparison Turkey post test whereby ****p<0.0001.
3.12.1 S1P measurement in cells expressing MPN associated oncogenes

Previously, several studies found that exogenous S1P inhibits motility in various cancer cells either through cell surface receptor binding or via intracellular mechanisms (Sadahira et al., 1992; Wang et al., 1999b). Having demonstrated that addition of S1P in the top well as well as in the bottom well partially reduced the number of MPL W515L cells migrating across the transwell, whilst addition of sphingosine kinase inhibitor substantially inhibited motility in Ba/F3 MPL W515L expressing cells, it was of interest to determine how S1P has its effects in MPL W515L oncogenes. Therefore, to link the S1P, THOC5 phosphorylation and motility in MPN associated oncogenes, the levels of S1P was measured in Ba/F3 and Ba/F3 MPL W515L expressing cells as well as in both Ba/F3 THOC5 Y225F and Ba/F3 MPL W515L THOC5 Y225F. The results are shown in Figure 3.28. Interestingly, this result demonstrated that the intracellular level of S1P was significantly higher in Ba/F3 MPL W515L (p<0.01) as compared to Ba/F3 and the mutant THOC5 Y225F expressing cells, which is in line with the reduction in chemokinesis observed in these cells (Figure 3.8). These findings correlate with the western blot result in Figure 3.24 showing that Ba/F3 MPL W515L treated with SKi display decreased expression of THOC5 Y225 phosphorylation. Activation of SK by a variety of agonists increases intracellular S1P, thus MPL W515L is apparently mimicking the effects of receptor mediated signalling activated by a number of different ligands (Takabe et al., 2008). From all the results obtained, it can be suggested that sphingosine governs THOC5 pathway and MYC activation via endogenous S1P which perhaps influences cell migration in MPL W515L oncogenes.
Figure 3.28: S1P analysis in whole cell lysates of Ba/F3 and Ba/F3 MPL W515L expressing cells. The levels of S1P were measured using LC/ESI-MS/MS and the results expressed as pmol/10^7 cells. Data shown are the mean ± SEM from six experiments (n=6). Ba/F3 MPL W515L expressing cells shows a significance differences from control as determined by One-way ANOVA with multiple comparison Turkey post test. *p<0.05 was considered significant. **p<0.01.

3.13 Validation of cell line based observations on MPL W515L patient material

Studies were performed to confirm and validate our cell line based observations on myelofibrosis patient material obtained from Cambridge Blood and stem cell Biobank, UK. Peripheral blood CD34^+ cells from healthy individuals/ normal group as well as from MPL W515L patient material were obtained using immunoaffinity chromatography with magnetic beads (MACS® cell isolation kits; Miltenyi Biotec). Due to limited sample, experiments were only performed to confirm the enhanced motility behavior and study the effect of inhibition of the TGFβ and MYC pathways on the colony forming ability of MPL W515L expressing cells. The results are shown in Figure 3.29-3.30. The motility behaviour of myelofibrosis patient material and that of the normal group (non-myelofibrosis patients) was assessed using a Boyden chamber method as previously described (Whetton et al., 2003). The number of cells migrating to the bottom well were counted after 6 hours incubation. The CD34^+ cells of MPL W515L patients showed a greater than 2-fold increase in chemokinesis as compared to normal group (Figure 3.29). This is in agreement with the motility result observed in Ba/F3 MPL W515L expressing cells versus Ba/F3 control cells (Figure 3.4). Having validated our cell line
model based observations in patient material, we interested to know whether the disrupted pathways offered any therapeutic value. This was assessed in colony forming assays in the presence of inhibitors to both TGFβ and MYC. These experiment demonstrated a marked and specific decrease in the colony forming ability of MPL W515L patient material in the presence of the MYC inhibitor JQ1 (Figure 3.30). Indeed, this corroborates the data I saw in the initial observations from MPL W515L cell line analyses to actual clinical material. However, TGFβ inhibition does not inhibit colony forming ability of CD34\(^*\) cells from MPL W515L patient material (Figure 3.30). Further work using more patient materials is underway to validate which pathway potentially offers novel therapeutic targets in myeloproliferative neoplasms.

![Figure 3.29: MPL W515L induced chemokinesis in patient material.](image)

Chemokinesis of CD34\(^*\) cells from MPL W515L positive and non-leukaemic patients was assessed using Boyden chamber assay in 96-transwell plates. 3 x 10\(^4\) CD34\(^*\) cells in 30 µL of media (Fisher 10% (v/v) horse serum) were place in the top well and 30 µL media in the bottom well. Plates were incubated in humidified 37°C, 5% (v/v) CO\(_2\) incubator for 6 hours and the number of cells migrating into the bottom well counted. Results shown are the number of cells migrating (mean +/- SEM, n=3). Significance differences from normal controls were determined by Student T-test whereby p<0.05 was considered significant.
Figure 3.30: Colony forming ability in patient material for TGFβ and JQ1 inhibitor. The colony forming assay of CD34+ cells from MPL W515L positive and non-leukaemic patients were assessed by resuspended 1 x 10^3 CD34+ in 1 mL human methylcellulose complete media with Epo (R&D systems). 250μL of CD34+ (in triplicates) were place in a 24 well plate. Plates were incubated in humidified 37°C, 5% (v/v) CO2 incubator. Results shown are the number of colonies of 50+ cells counted at 14 days (mean +/- SEM, n=3).
3.14 Discussion

Myelofibrosis and thrombocytosis are the hallmark of the mutated thrombopoietin receptor MPL W515L. A proteomic screen of MPL W515L oncogene expressing cells was undertaken. The proteomic data set revealed that within the proteins identified as changing due to MPL W515L expression, there was a marked enrichment of proteins involved in motility. Further analysis indicate a role for THOC5, a member of the TREX complex in mRNA splicing/export pathway involved in the leukaemic transformation process. Our group have previously published that THOC5 Y225 is a downstream target of many leukemogenic PTK (Pierce et al., 2008) and THOC5 Y225 phosphorylation was increased following CXCL12 stimulation (Griaud et al., 2013). In addition, our existing works has elucidated a novel pathway of THOC5 Y225 phosphorylation downstream of MPL W515L and JAK2 mutants. Taken together our previous result and present study, I am now moving forward in developing an understanding on THO complex modulation by tyrosine kinase oncogenes in leukaemias and chemotactic factors that control stem cell retention in the bone marrow.

In this study STAT5 was identified and quantitated as changing. The western blot results reported here support these studies for the involvement of STAT3/5 signal transduction downstream of MPL W515L activity. A number of previous studies suggest that the JAK2-STAT5 and STAT3 play an important role in the biology and evolution of MPL W515L driven thrombocytosis and myelofibrosis (Pikman et al., 2006; Tefferi, 2010). Recent studies suggest that the JAK2-STAT5 pathway has potential therapeutic value as a target in leukaemic stem cells (LSCs) in CML which have developed resistance against tyrosine kinase inhibitors such as imatinib (Gallipoli et al., 2014; Valent, 2014). Our proteomic and western blot results suggest that MCL-1 is also up-regulated by MPL W515L expression. MCL-1 or myeloid cell leukaemia-1 is a member of the antiapoptotic Bcl2 family and originally identified in the myeloid leukaemia (ML-1) of human myeloblastic leukaemia whereby its expression is increased in early myeloid differentiation (Bingle et al., 2000; Kozopas et al., 1993). The expression of MCL-1 is highly induced by multiple mechanisms and a number of previous studies have shown that MCL-1 serves as pro-survival agent in many neoplastic cells (Longo
et al., 2008; Puthier et al., 1999). MCL-1 is a STAT transcriptional target (Epling-Burnette et al., 2001). MCL-1 function is also controlled by phosphorylation resulting in increased expression and cytokine stimulation (Bingle et al., 2000). For instance IL-6 up-regulates MCL-1 transcription through a STAT3 pathway in multiple myeloma (Puthier et al., 1999). Recently, it has been reported that JAK2 V617F mutation increase MCL-1 transcription via STAT3 signalling since treatment with JAK2 inhibitor sensitised the cells to apoptosis (Guo et al., 2015).

This thesis demonstrated a major disruption of the CXCL12/CXCR4/CD45/Src axis by MPL W515L and this is associated with increased chemokinesis. This observation fits with the pathology of the disease in that patients with MF display a mobilisation of haemopoietic progenitors from the bone marrow and up-to a 200-fold increase in the number of circulating CD34+ cells have been reported (Barosi et al., 2001; Castro-Malaspina et al., 1981). The role of the CXCR4/CXCL12 axis in the directional movement of cells has been demonstrated in various cancers, including small-cell lung cancers, pancreatic cancers, myelomas, B cell lymphomas, chronic lymphocytic leukaemias, kidney cancer and rhabdomyosarcoma (Balkwill, 2004). It has been proposed that this increase results from several mechanisms including a reduced CXCR4 expression on CD34+ cells (Cho et al., 2010; Rosti et al., 2007) related to a hypermethylation of the CXCR4 promoter (Bogani et al., 2008). Further disruption of the CXCR4/CXCL12 trafficking has been attributed to dysregulation of the bone marrow environment in myelofibrosis owing to several events including over production of a variety of proteases such as neutrophil elastase, matrix metalloproteinase-2 (MMP-2), MMP-9 and cathepsin G. These abnormalities produce truncated CXCL12 which results in a reduction of CXCR4 leading to a constitutive mobilisation of CD34+ cells associated with MPN (Cho et al., 2010; Ciurea et al., 2007; Xu et al., 2005a). Gene expression analysis of the consequences of GivinoSTAT treatment (a drug with antiproliferative and proapoptotic activity against MPN cells which target STAT5), demonstrates that 25% of the change genes were associated with motility and adhesion (Amaru Calzada et al., 2012). The fact that MPL W515L influences motility can be viewed as fitting physiologic function of thrombopoietin as it stimulates migration in hepatoblastoma cells by a mechanism involving both chemotaxis and
chemokinesis (Romanelli et al., 2006). Of note from these studies however is that PI3K was required for the induction of migration in response to TPO which is not the case in our studies as inhibition of PI3K does not reduce the effects on motility. Furthermore, in this present study, I found that addition of CXCL12 in the top well of Ba/F3 MPL W515L cells has slightly reduced the cell migration. This indicates that the CXCL12 response is not lost in Ba/F3 MPL W515L expressing cells. Study by Zabel et al., who suggested that the reduction in CXCL12-triggered migration by additional CXCL12 within the cells could possibly be explained by the desensitization of CXCR4 or disruption of the chemokine gradient by ectopic CXCL12 (Boudot et al., 2014; Zabel et al., 2009). I have measured the number of CXCR4 positive cells by flow cytometry. MPL W515L expression led to a decrease in the CXCR4 expression. This is in line with studies on another MPL oncogene by Geay et al, who demonstrated that elevated expression of p210BCR/ABL inhibits CXCL12 chemotactic response via alteration and downregulation of CXCR4 expression. Furthermore, their studies also showed that BCR/ABL kinase inhibition rapidly reversed the down-regulation of CXCR4 in blast cells from CML patients (Geay et al., 2005). Our results indicate that MPL W515L expression alters the chemotactic response of cells via CXCL12/CXCR4 through a complex mechanism involving inhibitory effects on different signalling molecules. Given the importance of CD45 which lies downstream of CXCL12 induced motility (Shivtiel et al., 2011; Williamson et al., 2013), I analysed the effects of MPL W515L on CD45 expression. The MPL W515L expressing cells displayed a lower level of CD45 expression as compared to control cells. These counterintuitive observations prompted further investigation into the effects of MPL W515L on motility since our previous findings had shown that CD45 is required for CXCL12-induced chemotaxis as shown in CD45 null murine haematopoietic progenitor cells (Shivtiel et al., 2008; Williamson et al., 2013). It is feasible the remaining CD45 is highly active and contributes to the chemokinesis.

Given the decreased in CXCR4 and CD45 expression in MPL W515L expressing cells, I undertook an analysis of signal transduction pathways in an attempt to identify the pathways involved in the upregulation of the motile response of these cells. The results revealed the modulation of several pathways potentially involved in the MPL W515L induced change in
motility. Of the inhibitors studied, only JAK2 inhibitor, ruxolitinib, MEK inhibitor and TGFβ inhibitor displayed a significant inhibition in the number of cells migrating across the transwell membrane of a Boyden chamber. These results suggest the JAK-STAT and TGFβ pathway may contribute to the motile response to the MPL W515L oncogene. The mechanism underlying myelofibrosis associated with JAK2 induced MPN has been demonstrated to be associated with increased production of TGFβ by CD34+ progenitor cells that generates excessive number of megakaryocytes induced by thrombopoietin (Chagraoui et al., 2002; Le Bousse-Kerdilès et al., 1996) and also megakaryocytes in chronic myelogenous leukaemia (Martyré, 1995). However, the importance of TGFβ to the pathogenesis of myelofibrosis is still poorly understood. For example rather than a direct effect on fibroblasts, it has been suggested that TGFβ promotes tumorigenesis by the metabolic reprogramming of the tumour microenvironment changing the mitochondrial activity of adjacent cancer cells (Guido et al., 2012). Given its important in MPN myelofibrosis and motility, I investigated the potential upregulation of TGFβ in our model system. I showed that MPL W515L expressing cells display a significant increase in TGFβ secretion which plays a role in the increased chemokinesis observed. Further I showed that this TGFβ induced chemokinesis is achieved via phosphorylation of THOC5, a protein involved in RNA processing and export. Thus, MPL W515L oncogenes could induce a paracrine/autocrine set of effects via increased secretion of TGFβ leading to modulation of THOC5 phosphorylation and motility in this cell line studies. In addition, however, the studies on TGFβ SMAD2 receptor in MPL W515L expressing cells has no effect on phospho-SMAD2 expression (unpublished observation). Our group ongoing investigation into the inhibition of TGFβ production and secretion using our cell line model may hold promise for identifying compounds to target TGFβ production. Experiments are underway to study the activation of the changes in the CXCL12/CXCR4/CD45/Src/THOC5 pathway in primary cells using mass spectrometry.

Previously, our group and our collaborators (Teruko Tamura) have shown that THOC5 modulates MYC expression (unpublished observation). Given the initial observations of the effects of MPL W515L on THOC5 Y225 phosphorylation, I examined the link between THOC5 phosphorylation, MYC expression and motility. I demonstrated that MYC acts as modulator
regulating the THOC5 Y225 phosphorylation which in turn affects the motility in MPN associated oncogenes. MYC is an oncogenic transcription factor, which regulates cell growth and proliferation, cell cycle control, metabolic adaptation, survival and apoptosis. MYC is up-regulated in many cancer often as a result of gene amplification and chromosomal translocations. This aberrant expression leads to de-regulated MYC activity which contributes to leukaemia and lymphoma transformation (Albihn et al., 2010; Delgado and León, 2010; Hoffman et al., 1996; Ott et al., 2012). Given these facts, I attempted to define the mechanism and consequences of MYC regulation in the MPL W515L expressing cells to gain an insight into the therapeutic potential of targeting MYC in MPL mutant positive disease. Experiments were performed to confirm MYC regulation in MPL W515L oncogene expressing cells utilising the small molecule inhibitor thieno-triazolo-1,4-diazepine, JQ1, which has been reported to bind to the acetyl-lysine pocket of the conserved bromodomain and extra-terminal domain (BET) protein family (BRD2, BRD3, BRD4) (Filippakopoulos et al., 2010). Many studies confirmed that JQ1 treatment in MYC driven-malignancy such as in MLL-AF9-induced AML, Burkitt’s lymphoma, multiple myeloma, and osteosarcoma successfully abrogated the c-MYC regulation through displacement of BRD4 from the MYC gene promoter (Delmore et al., 2011; Lamoureux et al., 2014; Mertz et al., 2011; Zuber et al., 2011). JQ1 treatment of MPL W515L expressing cells reduced the oncogene induced elevation of c-MYC and THOC5 Y225 phosphorylation. Notably as JQ1 is not specifically a MYC inhibitor, I corroborate these observations by silencing c-MYC expression with esiRNA. As with JQ1 treatment, esiRNA targeting MYC led to reduced expression of c-MYC and THOC5 Y225 phosphorylation with inhibition of the MPL W515L induced chemokinesis. All the above findings demonstrated that the MYC pathway affects the motility mediated by THOC5 Y225 phosphorylation which may offer a novel therapeutic targets in MPN associated oncogene MPL W515L. A figure demonstrating our findings in Ba/F3 MPL W515L is shown below (Figure 3.3). As stated above I am now attempting to understand whether these effects occur in human disease.

Our group have previously published the differential effects of S1P on the motility of HSC populations (Whetton et al., 2003). Furthermore, another group have shown that there is cross talk between TGFβ signalling and S1P (Milara et al., 2012). In fact it appears that S1P is
involved in lung fibrosis as well as cardiac fibrosis via TGFβ activated S1P release (Gellings Lowe et al., 2009; Kono et al., 2007). Hence, to assess which other signalling pathways contribute towards the motility effects in MPN associated oncogenes, I investigated the sphingosine kinase/sphingosine-1-phosphate signalling pathway. I have shown earlier that expression of THOC5 Y225 was increased in MPL W515L oncogenes. In the present study, I am successfully showed that SKi treatment reduced the level of THOC5 Y225 phosphorylation as well as c-MYC expression in MPL W515L expressing cells. Strikingly, with the same treatment applied to the migration assay, SKi significantly inhibits motility. S1P has also been shown to have a crucial effect on motility, either strongly inducing or inhibiting cell migration in several cell lines (Van Brocklyn et al., 2003; Wang et al., 2008; Wang et al., 1999b). In this study, I found that MPL W515L expressing Ba/F3 cells treated with S1P reduced the THOC5 Y225 phosphorylation, c-MYC expression and from data with the THOC5 Y225F dominant negative mutation and MYC downregulation this is associated with chemokinesis. It still remains unclear how the S1P and SK regulates c-MYC expression, however, studies by Jiang et al. (2013) show that overexpression of SK stimulates MYC translation leading to increased intestinal epithelial cell proliferation (Jiang et al., 2013). Here I can conclude that S1P plays a role in MYC expression but does so via THOC5 phosphorylation and potentially contributes to leukaemogenesis by altering the motile behaviour of the MPL W515L expressing cells. Having identified the S1P effects on THOC5 and motility, I am interested in measuring the endogenous level of S1P in MPL W515L oncogenes by mass spectrometry. The result obtained shows that levels of S1P were significantly higher in Ba/F3 MPL W515L expressing cells compared to control cells. Perhaps this suggest that sphingosine kinase actively produced S1P intracellularly. This result is in contrast with the previous studies which demonstrated that an increase of endogenous S1P in human breast cancer cell lines; MCF-7 and MDA-MB-231, inhibits motility via overexpression of sphingosine kinase. In addition, they also indicated that a breast cancer cell line with high levels of intracellular S1P was associated with inhibited motility (Wang et al., 1999a; Wang et al., 1999b). In different studies, it was also found that at micromolar concentration, S1P inhibits motility of human breast cancer cell lines (MCF-7 and MDA-231) (Wang et al., 1999a). Furthermore, Yamamura et al. (1997) showed that addition of S1P also inhibits motility via cell surface receptor mediated effects in
melanoma cells studies. These differences highlight the possibility that S1P controlling cell motility in MPL W515L could be via sphingosine kinase produced S1P-induced intracellular action independent of cell surface receptors, or perhaps involving other signalling pathways that are as yet unclear. Therefore, it is important to explore further the S1P effect on motility to determine whether it is either mediated intracellularly or extracellularly by the MPN associated oncogene MPL W515L. Of note, however, it remains poorly understood whether S1P-induced inhibition of cell motility involves a receptor-mediated process and, if so, which S1P receptor mediates modulation of cell migration in response to S1P and what cellular mechanisms underlie the inhibitory actions of S1P on cell motility (Okamoto et al., 2000). Several studies have shown that S1P receptors such as S1P2/EDG5 induce inhibition in cell migration (Okamoto et al., 2000; Sugimoto et al., 2003; Young and Van Brocklyn, 2007). It has been reported that overexpression of S1PR1 in haematopoietic progenitor cells resulted in suppression of their migration towards CXCL12, and that this was associated with a 1.8 fold decrease in CXCR4 expression (Ryser et al., 2008). Taken together these facts suggest further investigation into the levels of S1P receptors and their specific biological effects will be of value in understanding the motility effects MPL W515L oncogenes. In addition studies on the level of sphingosine kinase activity and other potential signalling pathway activation processes will more clearly define the role of S1P in MPN associated oncogenes.

Importantly we have been able to extend some observations made in our cell line based observations to myelofibrosis patient material. We have shown an elevated motility in CD34+ cells from MPL W515L patients compared to apparently normal CD34+ cells. Further the inclusion of either MYC inhibitors in colony forming assays revealed a marked specific inhibition of colony forming ability in MPL W515L patient samples. However, TGFβ inhibition does not inhibit colony number. Further investigation of the effects of SK inhibition on myelofibrosis patient material is underway since sphingosine pathway plays a role in cell survival, proliferation as well as migration of cancer cells (Calise et al., 2012; Donati and Bruni, 2006; Spiegel and Milstien, 2000). Our preliminary validation studies demonstrate very promising results, therefore, further validation using large numbers of MPL W515L patient material on the novel disrupted pathway observed will be interested in identifying therapeutic
targets in myeloproliferative neoplasms. Overall, I have successfully delineated a novel pathway of MPN leukaemogenic PTK mediated by TGF-β, MYC, and sphingosine induced motility via THOC5 (Figure 3.31).

Figure 3.31: Schematic representation of the MPL W515L and JAK2 mutant mediated pathway of THOC5 induced motility via TGF-β, MYC, and S1P. MPL W515L increased in chemokinesis modulated by THOC5 Y225 phosphorylation. MPL W515L induced THOC5 phosphorylation was linked to elevated TGFβ, MYC and S1P expression. I have identified a novel pathway disrupted in MPN and are starting to understand the mechanisms by which the phosphorylation of THOC5 may contribute to leukaemogenic transformation through links to TGFβ, MYC, and S1P biology.
Chapter 4

Proteomic effects on motility induced by the myeloproliferative neoplasm associated oncogene MPL W515L via THOC5
Chapter 4

Proteomic effects on motility induced by the myeloproliferative neoplasm associated oncogene MPL W515L via THOC5

4.1. Introduction

The development of systems biology offers new opportunities for the study of oncogenic effects of pathways de-regulated in patients with leukaemia. Investigation of protein levels in such cells is important as in haematopoietic cells, changes in the levels of mRNA do not necessarily act as a predictor for changes in the proteome (Unwin et al., 2006b). Quantitative proteomics based on mass spectrometry (MS) is an important methodology for biological and clinical research allowing for instance, the identification of functional pathways, and monitoring disease biomarkers (Trinh et al., 2013). Relative quantification proteomics using higher sensitivity mass spectrometry techniques now offers the potential to compare and contrast the effects of leukaemogenic PTKs. Currently an isobaric tag-based methodology for both relative and absolute quantification (iTRAQ) coupled to multidimensional liquid chromatography and tandem mass spectrometry simultaneously enables the assessment of protein levels up to eight samples in one run (Pierce et al., 2008b; Ross et al., 2004). The iTRAQ labels consist of a protein-reactive group that labels all free amines at the N terminus and also the side chain of internal lysine residues at C-terminal, a balance group and a reporter group. The labels are isobaric, with a different distribution of isotopes between the reporter and balance groups. Hence, each labeled peptide appears at the same mass in an MS scan. Fragmentation of the labelled peptide in the tandem MS/MS allows the identification of the protein and generates distinct low-mass reporter ions at m/z 113.1-119.1, and 121.1 for 8-channel (Eyrich et al., 2011; Gafken and Lampe, 2006; Pierce et al., 2008c). The mass at 120.1 is omitted to avoid contamination from phenylalanine immonium ion (m/z 120.8). As the reporter groups differ in mass, the balance group is used to equilibrate the total mass of the iTRAQ™ reagent. However, upon fragmentation and collision-induced dissociation (CID), the mass balancing
carbonyl group dissociates and releases the reporter group that provides relative intensities information for protein quantification. Relative peak area indicates the contribution of each sample to total peptide present, providing a measure of relative abundance (Drabik et al., 2013; Unwin et al., 2005a). I have demonstrated that Ba/F3 MPL W515L expression leads to an increase in chemokinesis and this is linked to THOC5 phosphorylation. Given the differential chemokinesis effects in Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F oncogenes (Chapter 3; Figure 3.8), I undertook a discovery proteomics study to gain insight into the role of THOC5 in the induced motility effects of MPL W515L. Here I reported the differences in the global proteome changes which are linked to motility in MPL W515L expression of a potentially dominant negative THOC5 Y225F utilising eight-channel isobaric tagging for relative quantification (iTRAQ) strategy.

4.2. Results

4.3. Characterisation of Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cell lines by eight-channel isobaric tagging

The differences in global proteome changes linked to motility as outlined in Figure 4.1. Biological replicates of Ba/F3, Ba/F3 MPL W515L as well as Ba/F3 MPL W515L THOC5 Y225F expressing cells were included in the eight-channel isobaric tagging for relative quantification (iTRAQ™, ABSCIEX) strategy. The inclusion of biological replicates is important to measure reproducibility and allow a statistical calculation to define a change in protein expression with high confidence. The proteomic dataset was analysed to identify the common and/or differences of protein changes which affect motility (Pierce et al., 2008b; Unwin et al., 2005a).
Figure 4.1: Overview of iTRAQ™ reagent eight-channel methodology for Ba/F3, Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cells.
4.4. Data analysis using ProteinPilot™ software

Data was processed against the UniProt/SwissProt mouse database containing 532146 sequence entries using ProteinPilot™ Software 4.1, revision number 460, using the Paragon™ Algorithm 4.0.0.0, 459 as a search engine. The search parameters allowed for one missed or nonspecific cleavage (AB SCIEX, Framingham, USA), methyl methanethiosulfonate (MMTS) and 8-channel iTRAQ fixed modifications as default setting. False discovery rate (FDR) was calculated using a reverse database and the ProteinPilot v.2.0 software (Applied Biosystems, Warrington, UK). Ratio values for each protein were obtained from weighted averages of multiple spectra when appropriate, similarly to the way ProteinPilot™ calculate ratios for proteins. iTRAQ labelling efficiency of the reagents was estimated to be greater than 98% in this experiment by comparing the total number of identified match count with the number of N terminal and lysine count. The minimum iTRAQ™ reporter ion area used for quantification was set at 20 (arbitrary units). ProteinPilot™ biases were used to correct for any sampling error so that the median value of log$_2$ (ratios) of the protein distribution is equal to 0 (Lu et al., 2012). Figure 4.2 shows the distribution of relative protein measurement for two biological replicates Ba/F3 control samples. The protein frequency showed the Gaussian distribution demonstrated that iTRAQ™ labelling had been performed efficiently between replicates samples.

![Graph](image.png)

**Figure 4.2: Distribution of protein quantification ratios.** The histogram depicts the relative protein ratios after normalisation for the two biological replicates of Ba/F3 control samples.
4.5. Identification of differentially expressed proteins

To understand the global proteomic alterations that could induce the changes in motility observed, the ProteinPilot™ dataset of the above replicates were analysed and 2717 proteins were identified across the biological replicates. Using a 95% confidence interval for the internal replicates a change in protein expression was defined as a protein ratio of >1.34 and <0.74. Then, by using the Venny OMIC web (http://bioinfogp.cnb.csic.es/tools/venny), the protein identification and quantification was performed on 2717 proteins and a Venn diagram was generated across the three replicates of Ba/F3 MPL W515L versus Ba/F3, Ba/F3 MPL W515L THOC5 Y225F versus Ba/F3 as well as Ba/F3 MPL W515L versus Ba/F3 MPL THOC5 Y225F expressing cells. To be included as changing, the list of proteins either being up or down regulated, with a ratio >1.34 or <0.74 (p-value <0.05) were selected in at least two of the three replicates. The log₂ value ratio for the protein ratio was then calculated and averaged across the three replicates.

As the Ba/F3 MPL W515L cells display over a 3-fold increase in chemokinesis to Ba/F3 cells which was markedly reduced upon expression of THOC5 Y225F (Figure 3.8-chapter 3), we eliminated the common proteins identified as changing in Ba/F3 MPL W515L THOC5 Y225F versus Ba/F3 as well as those in Ba/F3 MPL W515L versus Ba/F3 MPL THOC5 Y225F expressing cells as shown in Figure 4.3 and 4.4. This allows the potential identification of proteins associated with increased motility observed in the MPL W515L expressing cells. By the above criteria, 24 proteins that were up-regulated and 24 proteins that were down-regulated as shown in blue-shaded area of Venn diagram were identified (Figure 4.3 and 4.4). These 48 proteins are listed in Table 4.2 and Table 4.3 and are potentially responsible for the changes in motility seen in the MPL W515L expressing cells compared to control Ba/F3 cells. From the data accumulated, the number of the overlapping protein is not important. We were interested in selecting and validating proteins identified by working on the up-regulated and/or down-regulated proteins induced increased motility in MPL W515L expressing cells. Validation of the iTRAQ dataset was performed by western blot and any protein interactions further
Figure 4.3: Venn diagram of up-regulated proteins compared to Ba/F3 cells. The Venn diagram showing the relationship of protein identified as changing due to THOC5 Y225F expression in MPL W515L with a protein ratio >1.34 at p-value 0.05. The analysis was performed using Venny OMIC web (http://bioinfogp.cnb.csic.es/tools/venny).

Figure 4.4: Venn diagram of down-regulated proteins compared to Ba/F3 cells. The Venn diagram showing the relationship of protein identified as changing due to THOC5 Y225F expression in MPL W515L with a protein ratio <0.74 at p-value 0.05. The analysis was performed using Venny OMIC web (http://bioinfogp.cnb.csic.es/tools/venny).
Table 4.2: Proteins up-regulated by the action of MPL W515L oncogene altered by THOC5 Y225F expression. The table lists of proteins up-regulated in Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F cells when compared to Ba/F3 cells. To be included as changing, the protein had a ratio >1.34 at p-value 0.05 compared to Ba/F3 cells in at least two of the three replicates. The relative ratios of the significantly altered proteins from each of Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F against Ba/F3 data set were calculated as a weighted value combining averages from three replicates. Log2 values are shown in brackets.

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<th>Accession No.</th>
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<th>Gene names</th>
<th>Average protein ratio Ba/F3 MPL W515L vs Ba/F3 (control)</th>
<th>Average protein ratio Ba/F3 MPL W515LTHOC5 Y225F vs Ba/F3 (control)</th>
<th>No. of observation</th>
<th>Peptides used</th>
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<td>ENSMUSP00000022256</td>
<td>Psmd6</td>
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<td>Macoillin</td>
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<th>Gene names</th>
<th>Average protein ratio Ba/F3 MPL W515L vs Ba/F3 (control)</th>
<th>Average protein ratio Ba/F3 MPL W515LTHOC5 Y225F vs Ba/F3 (control)</th>
<th>No. of observation</th>
<th>Peptides used</th>
</tr>
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<tr>
<td>ENSMUSP00000074387</td>
<td>Preb</td>
<td>Prolactin regulatory element-binding protein</td>
<td>1.41 (0.49)</td>
<td>1.36 (0.44)</td>
<td>3</td>
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<tr>
<td>ENSMUSP00000037970</td>
<td>Bmp2k</td>
<td>BMP-2-inducible protein kinase</td>
<td>1.32 (0.39)</td>
<td>1.09 (0.12)</td>
<td>3</td>
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</tr>
<tr>
<td>ENSMUSP000000103012</td>
<td>Acly</td>
<td>ATP citrate lyase; ATP-citrate synthase</td>
<td>1.37 (0.46)</td>
<td>1.28 (0.36)</td>
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<tr>
<td>ENSMUSP00000113396</td>
<td>Itgal</td>
<td>Integrin alpha-L</td>
<td>1.31 (0.38)</td>
<td>1.35 (0.41)</td>
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<td>ENSMUSP00000029266</td>
<td>Anxa5</td>
<td>Annexin A5</td>
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<td>ENSMUSP00000018333</td>
<td>Uchl5</td>
<td>Ubiquitin carboxyl-terminal hydrolase isozyme L5</td>
<td>1.34 (0.42)</td>
<td>1.27 (0.29)</td>
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<tr>
<td>ENSMUSP00000115078</td>
<td>Svil</td>
<td>Supervillin</td>
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<td>1.32 (0.39)</td>
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<tr>
<td>ENSMUSP00000059143</td>
<td>2310047M10Rik</td>
<td>Uncharacterized protein C17orf59 homolog</td>
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<td>1.08 (0.07)</td>
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<tr>
<td>ENSMUSP00000028848</td>
<td>Fahd2a</td>
<td>Fumarylacetoacetate hydrase domain-containing protein 2A</td>
<td>1.43 (0.51)</td>
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<td>ENSMUSP00000031841</td>
<td>Tra2a</td>
<td>Transformer-2 protein homolog alpha</td>
<td>1.35 (0.43)</td>
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<tr>
<td>ENSMUSP00000096354</td>
<td>I830077J02Rik</td>
<td>Transmembrane protein C1orf162 homolog</td>
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<td>1.37 (0.52)</td>
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<tr>
<td>ENSMUSP00000032841</td>
<td>Mrpl46</td>
<td>39S ribosomal protein L46, mitochondrial</td>
<td>1.38 (0.46)</td>
<td>1.24 (0.30)</td>
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Table 4.3: Proteins down-regulated by the action of MPL W515L oncogene altered by THOC5 Y225F expression. The table lists proteins down-regulated in Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F cells when compared to Ba/F3 cells. To be included as changing, the protein had a ratio <0.74 at p-value 0.05 as compared to Ba/F3 in at least two of the three replicates. The relative ratios of the significantly altered proteins from each of Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F against Ba/F3 data set were calculated as a weighted value combining averages from three replicates. Log₂ values are shown in brackets.

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Gene Symbol</th>
<th>Gene names</th>
<th>Average protein ratio Ba/F3 MPL W515L vs Ba/F3 (control)</th>
<th>Average protein ratio Ba/F3 MPL W515LTHOC5 Y225F vs Ba/F3 (control)</th>
<th>Peptides used</th>
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<td>ENSMUSP00000020099</td>
<td>Cdk1</td>
<td>Cyclin-dependent kinase 1</td>
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<td>ENSMUSP00000125731</td>
<td>Bid</td>
<td>BH3-interacting domain death</td>
<td>0.70 (-0.51)</td>
<td>0.80 (-0.32)</td>
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<td>ENSMUSP00000063825</td>
<td>Pcx</td>
<td>Pyruvate carboxylase</td>
<td>0.71 (-0.51)</td>
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<tr>
<td>ENSMUSP00000106468</td>
<td>Kcnh1</td>
<td>Potassium voltage-gated channel subfamily H member 1</td>
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<td>0.76 (-0.39)</td>
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<td>ENSMUSP00000037341</td>
<td>Ndufb7</td>
<td>NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7</td>
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<td>0.73 (-0.49)</td>
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<tr>
<td>ENSMUSP00000047562</td>
<td>Trappc11</td>
<td>Trafficking protein particle complex subunit 11</td>
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<td>0.97 (-0.04)</td>
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<td>ENSMUSP0000001672</td>
<td>Ifrd1</td>
<td>Interferon-related developmental regulator 1</td>
<td>0.83 (-0.27)</td>
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<td>ENSMUSP00000045873</td>
<td>Anln</td>
<td>Actin-binding protein anillin</td>
<td>0.82 (-0.33)</td>
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<tr>
<td>ENSMUSP00000034388</td>
<td>Vps4a</td>
<td>Vacular protein sorting-associated protein 4A</td>
<td>0.75 (-0.42)</td>
<td>0.77 (-0.38)</td>
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<td>ENSMUSP00000032425</td>
<td>Emc3</td>
<td>ER membrane protein complex subunit 3</td>
<td>0.73 (-0.46)</td>
<td>0.91 (-0.21)</td>
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Table 4.3. Continued

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<tr>
<th>Accession No.</th>
<th>Gene Symbol</th>
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<th>Average protein ratio Ba/F3 MPL W515L vs Ba/F3 (control)</th>
<th>Average protein ratio Ba/F3 MPL W515LTHOC5 Y225F vs Ba/F3 (control)</th>
<th>Peptides used</th>
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<tr>
<td>ENSMUSP00000021148</td>
<td>Ube2g1</td>
<td>Ubiquitin-conjugating enzyme E2 G1</td>
<td>0.74 (-0.43)</td>
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<tr>
<td>ENSMUSP00000027642</td>
<td>Nek7</td>
<td>Serine/threonine-protein kinase</td>
<td>0.73 (-0.46)</td>
<td>0.80 (-0.33)</td>
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</tr>
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<td>ENSMUSP00000049605</td>
<td>Pycr1</td>
<td>Pyrroline-5-carboxylate reductase 3</td>
<td>0.74 (-0.43)</td>
<td>0.74 (-0.43)</td>
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<td>ENSMUSP00000121042</td>
<td>Dda1</td>
<td>DET1- and DDB1-associated protein 1</td>
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<td>0.94 (-0.15)</td>
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<td>ENSMUSP00000025904</td>
<td>Prdx5</td>
<td>Peroxiredoxin-5</td>
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<td>ENSMUSP00000081141</td>
<td>Pgd</td>
<td>6-phosphogluconate dehydrogenase, decarboxylating</td>
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<tr>
<td>ENSMUSP00000022894</td>
<td>Ywhaz</td>
<td>14-3-3 protein zeta/delta</td>
<td>0.76 (-0.41)</td>
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<td>ENSMUSP0000099739</td>
<td>H2-T23</td>
<td>H-2 class I histocompatibility antigen, D-37 alpha chain</td>
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<td>ENSMUSP0000048537</td>
<td>Smyd5</td>
<td>SET and MYND domain-containing protein 5</td>
<td>0.63 (-0.72)</td>
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<tr>
<td>ENSMUSP0000115346</td>
<td>Anxa4</td>
<td>Annexin 4</td>
<td>0.76 (-0.41)</td>
<td>0.80 (-0.32)</td>
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<tr>
<td>ENSMUSP0000123605</td>
<td>Ywhaq</td>
<td>14-3-3 protein theta</td>
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<td>0.74 (-0.43)</td>
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<td>ENSMUSP0000092409</td>
<td>Sae1</td>
<td>SUMO-activating enzyme subunit 1</td>
<td>0.72 (-0.48)</td>
<td>0.75 (-0.42)</td>
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<tr>
<td>ENSMUSP0000052086</td>
<td>Actbl2</td>
<td>Beta-actin-like protein 2</td>
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<td>0.64 (-0.71)</td>
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<tr>
<td>ENSMUSP0000033880</td>
<td>Eif4ebp1</td>
<td>Eukaryotic translation initiation factor 4E-binding protein 1</td>
<td>0.73 (-0.45)</td>
<td>0.88 (-0.18)</td>
<td>4</td>
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</table>
4.6. Validation of iTRAQ™ changes in protein levels by western blot analysis

As part of the validation of the proteins identified as changing in the iTRAQ™ experiments, western blot analysis was performed. Based on their average ratio differences from Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cells; EZH2, TOP1 and TMEM57 were selected for western blot analysis. TMEM57 was chosen since the iTRAQ output shows the biggest change of 24 proteins that were up-regulated, whilst TOP1 was chosen since our collaborator has reported that TOP1 was one of the THOC5 interactome proteins involved in macrophage differentiation (Tran et al., 2014a). Furthermore, it has been reported that EZH2 is involved in the misregulation of the polycomb repressive complex 2 (PRC2) which confers sensitivity to EZH2 inhibition in CML patients (Scott et al., 2013). Our initial studies have shown that the expression of EZH2 (98 kDa), TOP1 (91 kDa) and TMEM57 (76 kDa) were found to be up-regulated in Ba/F3 MPL W515L expressing cells when compared to Ba/F3 and reduced in the presence of THOC5 Y225F expression in MPL W515L cells (Figure 4.5). The densitometry from western blots of 4 biological replicates were confirmed as shown in Figure 4.6. These western blot analysis confirmed the iTRAQ™-coupled LC MS/MS analysis as reported in Table 4.2-4.3.
Figure 4.5: Western blot analysis of EZH2, TOP1, and TMEM57 expression. A representative western blot validation using the whole cell lysates of Ba/F3, Ba/F3 MPL W515L & Ba/F3 MPL W515L THOC5 Y225F expressing cells for EZH2, TOP1 and TMEM57. Actin was used as a loading control. Representative result from four independent biological replicates.

Figure 4.6: Total EZH2, TOP1 and TMEM57 of western blot analysis normalised to Ba/F3. The histogram was calculated using densitometry and the amount of EZH2, TOP1 and TMEM57 of Ba/F3, Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F were normalised by the ratio intensity of the actin and expressed as a Ba/F3 control cells. The error bars depict the standard error of the mean from four biological replicates (n=4).
4.7. Ingenuity pathway analysis

To better understand the data set generated, the list of significantly altered proteins was uploaded into Ingenuity Pathway Analysis (IPA) software server (http://www.ingenuity.com) (Ingenuity system) and analysed using the Core Analysis module to rank the proteins into their top biological functions as well as canonical pathways involved. The analyse network algorithm builds biological networks from the uploaded 48 protein list and assigns a biological functions and top diseases process to each network. The significance of associations between the networks and biological process is represented by a p-value.

The 48 differentially abundant proteins between Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cells represent diverse biological processes associated with cell motility/cellular movement (p-value: 1.6 x 10^-4), cellular assembly cytoskeletal organization (p-value: 6.35 x 10^-5) and cell-to-cell signalling and interaction (p-value: 1.7 x 10^-4). These results suggest nine proteins of the 48 are involved in cellular movement including Histone-lysine N-methyltransferase; enhancer zester homolog 2 (EZH2), Annexin A5, Supervillin, Integrin alpha-L, Actin-binding protein anillin, Cyclin-dependent kinase 1 (Cdk1), Serine/threonine-protein kinase (Nek7), 14-3-3 protein theta (Ywhaq), and vacuolar protein sorting-associated protein 4A (Vps4A). The pathway analysis on proteins identified as changing support the motility effects seen in MPL W515L expressing cells. Figure 4.7 shows the canonical pathways of 48 significantly altered proteins on IPA analysis. For instance, Myc-mediated apoptosis signalling and ERK/MAPK signalling were significantly ranked (p <0.01) within the top 10 pathways. Most of these pathways have been reported to play roles in cell-cycle regulation, cell survival and cell apoptosis. Constitutive activation of the ERK/MAPK signalling pathway has been observed in many tumors with consequences including increased cell proliferation, motility and invasion, and inhibition of apoptotic mechanisms (Modjtahedi and Essapen, 2009). This top highlighted pathways could be involved in the chemokinesis observed due to the expression of THOC5 Y225F in MPL W515L expressing cells.
Figure 4.7: Ingenuity Pathway Analysis. The 48 significantly proteins found to change uniquely due to the expression of THOC5 Y225F in MPL W515L were subject to pathway analysis using the Ingenuity software. The figure shows the top canonical pathways with which significantly altered proteins are associated that could involved in the chemokinesis observed due to the expression of THOC5 Y225F in MPL W515L expressing cells. The p-value of the ratio (p<0.01) was calculated by IPA using Fischer’s exact test.
4.8. Protein interactions analysis of differentially expressed proteins identified in Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cells

The network analysis based on the 48 proteins up-and down-regulated in a comparison between Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cells is shown in Figure 4.8. Our results developed an interactions network of proteins modulated by THOC5 Y225F expression. This protein has a linkage to CDK1, EZH2, TOP1, Anilin, and Psmd6. EZH2 has been reported to interact with CDK1 through phosphorylation at threonine T345 which promotes cell migration and proliferation in cancer (Chen et al., 2010; Simon and Lange, 2008; Yamaguchi and Hung, 2014). These sub-networks identified provides a set of candidate genes and further validation on this target genes may help to further understand of the pathways that altered motility contribute to myelofibrosis in MPN diseases. This may potentially offer future therapeutic intervention in MPL W515L patients.

Figure 4.8: The protein interactions modulated by THOC5 Y225F expression. The 48 proteins identified as changing due to THOC5 Y225F expression in MPL W515L were analysed with STRING software to identify any potential protein interactions. An edge was drawn with up to seven differently colored lines, representing the existence of the seven types of evidence used for predicting the associations: a red line indicates the presence of fusion evidence; a yellow line indicates text mining evidence; a purple line indicates experimental evidence; a blue line indicates cooccurrence evidence; a light blue line indicates database evidence; a green line indicates neighborhood evidence; a black line indicates coexpression evidence.
4.9. Discussion

Advanced mass spectrometry-based proteomics has become a powerful tool for the comprehensive understanding of signal transduction networks at the system level. It is now accepted that changes in the proteome do not necessarily occur as a result of changes in transcription (Unwin et al., 2005a). To look for common proteomic targets, I utilised the mass spectrometry approach to simultaneously assess protein expression and relative quantification of MPL W515L and MPL W515L THOC5 Y225F expressing Ba/F3 cells using 8-channel iTRAQ™ coupled to LC-MS/MS. iTRAQ™ reagents label all peptides at their N terminus, along with free amines in lysine side chains, hence all of the peptide population is labeled, allowing more peptides to be quantified from each protein and increasing the quality of the data obtained by this approach. The labeling protocol for iTRAQ™ reagents is simple, so there are few opportunities of sample loss or contamination. Another advantage of iTRAQ™ reagent approach is that the peptides from all samples appear as one peak in MS increasing the total ion current for that peptide, simplifying the spectra, and requiring only one MS/MS experiment per peptide (Unwin et al., 2005a).

In this study, a total of 220 proteins were identified as significantly changing, however based on motility results we observed, our interest was in specific biological questions such as MPL W515L mediated changes on motility or the effects of a putative dominant negative THOC5 Y225F. As such, 48 proteins were identified as potentially being involved in the induced chemokinesis in Ba/F3 MPL W515L. Of these 48 proteins, 24 were up-regulated and 24 down-regulated between Ba/F3 MPL W515L and Ba/F3 MPL W515L THOC5 Y225F expressing cells. The reliability of iTRAQ™-LC MS/MS dataset was confirmed via western blot. In this study, we demonstrated that EZH2, TOP1 and TMEM57 have altered levels of expression in MPL W515L expressing cells and by the fact that this change in expression is counteracted by the expression of THOC5 Y225F suggest these proteins are candidates that may involved in the increase in chemokinesis observed in MPL W515L effects. EZH2, a polycomb-group (PcG) protein, is increased in MPL W515L expressing cells. I am now keen to investigate its potential to offer therapeutic benefit for the treatment of myelofibrosis. PcG
proteins are essential regulators of haematopoietic stem cells through chromatin modifications associated with gene silencing and deregulation of PcG are implicated in tumorigenesis (Chang and Hung, 2012; Chase and Cross, 2011; Lund et al., 2014; Yamaguchi and Hung, 2014). PcG is composed of 2 main groups, polycomb repressive complex 1 and 2. The polycomb repressive complex 1 (PRC1) consists of the core protein component RING2, B lymphoma Mo-MLV insertion region 1, BMI1, poly homeotic, HPH, and chromobox, CBX. The polycomb repressive complex 2 (PRC2) contains 4 components, EZH2, embryonic ectoderm development, EED, Suppressor of zeste 12, SUZ12, and the histone-binding proteins, RBAP48 (Figure 4.9) (Au et al., 2013; Kirmizis et al., 2004). EZH2 is frequently overexpressed in many cancer types including melanoma, prostate, breast, bladder, liver, lung, brain, and kidney and has been reported to promote cancer initiation, development, migration and invasion (Bachmann et al., 2006; Kondo et al., 2007; Lee and Choe, 2012; Vieira et al., 2014; Yang and Yu, 2013). Further studies have shown that EZH2 supports ovarian cancer cell invasion and/or metastasis via TGFβ. EZH2 knockdown was found to reduce TGFβ expression (Rao et al., 2010). Our data have shown that inhibition of TGFβ led to a partial reduction in the chemokinesis (Figure 3.14) and TGFβ displayed a marked increase in conditioned media (Figure 3.15) from MPL W515L expressing cells, therefore, it is our interest to further studies on the effects of TGFβ and EZH2 in MPL W515L expressing cells linked to motility. TOP1 has been reported to be involved in THOC5-dependent cellular development of macrophages (Tran et al., 2013).

![Figure 4.9: Composition of the PRC2 complex and domain structure of EZH2.](image)

(A) The four core subunits of human PRC2 are EZH2, EED, SUZ12 and RBAP48. (B) Five functional domains in EZH2/E(Z) are depicted, with % identities between the human and fly versions indicated. EZH2/E(Z) is the catalytic subunit that contains a SET domain. The SET domain houses the histone methyltransferase active site and the CXC domain also contributes to activity. Robust methyltransferase requires EZH2 assembly with both EED/ESC and SUZ12, and domains required for binding these noncatalytic subunits are indicated. (Taken from: Simon and Lange, 2008)
Given our data, further confirmation of the involvement of EZH2, TOP1 and TMEM57 in the induced motility is required. Further analysis on Ingenuity pathway analysis has demonstrated 9 proteins including EZH2 were involved in cell motility/cellular movement, cellular assembly cytoskeletal organization, and cell-to-cell signalling, suggesting their possible role in the emergence of oncogenic pathways leading to aggressive cellular behavior in MPL W515L. Further studies on the other proteins either up- or down-regulated found to be involved in motility will be of valuable to increase our understanding on MPL W515L induced increase in chemokinesis. Thus, discovery proteomics can direct insights into leukaemias via highlighting proteins of interest, which in turn leads to identification of pathways and associated proteins that define primary cell transformation events. Consequently, mass-spectrometry-based proteomics will pave the way to evaluate molecular hubs in signalling systems and to develop novel targets for treatment of various diseases caused by signalling aberration. Given the effect of THOC5, MYC, TGFβ and sphingosine-1-phosphate in MPL W515L and EZH2 expression increases in MPL W515L, it would be interesting to see the interaction between EZH2 expression and TGFβ, MYC, and sphingosine-1-phosphate signalling. Further exploration on EZH2 inhibition treatment using GSK126 on MPL W515L and assess the motility will be great of interest. Since the current status of EZH2 downstream effectors in MPL W515L expressing cells are unknown, further studies on EZH2 as a potential new therapeutic target in MPN myelofibrosis need to be explored and this will be discussed further in chapter 6.
Chapter 5

Regulation of mRNA export complex and THOC5 function in myeloproliferative neoplasms
Chapter 5

Regulation of mRNA export complex and THOC5 function in myeloproliferative neoplasms

5.1 Introduction

The ability of a cell to respond in a timely coordinated manner to any changes in the environment is essential in all living organisms. Recently many spliceosome genes have been associated with disease. This often means changes in gene expression. Perturbation in the mRNA processing, transcription and translation are important in homeostasis but also in diseases such as cancer (Culjkovic-Kraljacic and Borden, 2013; Siddiqui and Borden, 2012). Using systems biology approaches our group has previously shown that mRNA level changes do not correspond to protein level changes in a range of cell types from embryonic stem cells (Lu et al., 2009) to haematopoietic stem cells (Pierce et al., 2012). By using our Ba/F3 model system, I am therefore keen to investigate whether the mRNA export pathway is modulated and targetable in leukaemogenic oncogene expressing cells. Messenger RNA (mRNA) export adaptors play an important role in the transport of mRNA from the nucleus to the cytoplasm (Hautbergue et al., 2009). mRNA processing in the nucleus is co-transcriptionally processed whereby the mRNA transcripts are capped, spliced and polyadenylated before becoming export competent (mRNP) (Carmody and Wente, 2009; Masuda et al., 2005). These mRNA processing events are all coupled and result in the deposition of different export stimulating proteins onto the mRNA molecules. The order of deposition is tightly regulated and imparts directionality on the mRNA movement through the nuclear pore complex (NPC) (Hautbergue et al., 2009; Aguilera, 2005). Different export pathways exist for RNA transport (Figure 5.1). SnRNA, tRNA and microRNA are exported via the exportins and chromosome region maintenance 1 (CRM1) pathways, for instance eukaryotic initiation factor-4E (eIF4E) is dependent on CRM1 pathway (Culjkovic et al., 2006) whilst the bulk of mRNA is release via TAP/p15 mediated export (Figure 5.1).
Figure 5.1: Overview of mRNA export pathway. Difference export pathways exist for mRNA transport. The bulk of mRNA was exported via TAP/p15 whilst the subset of RNA is depend on chromosome region maintenance 1 (CRM1) export pathway. TREX consists of multisubunits of THO complex (THOC1, THOC2, THOC5, THOC6, THOC7) as well as UAP56, ALY/REF. With ALY/REF, TREX/THO complex bridge the interaction of mRNA cargoes and TAP/p15 receptor. These complexes are transported out to the nuclear pore complex and released from cytoplasmic via DDX19/Gle1-Insp16. For the subsets of mRNA and eIF4E are exported to the cytoplasm via exportin and CRM1 together with RAN-GTP. Upon arrival to the cytoplasm, all mRNA export factors recycled for future round of export. (Taken from: Culjkovic-Kraljacic and Borden, 2013; Siddiqui and Borden, 2012).

In the cytoplasm, eIF4E binds to the methyl 7 guanine (m7G) that caps all mRNAs, but in the nucleus, eIF4E, recognises a structural element (a complex pair of stem loops) within the 3’ UTRs of its specific export targets (LeBrasseur, 2006). Thus, eIF4E plays a complex role participating in recruitment of mRNA to ribosomes which control translation (Rhoads, 2009). This enables regulation of eIF4E by numerous pathways such as growth factors; insulin, PDGF, and insulin-like growth factor 1 (IGF-1) stimulation (Sonenberg and Gingras, 1998). The highly conserved TREX (transcription/export) complex, which contains the proteins ALY/REF, UAP56, UIF, chTOP, and THO complex (THOC), is required to couple transcription and nuclear export of bulk mRNAs (Aguilera, 2005; Reed and Cheng, 2005; Reed and Hurt, 2002). Hence, perturbations in these mRNA nuclear export factors are linked
to different diseases as summarised in Table 5.1. THOC5/FMIP is a sub-member of THO complex found to be involved in mRNA export pathway (Reed and Cheng, 2005; Strässer et al., 2002). In human and *Drosophila*, various studies have shown that THO is required for the export of heat shock mRNAs (Katahira et al., 2009). Indeed, recent studies has demonstrated that THOC5 influenced more than 90% of mRNA export induced by growth factors and cytokines, which therefore contributes to the 3'-end processing and/or export of immediate-early genes induced to be expressed by extracellular stimuli (Tran et al., 2014a).

UAP56 interacting factor (UIF) is an export adaptor which binds TAP/p15 & delivers mRNA to the nuclear pore complex (NPC). UIF associates with TREX complex and binds UAP56 with a short peptide that is also found in ALY/REF. However, in addition, UIF recruitment to mRNA is dependent on the histone chaperone FACT which facilitates transcription by RNA polymerase through chromatin which together with ALY/REF ensures efficient mRNA export (Hautbergue et al., 2009). ALY/REF is a substrate for the PRMT1 enzyme (Hung et al., 2010). chTOP/SRAG/Fop is a chromatin target of Prmt1. chTOP/SRAG/Fop requires methylation to be activated via numerous arginines to bind TAP/p15 for mRNA export processes. However, post-translational arginine methylation prevents chTOP bindings to ALY/REF ultimately decreasing RNA binding. Epigenetic modification of histones by PRMT leads to various biological responses such as transcriptional activation/repression (Cha and Jho, 2012). The TREX complex appears to be highly dynamic with the post-translational modifications such as phosphorylation (Dufu et al., 2010) and methylation (Chang et al., 2013) affects the activity of mRNA binding and processing contribute to the pathogenesis of leukaemia. Therefore, studies on inhibition of post-translational arginine methylation of mRNA export factors may yield new insight into cancer therapy. Arginine methylation appears to be important to finely regulate RNA-protein and protein-protein interactions for effective of mRNA transfer to the export receptor TAP/p15. Studies by (Hautbergue et al., 2009) who showed that UIF and ALY/REF appear to have overlapping functions since knockdown of either UIF or ALY/REF or both severely results in blocking mRNA export. However, their recruitment to mRNA occurs in mutually exclusive manner (Hautbergue et al., 2009). Knockdown studies on RNAi F1p-In 293 expressing cell line shows that chTOP
increased significantly with ALY/REF knockdown (Chang et al., 2013). In addition, loss of ALY/REF or THOC5 in human cells also lead to severe reduction in mRNA export (Chi et al., 2013).

Chromatin target of Prmt1 (chTOP) is also termed as SRAG/Fop (Friend of PRMT1) (Chang et al., 2013). chTOP/SRAG/Fop act as a substrate for arginine methylase protein methyltransferase 1 (PRMT1) and their methylation enhances the binding of mRNA to UAP56 and TAP/p15 (Chang et al., 2013; van Dijk et al., 2010). Moreover, chTOP/SRAG/Fop binds UAP56, in a mutually exclusive manner with ALY/REF and chTOP/SRAG/Fop binds TAP/p15 in mutually exclusive manner with THOC5 (Chang et al., 2013). For instance, knockdown studies on RNAi F1p-In 293 expressing cells shows that chTOP increased significantly with ALY/REF knockdown. However, knockdown of chTOP and THOC5 does not cause a major mRNA export block (Chang et al., 2013). All of these data show a set of complex regulatory mechanisms important for RNA metabolism. Thus these protein will be explored to understand any differences using our Ba/F3 model system and leukaemogenesis.

Until recently, the mRNA export had not been considered as a target for treatment in cancers, however many studies have shown that mRNA export components are de-regulated in many cancer cells such as eIF4e, ALY/REF and THOC (Hautbergue et al., 2009; Chang et al., 2013). This suggest that the mRNA export process offers potential therapeutic targets for cancer treatment (Table 5.1) (Siddiqui and Borden, 2012). Our group have shown that the MPN associated oncogene MPL W515L has profound effects on THOC5 and given our SILAC dataset in JAK2 V617F showed changes in two transcription factors MYC as well as p53 (A.Pierce, unpublished observations), my aim was to understand the consequences of this perturbation on the whole mRNA export complex that could be involved in leukaemic transformation. In this study, I examined and investigated whether the mRNA export complex is targetable in MPN associated oncogenes with a view to identifying therapeutic targets in MPN patients.
Table 5.1: Components of transcription export complex

<table>
<thead>
<tr>
<th>mRNA export factors</th>
<th>Role in export pathway</th>
<th>Sources</th>
</tr>
</thead>
</table>
| eIF4E               | • Acting in the rate-limiting steps of cap-dependent translation initiation in the cytoplasm.  
• Function is controlled by growth factors such as insulin, PDGF, and insulin-like growth factor 1 (IGF-1) stimulation.  
• Overexpression and dysregulation leads to increased in tumor development, invasion and metastasis in mouse models.  
• Elevated in acute myeloid leukaemia and breast cancers. | (Assouline et al., 2009; Topisirovic et al., 2004; Graff and Zimmer, 2003; Sonenberg and Gingras, 1998) |
| SRAG                | • Alternatively known as chTOP/Fop  
• Controlled by RNA-binding proteins, nuclear matrix proteins, cytokines, and transcriptional regulators.  
• Methylation by Prmt1 enhances binding to TAP-p15.  
• Overexpressed in cervical, glioma, ovarian, pancreatic cancers. | (Chang et al., 2013; Bedford and Clarke, 2009) |
| TAP-p15             | • Alternatively known as NXF1–NXT1.  
• The hetero-dimeric mRNA export receptor.  
• assist in export of the mature mRNA transcripts to the cytoplasm.  
• Overexpression of TAP-p15 stimulate mRNA export. | (Chi et al., 2013; Katahira et al., 2009; Erkmann and Kutay, 2004; Kang and Cullen, 1999) |
| THOC1               | • Alternatively known as Hpr1 or p84.  
• component of the THO subcomplex of the TREX which couple mRNA transcription, processing and nuclear export.  
• Requires Sub2p for stable mRNA formation and export.  
• Elevated in ovarian, lung and breast cancer. | (Domínguez-Sánchez et al., 2011; Wang et al., 2006; Li et al., 2005; Strasser et al., 2002; Zenklusen et al., 2002) |
| THOC2               | • component of the THO subcomplex of the TREX which couples mRNA transcription, processing and nuclear export. | (Domínguez-Sánchez et al., 2011; Masuda et al., 2005) |
| THOC3               | • component of the THO subcomplex of the TREX complex which couples mRNA transcription, processing and nuclear export. | (Domínguez-Sánchez et al., 2011; Masuda et al., 2005) |
| THOC4               | • Alternatively known as ALY/REF.  
• Adaptor mRNA binding protein involved in multiple processes including RNA export, nuclear RNA stability and transcription.  
• Recruits the essential mRNA export factor TAP.  
• Dysregulated in colon, stomach, pancreas. | (Katahira, 2012; Domínguez-Sánchez et al., 2011; Hautbergue et al., 2009) |
<table>
<thead>
<tr>
<th>Protein</th>
<th>Summary</th>
<th>References</th>
</tr>
</thead>
</table>
| **THOC5/FMIP** | • Alternatively known as FMIP.  
• Component of the THO subcomplex of the TREX which couple mRNA transcription, processing and nuclear export.  
• THOC5 is a substrate and binding partner for the Macrophage Colony Stimulating Factor receptor (M-CSF) tyrosine kinase (FMS).  
• Target for leukaemogenic PTKs.  
• Elevated in lung, ovarian and colon cancer. | (Culjkovic-Kraljacic and Borden, 2013; Masuda et al., 2005; Rehwinkel et al., 2004; Tamura et al., 1999) |
| **THOC6** | • Component of the THO subcomplex of the TREX which couple mRNA transcription, processing and nuclear export.  
• Elevated in breast cancer. | (Domínguez-Sánchez et al., 2011; Masuda et al., 2005; Rehwinkel et al., 2004) |
| **THOC7** | • Component of the THO subcomplex of the TREX which couple mRNA transcription, processing and nuclear export.  
• THOC7/THOC5 interaction is required for nuclear localization of THOC7.  
• Elevated in cervical cancer. | (Lando et al., 2013; El Bounkari et al., 2009) |
| **UAP56** | • Member of the DEAD-box helicase family.  
• Involved in spliceosome assembly and recruitment of adaptors.  
• Essential for mRNA export. | (Luo et al., 2001) |
| **UIF** | • Also known as Uap56-Interacting Factor 1.  
• An mRNA export adaptor which bind TAP-p15 & delivers mRNA to the nuclear pore complex (NPC).  
• Functions along with THOC4/Aly as an additional adaptor mRNA binding protein.  
• FACT is specifically required for the recruitment of UIF to mRNAs | (Katahira, 2012; Hautbergue et al., 2009) |
5.2 Results

5.3 Analysis of mRNA transcript level by qRT-PCR

To assess and understand the involvement of TREX complex in Ba/F3 MPL W515L and Ba/F3 JAK2 mutant expressing cells, we evaluated the expression of the components of the human THO complex; THOC1, THOC2, THOC3, THOC5, THOC6 and THOC7, as well as the expression levels of the adaptor proteins such as UIF, SRAG and ALY in MPL W515L expressing cell line as well as activated JAK2 mutant expressing Ba/F3 cells. Total mRNA levels were analyzed by TaqMan® qRT-PCR assays and compared to the control Ba/F3 cell line (Table 5.2). From the results obtained, THOC6 mRNA levels shows significantly increased in the expression levels in Ba/F3 MPL W515L and Ba/F3 JAK2 V617F expressing cells whilst not in Ba/F3 JAK2 K539L. In addition, THOC5, chTOP, ALY/REF and eIF4E demonstrate the largest change with increase in the expression levels in Ba/F3 MPL W515L expressing cells as compared to control cells (Table 5.2).

Table 5.2: Quantitation of mRNA expression in Ba/F3 MPL W515L as well as JAK2 mutant expressing Ba/F3 cells. qRT-PCR assays were performed in triplicate on a 7900HT Fast Real-Time PCR System. ΔΔCt values were calculated for each sample against the average of the two housekeeping genes that were used to calculate fold change using the 2^ΔΔCt method. The values presented in this table are log2 of fold increase of each gene expression level, between the oncogene-transfected cell lines and the respective controls.

<table>
<thead>
<tr>
<th></th>
<th>Ba/F3 MPL W515L</th>
<th>Ba/F3 JAK2 K539L</th>
<th>Ba/F3 JAK2 V617F</th>
</tr>
</thead>
<tbody>
<tr>
<td>THOC1 or hHpr1 or p84</td>
<td>0.22</td>
<td>0</td>
<td>-0.46</td>
</tr>
<tr>
<td>THOC2</td>
<td>-0.08</td>
<td>0.48</td>
<td>0.21</td>
</tr>
<tr>
<td>THOC3</td>
<td>0.02</td>
<td>0.33</td>
<td>-0.02</td>
</tr>
<tr>
<td>THOC4/ALYREF</td>
<td>0.34</td>
<td>0.31</td>
<td>0.06</td>
</tr>
<tr>
<td>THOC5</td>
<td>0.54</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>THOC6</td>
<td>0.75</td>
<td>-0.48</td>
<td>0.41</td>
</tr>
<tr>
<td>THOC7</td>
<td>-0.49</td>
<td>-0.64</td>
<td>-0.62</td>
</tr>
<tr>
<td>chTOP/SRAG</td>
<td>0.48</td>
<td>-0.54</td>
<td>-0.21</td>
</tr>
<tr>
<td>UIF</td>
<td>-0.46</td>
<td>0.04</td>
<td>0.72</td>
</tr>
<tr>
<td>eIF4E</td>
<td>0.55</td>
<td>0.45</td>
<td>-0.13</td>
</tr>
<tr>
<td>NXF1/TAP</td>
<td>0.12</td>
<td>-0.04</td>
<td>-0.91</td>
</tr>
</tbody>
</table>

- increased, p<0.05
- increased, p<0.01
- increased, p<0.001
- decreased, p<0.05
5.4 Western blot analysis of mRNA export protein in MPN oncogenes

To study the protein expression levels of the above mRNA export factors, the western blot analysis was performed on Ba/F3 MPL W515L and Ba/F3 JAK2 K539L and Ba/F3 JAK2 V617F expressing cells. All assessments were made on multiple cell lysates and to avoid misrepresentation of the results by choosing a single image, the results are displayed as average densitometry value for all experiments. Protein expression levels were normalised to actin and expressed as a ratio to specific protein expression in Ba/F3 control cells (Figure 5.2-5.5). Figure 5.2 shows the average densitometry results for SRAG protein. SRAG expression is unaltered by MPL W515L (p<0.45) expressing cells, whilst the JAK2 mutants induce an increase in the SRAG expression. In addition, the densitometry average for UIF was significantly decreased in Ba/F3 MPL W515L expressing cells (p<0.05) (Figure 5.3) which is in agreement with the result of mRNA transcript level in Ba/F3 MPL W515L (p<0.05) whilst not in JAK2 K539L and JAK2 V617F expressing Ba/F3 cells. Furthermore, the average densitometry of eIF4E (Figure 5.4) and ALY/REF (p<0.05) (Figure 5.5) shows increased in the level of expression either in Ba/F3 MPL W515L and Ba/F3 JAK2 K539L which is in agreement with the result of mRNA transcript level (p<0.001) (Figure 5.8-5.9). These results suggest that there is differential patterns in expression of mRNA export factors in MPL W515L expressing cells.
Figure 5.2: SRAG protein level of Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells. The histogram was calculated using densitometry and the amount of SRAG/chTOP was normalised by the intensity of the actin which expressed as a ratio of Ba/F3. The error bars depict the standard error of the mean from five biological replicates (n=5). Ba/F3 JAK2 K539L expressing cells shows a significance differences (p<0.05) from controls as determined by Student T-test. ns; no significant difference between control cell tested.

Figure 5.3: UIF protein level of Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells. The histogram was calculated using densitometry and the amount of UIF was normalised by the intensity of the actin which expressed as a ratio of Ba/F3. The error bars depict the standard error of the mean from 8 biological replicates. Ba/F3 MPL W515L expressing cells shows a significance differences (p<0.05) from controls was determined by Student T-test. ns; shows no significant difference between control cell tested.
Figure 5.4: eIF4E protein level of Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells. The histogram was calculated using densitometry and the amount of eIF4E was normalised by the intensity of the actin which expressed as a ratio of Ba/F3. The error bars depict the standard error of the mean from 8 biological replicates. Ba/F3 MPL W515L expressing cells shows a significant differences (p<0.05) from controls as determined by Student T-test. ns; no significant difference between control cell tested.

Figure 5.5: ALY/REF protein level of Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells. The histogram was calculated using densitometry and the amount of ALY/REF was normalised by the intensity of the actin which expressed as a ratio of Ba/F3. The error bars depict the standard error of the mean from three biological replicates (n=3). ns; no significant difference between the control cell tested.
Figure 5.6: Comparison of SRAG protein change against mRNA change for Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells. The graph was calculated from the average of protein changes (%) and the average of mRNA fold change (%) of SRAG expression in Ba/F3 MPL W515L, Ba/F3 JAK2 K539L and Ba/F3 JAK2 V617F expressing cells. The error bars depict the standard error of the mean from 8 biological replicates (n=8).

Figure 5.7: Comparison of UIF protein change against mRNA change for Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells. The graph was calculated from the average of protein changes (%) and the average of mRNA fold change (%) of UIF expression in Ba/F3 MPL W515L, Ba/F3 JAK2 K539L and Ba/F3 JAK2 V617F expressing cells.
Figure 5.8: Comparison of eIF4E protein change against mRNA change for Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells. The graph was calculated from the average of protein changes (%) and the average of mRNA fold change (%) of eIF4E expression in Ba/F3 MPL W515L, Ba/F3 JAK2 K539L and Ba/F3 JAK2 V617F expressing cells.

Figure 5.9: Comparison of ALY/REF protein change against mRNA change for Ba/F3, Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells. The graph was calculated from the average of protein changes (%) and the average of mRNA fold change (%) of ALY/REF expression in Ba/F3 MPL W515L, Ba/F3 JAK2 K539L and Ba/F3 JAK2 V617F expressing cells.
5.5 Cell proliferation studies for mRNA export factor inhibition in MPN associated oncogene expressing Ba/F3 cells

Given we identified differences in the expression of key proteins in mRNA metabolism, we investigated whether oncogene expressing cells were more susceptible to disruption of a perturbed mRNA pathway using inhibitors in the Ba/F3 cells model. To answer this question, we treated MPN associated oncogenes with the inhibitors of different mRNA export proteins; eIF4E (ribavirin), arginine methylase inhibitor; adenosine dialdehyde (AdOx) and curaxin (Figure 5.6). AdOx caused inhibition in the methylation reactions that blocks the transfer group of methyl to the PRMT substrate of SRAG (Chang et al., 2013). The use of curaxin (that binds to UIF) causes functional inactivation of FACT and sequesters the FACT complex on chromatin. Ribavirin, a guanosine ribonucleoside analogue acts as an eIF4E inhibitor by competing with 7-methylguanosine of the 5’-mRNA (Kentsis et al., 2004; Kentsis et al., 2005).

Figure 5.10: Schematic representation of mRNA export for cell proliferation studies. The figure depicts the alternate mRNA export pathway highlighting potential points of intervention.
The cell proliferation studies were performed using WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2 h-5-tetrazolio]-1,3-benzene disulfonate) assays. The concentrations of inhibitors used in this assay are based on the half maximal effective concentration (EC50) as recommended by manufacturers. The measured absorbance was quantitated and the rate of cellular proliferation was calculated. Results are shown in Figure 5.11-5.13. Over a 24 hours time period Ba/F3, Ba/F3 MPL W515L and Ba/F3 JAK2 V617F expressing cells displayed a concentration dependent inhibition of proliferation to curaxin (0 nM-1 µM) (Figure 5.11). There was however no significant difference between the Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 and control cells. Similar observations were made with ribavirin (0.2 µM-200 µM) (Figure 5.12) as well as methylation inhibition by AdOx (0.2 µM -200 µM) (Figure 5.13). The result suggest that targeting mRNA export by inhibiting UIF, SRAG or eIF4E does not offer any therapeutic value in MPL W515L expressing cells or JAK2 mutant expressing cells.

![Figure 5.11: Effects of curaxin on cell growth in MPN associated oncogenes expressing Ba/F3 cells.](image)

Ba/F3, Ba/F3 MPL W515L and Ba/F3 JAK2 V617F expressing cells were treated for 24 hours with the doses of curaxin as shown and the rate of cellular proliferation measured using the WST-1 assay. Results are expressed as percentage of no addition for each cell line and are the mean of three biological replicates +/- SEM.
Figure 5.12: Effects of ribavirin on cell growth in MPN associated oncogenes expressing Ba/F3 cells. Ba/F3, Ba/F3 MPL W515L and Ba/F3 JAK2 V617F expressing cells were treated for 24 hours with the doses of ribavirin as shown and the rate of cellular proliferation measured using the WST-1 assay. Results are expressed as percentage of no addition for each cell line and are the mean of three biological replicates +/- SEM.

Figure 5.13: Effects of AdOx on cell growth in MPN associated oncogenes expressing Ba/F3 cells. Ba/F3, Ba/F3 MPL W515L and Ba/F3 JAK2 V617F expressing cells were treated for 24 hours with the doses of AdOx as shown and the rate of cellular proliferation measured using the WST-1 assay. Results are expressed as percentage of no addition for each cell line and are the mean of three biological replicates +/- SEM.
5.6 Discussion

In recent years, it has become evident that mRNA processing does not proceed through a single pathway but that there are alternative pathways in the regulation of mRNA processing (Siddiqui and Borden, 2012). Disruption of mRNA export mechanisms has potential to contribute to the development of a variety of cancers. Numerous studies have shown that mRNA export components (UIF, SRAG, ALY/REF and THO complex) are dysregulated in many cancer cells (Assouline et al., 2009; Chang et al., 2013; Hautbergue et al., 2009). Studies have shown that THOC5 Y225 phosphorylation modulates mRNA binding hence translational control in cell development and haematopoiesis (Griaud et al., 2013; Guria et al., 2011). Given my knowledge on THOC5 phosphorylation by the oncogenes, I investigated the modulation of mRNA processing of THOC5 and other members of TREX complex that may be deposited on the mRNA of MPN associated oncogenes MPL W515L and JAK2 mutants. THOC5 is a member of THO complex which is highly conserved TREX (Transcription/Export) complex in higher eukaryotes (Katahira et al., 2009; Reed and Cheng, 2005). Components of TREX complex are formed by assembly of UAP56, adaptor proteins UIF, ALY/REF, ChTOP and THO complex including THOC1, THOC2, THOC3, THOC4, THOC5, THOC6, and THOC7 (Masuda et al., 2005; Tamura et al., 1999). The western blot results show a decrease in the expression of UIF and SRAG in MPL W515L expressing cells whilst increased expression of eIF4E. Apart from THOC5, THOC6 also significantly increased in qRT PCR analysis from Ba/F3 MPL W515L expressing cell. Differential protein expression data for those key proteins obtained indicated that these adaptor proteins may play a role in the altered of mRNA processing in Ba/F3 MPL W515L oncogene.

Curaxin inhibits the transcription of both NF-kappa β and heat shock transcription factor 1 (HSF1) and modulates p53 activation without inducing genotoxicity to the cells (Draetta and Depinho, 2011; Gasparian et al., 2011). For instance, FACT expressed at higher level in cancer cell lines as compared to normal cells and further RNAi-mediated knockdown of FACT reduced the growth and survival of cancer cells (Gasparian et al., 2011). For instance, FACT is an accelerator in tumor transformation of in vitro H-RasV12 induced in fibroblast cells.
In addition, curaxin has been shown to suppress tumorigenesis on Her2/neu of mammary transgenic mice and offers a potential therapeutic target for breast cancer (Koman et al., 2012). Ribavirin potently suppresses eIF4E-mediated oncogenic transformation of an in vivo mouse model of eIF4E-dependent human squamous cell carcinoma, and also colony formation of eIF4E-dependent acute myelogenous leukaemia cells derived from human patients (Kentsis et al., 2004; Kentsis et al., 2005). WST-1 cell proliferation assays in the presence of different inhibitors to mRNA processing pathways have been used in this study to identify any potential targets in the mRNA export system. Therefore, studies were performed in the presence of inhibitors; AdOx, curaxin and ribavirin demonstrated that all the oncogenes tested display dose response curve to the inhibitors used, but there is no significant difference in the effects between the Ba/F3 MPL W515L and JAK2 mutant expressing Ba/F3 cells and control cell. This suggests that inhibiton of SRAG, UIF or eIF4E does not induce cell death specifically in Ba/F3 MPL W515L, Ba/F3 JAK2 K539L and Ba/F3 JAK2 V617F, thereby mRNA export complex probably does not offer therapeutic advantage via the use of AdOx, curaxin and ribavirin.

mRNA translation is regulated by various initiation factors such as eIF4E (Spilka et al., 2013). Of particular interest, based on our observation using western blot and mRNA level assay we showed that eIF4E level increases in the presence of MPL W515L oncogenes. Several studies have demonstrated that elevated level of eIF4E seen in numerous human cancers such as in AML (Tamburini et al., 2009) and their upregulation correlates with poor prognosis and disease progression (Graff et al., 2008; Montanaro and Pandolfi, 2004; Robichaud et al., 2015). Therefore, further studies on eIF4E inhibiton were thought to be useful to gain an advance in knowledge on MPL W515L mediated transformation. I have shown that Ba/F3 MPL W515L expressing cells increased in MYC and MCL-1 expression. Studies have shown that increase in MYC expression induces eIF4E upregulation (Lin et al., 2008). Furthermore, MCL-1 in gastric cancer are sensitive towards histone deacetylase inhibitor (Labisso et al., 2012). c-MYC cooperates with eIF4E to promote lymphoma formation (Ruggero et al., 2004). Given our results, it would be of interest to further understand the role of eIF4E and links to our novel pathway. I have shown that inhibiton of
Ba/F3 MPL W515L expressing cells with JQ1, MYC inhibitor reducing the level of MYC and THOC5 Y225 phosphorylation. Therefore, further experiment could be done for example treat the MPL W515L expressing cells with JQ1 and assay for elF4E and MCL-1 expression levels or inhibit elF4E and look at MYC expression levels.

The elevated level of elF4E in leukaemias are associated with the mutation in mTOR and PI3K/AKT pathways (Ilic et al., 2011; Sun et al., 2005). Study using human mammary epithelial cells (HMECs) shows that MYC and elF4E overexpression are resistant to PI3K and rapamycin (mTOR inhibitor) inhibition. This resistance leading to an increase in 5’-cap dependent translation (Ilic et al., 2011; Sun et al., 2005). In the future, treating Ba/F3 MPL W515L cells as well as activated JAK2 mutants expressing Ba/F3 cells with rapamycin/PI3K inhibitor and measuring the phosphorylation of elF4E, c-MYC and MCL-1 expression levels will help to understand the role of elF4E in MPN associated oncogenes cells. In conclusion, this study could offer the possibility of defining mRNP biogenesis that may contribute to pathogenesis of myelofibrosis in MPL W515L patients.
Chapter 6

General Discussion, Future Work and Conclusion
Chapter 6

General Discussion, Future Work and Conclusion

6.1. General discussion

MPL is the thrombopoietin receptor. A point mutation in MPL can be the driver mutation in myelofibrosis. MPL in its mutated form can activate Janus kinase-2 (JAK2), which mediates signalling by various cytokines, including erythropoietin and thrombopoietin, and growth factors such as GM-CSF. JAK2 possesses tandem pseudokinase and tyrosine-kinase domains. Mutations in the kinase domain are also linked to myeloproliferative neoplasms (MPNs); which are clonal proliferative disorders affecting different myeloid lineages (Shan et al., 2014). Thus, MPNs are associated with constitutive activation of the JAK-STAT pathway due to JAK2 mutation (JAK2 K539L, JAK2 V617F), MPL mutation (MPL W515L) or chromosomal translocations (BCR/ABL). The consequences of these somatic mutations leads to diverse phenotypes including polycythaemia vera, myelofibrosis, essential thrombocythemia and chronic myeloid leukaemia. The identification of activating mutations in JAK2 and in the thrombopoietin receptor gene (MPL W515L) in most patients with MPN made them promising targets for drug discovery. However, the use of inhibitors to oncogenes such as JAK2 V617F has been argued to offer little improvement on chemotherapeutic agents, such as hydroxyurea (Antonioli et al., 2010; Ricksten et al., 2008). The use of protein tyrosine kinase inhibitors (TKIs) such as JAK2 inhibitors have been developed do not eradicate the leukaemic stem cells (Hochhaus et al., 2007), similarly in CML ABL TKIs do not extinguish the leukaemic stem cell. Hence, understanding the effects of PTKs is still important for the development of a firm knowledge on the molecular pathogenesis of the leukaemias as well as the development of new therapeutic intervention strategies. Following all results in Chapter 3, we have produced a pathway of the proteomic effects of the JAK2 activating mutant MPL W515L using a mass spectrometric approach.
Within the proteins identified as changing due to MPL W515L expression, we saw an enrichment of proteins involved in motility. Further analysis of these data sets indicated a role for THOC5, a member of the THO complex involved in mRNA splicing/export pathway in the transformation process. THOC5 is essential to maintain the haematopoietic stem cells (Mancini et al., 2010). THOC5 is phosphorylated on Y225 following CXCL12 stimulation and this has been found to be involved in the motility response (Griaud et al., 2013). My work has revealed a novel pathway inclusive of the induced THOC5 Y225 phosphorylation downstream of MPL W515L and JAK2 mutant. Subsequent work showed a major disruption of the CXCL12/CXCR4/CD45/Src axis by MPL W515L yet the MPLW515L expressing Ba/F3 cells display increased chemokinesis in the absence of any stimulus. This observation fits with the pathology of the disease in that patients with myelofibrosis display a mobilisation of haemopoietic progenitors from the bone marrow and up-to a 200-fold increase in the number of circulating CD34+ cells have been reported (Barosi et al., 2001; Castro-Malaspina et al., 1981). It has been proposed that the increase in motility results from several mechanisms including a reduced CXCR4 expression on CD34+ cells (Rosti et al., 2007) shown to be related to hypermethylation of the CXCR4 promoter (Bogani et al., 2008). This fits with our observation that shows an increase in chemokinesis observed in MPL W515L without any addition of CXCL12. It is not clear how CXCR4 decrease governs motility increases. Whilst the mechanism underlying myelofibrosis associated with MPL W515L or JAK2 mutant induced myeloproliferative neoplasms is poorly understood, it has been reported to involve the excess production of TGFβ by CD34+ cells. Interestingly, the MPL W515L expressing cells in our model secreted TGFβ, which is implicated not only in myelofibrosis but also motility. Addition of TGFβ to Ba/F3 cells demonstrated that TGFβ stimulates the phosphorylation of THOC5. Inhibition of TGFβ reduced the MPL W515L induced increase in chemokinesis demonstrating a role for the secretion of TGFβ in the increased chemokinesis we observed. These data infer targeting THOC5 may affect the myelofibrosis process. Given the effect of MPL W515L on THOC5 phosphorylation, and that THOC5 modulates MYC expression (Griaud et al., 2013), it was of interest that MPL W515L expression led to elevated MYC expression. My subsequent studies on MYC inhibition and MYC gene
silencing, reduced the MPL W515L induced increase in chemokinesis as well as THOC5 Y225 phosphorylation. Having previously published on the differential effects of sphingosine-1-phosphate (S1P) on the motility of HSC populations (Whetton et al., 2003) and given the fact that TGFβ upregulates and activates S1P which contribute to fibrosis (Milara et al., 2012), I investigated the potential role of S1P in our pathway. Inhibition of sphingosine kinase reduced the elevated chemokinesis and THOC5 Y225 phosphorylation in MPL W515L expressing cells and in accordance with our observations on the role of THOC5 and MYC led to a dramatic decrease in the level of THOC5 Y225 phosphorylation and MYC expression. This is despite the fact that S1P added exogenously did not act as a chemo-attractant for Ba/F3 or MPL W515L expressing Ba/F3 cells. A possible explanation for this comes from the report that whilst extracellular S1P is a chemoattractant intracellular S1P acts to induce non-directional cell movement (Spiegel and Milstien, 2011; Strub et al., 2010; Takabe et al., 2008). In accordance with these reports we demonstrated an increase in intracellular S1P in the MPL W515L expressing cells. From all the results obtained, it can be suggested that sphingosine-1-phosphate governs the THOC5 pathway and MYC activation, perhaps influencing cell migration in MPL W515L oncogenes.

To further understand the role of THOC5 in the MPL W515L induced effects on motility, I undertook an analysis on the global effects on the proteome of expression of MPL W515L in the presence of wild type and the mutant Y225F THOC5 (Chapter 4). Amongst the proteins shown to be up-regulated, I have identified enhancer zester homolog 2 (EZH2). Further validation showed that EZH2 expression is elevated in MPL W515L but reduced to basal levels by co-expression of THOC5 Y225F. This is in line with the motility behaviour of these cells in that MPL W515L enhance chemokinesis that is reduced to basal levels by co-expression of THOC5 Y225F. This implicates such proteins in increased motility. Growing evidence shows that EZH2 activity is regulated by post translational modifications which are critical for biological function of polycomb repressive complex (PRC2) (Kondo, 2014; Tiffen et al., 2015). Here I focussed on histone methyltransferase, EZH2, which is the catalytic enzyme that is involved in repressing gene expression through methylation of histone H3 on lysine 27 (H3K27) ultimately silencing the genes that promote transcription and restrain
proliferation (Simon and Lange, 2008; Yamaguchi and Hung, 2014). EZH2 is frequently overexpressed in many cancer types including melanoma, prostate, breast, bladder, liver, lung, brain, and kidney which have been reported to promote cancer initiation, development, migration and invasion (Bachmann et al., 2006; Kondo et al., 2007; Lee and Choe, 2012; Vieira et al., 2014; Yang and Yu, 2013). Further studies have shown that EZH2 supports ovarian cancer cell invasion and/or metastasis via TGFβ: EZH2 knockdown was found to reduce TGFβ expression (Rao et al., 2010). CDK1 phosphorylates EZH2 at T345 to promote cell migration and proliferation, however phosphorylation at T487 inactivates EZH2 and reduces cell invasion. In contrast, some studies reported that EZH2 acts as a tumor suppressor through inactivating mutations such as in myeloid malignancies associated with poor prognosis in myelodysplastic syndrome as well as in MPN (Ernst et al., 2010; Nikoloski et al., 2010). This indicates that deregulation of EZH2 in cancer may not be generalised into one simple mechanism. Hence, changes in epigenetic modifications caused by defective EZH2 may result in tumor promotion by different mechanisms depending on the cellular context and the oncogenic pathways that are activated (Lund et al., 2014; Martinez-Garcia and Licht, 2010).

THOC5 is part of the transcription/export (TREX) complex and plays a role in transcription regulation and mRNA export. My data suggest that THOC5 Y225 phosphorylation was increased in MPL W515L and THOC5 is required for the 3' end processing and/or export of growth factor/cytokine induced genes (Tran et al., 2014a). The cell proliferation studies in the presence of inhibitors; AdOx, curaxin and ribavirin demonstrate that all the oncogenes tested (e.g MPL W515L, JAK2 V617F) have an effects on dose response curve to all the inhibitors used, but there is no significant difference in the effects between the oncogenes and control cells. This suggests that mRNA export complex UIF, SRAG and eIF4E are not targetable and may not offer therapeutic advantage in myelofibrosis. Further interaction studies need to be done to further understand the THOC5 function and regulation of mRNA export complex in MPN associated oncogenes.
In addition, our preliminary result (in collaboration with Teruko Tamura’s group in Hannover, Germany) on the THOC5 interactome studies (Saran et al., 2013) have led to it being shown that SOX9 is involved in transcriptional activation following THOC5 activation and this may be a therapeutic target in MPN myelofibrosis. SRY-box containing gene 9 (SOX9) a member of the SOX (SRY–related high-mobility group box) characterized by the presence of a 79 amino acid HMG-type DNA-binding domain (Wegner, 1999). In vitro studies observed that SOX9 activates transcription and cell differentiation (Bastide et al., 2007; Blache et al., 2004). SOX9 may as a part of THOC5 mRNA complex contribute to processing of a subset of wingless/integrated (Wnt) target mRNAs in the small intestine (Guria et al., 2011). Saran et al, 2013 demonstrated that THOC5 depletion in intestinal tissue caused a reduction in SOX9 transcription (Saran et al., 2013). Moreover, they also showed that unspliced SOX9 mRNA accumulated in the nucleus upon depletion of THOC5. This suggests THOC5 contributes to both mRNA processing and export as well as in degradation of unspliced SOX9 mRNA (Saran et al., 2013). In addition, SOX9 expression can be activated by TGFβ family members; bone morphogenetic proteins (BMPs) through mitogen-activated protein (MAP) kinases pathway to induce human marrow-derived mesenchymal stem cell (hMSC) differentiation (Chang et al., 2008; Zehentner et al., 1999). Another study by Guang et al, (2012), showed that blocking of the SDF-1/CXCR4 pathway inhibits the BMP2-induced SOX9 (regulator of early phase of chondrogenic differentiation) and Runx2 (transcription factor for late phase of chondrogenic differentiation) expression in ATDC5 chondrogenic cells and mouse bone marrow-derived mesenchymal stromal cells (BMSCs) (Guang et al., 2012). This, therefore may provide links for our further studies on MPL W515L action.

In conclusion THOC5 is a downstream target of MPL W515L and its phosphorylation can affect motility (Figure 6.1). The future challenge remains, however, to understand how the multifaceted signalling pathways regulated by THOC5 interplay and how TGFβ, MYC, and sphingosine-1-phosphate deregulation lead to in MPN if indeed they do, in primary cells. This work will need to include validation studies on a broad range of myelofibrosis patient material and in-vivo mouse models if opportunities for future therapeutic improvements are to be identified.
Figure 6.1: Schematic representation depicting the MPL W515L and JAK2 mutants potentiates THOC5 induced motility. The substantial analysis elucidated a pathway from CXCL12/CXCR4/CD45 mediated Src activation to THOC5 Y225 phosphorylation that had been compromised by MPL W515L. The MPL W515L increase in chemokinesis is modulated by THOC5 Y225 phosphorylation. MPL W515L induced THOC5 phosphorylation was linked to elevated TGFβ, MYC and S1P expression. Inhibition of TGFβ, MYC, SK and S1P induced decrease in THOC5 Y225 phosphorylation and also increased chemokinesis. I have identified a novel pathway disrupted in MPN associated oncogene MPL W515L and allows to understand the mechanisms by which the phosphorylation of THOC5 may contribute to leukaemogenic transformation through links to TGFβ, MYC, and S1P biology. A discovery proteomics screen shows an increase in EZH2 expression level in MPL W515L expressing cells possibly dependent on THOC5 phosphorylation and this may be linked to the increases in chemokinesis observed. SOX9, a THOC5 protein interactor is involved in transcriptional activation following THOC5 activation. Further studies on the EZH2, SOX9 and other proteins of interest (of our proteomic output) will be useful for future analysis in identifying a novel therapeutic target in MPN myelofibrosis.
6.2. Future work

Despite identifying a novel pathway and mapping certain aspects further work is required to better understand the interplay between THOC5 and the TGFβ, MYC, and S1P pathways and how they contribute to MPN leukaemic transformation. Currently, we are in the process of validating our observations on motility and TGFβ secretion using a broad range of myelofibrosis patient material. To achieve our goal of improving patient treatment we are hoping to exploit the novel pathway I have identified. I believe that this may be possible by using interdisciplinary approaches of rational drug design and/or high throughput screening (Drug Discovery Group Paterson Institute) or by optimization of various combination therapies based around our observations. Our collaborator (Tim Sommervaille, Manchester Cancer Research Centre, UK) is currently developing a MPL W515L mouse model of myelofibrosis which we wish to exploit to investigate the potential of TGFβ, MYC and S1P inhibition, alone and in combination, for the treatment of myelofibrosis patients.

Our group have previously shown that THOC5 is a downstream target of CD45 and also THOC5 phosphorylation is linked to motility (Griaud et al., 2013). There are studies reported that disruption and down regulation of CXCR4/CXCL12 leads to stem cell mobilisation into peripheral blood (Gazitt and Liu, 2001; Gazitt et al., 2001). Our group has also identified a novel phosphorylation event on CD45 that is required for CXCL12-induced chemotaxis in primitive haematopoietic cells (Williamson et al., 2013). As our group suggest the importance of CD45 (Williamson et al., 2013), CXCL12/CXCR4 axis in motility (Griaud et al., 2013), and the down regulation of their expression in MPL W515L expressing cells, it is imperative that we confirm the reduction of CD45 expression in myelofibrosis patient material and examine the phosphorylation status of CD45 on the site we discovered. In addition, it would be particular interesting to look into the in vivo studies on CD45<sup>null</sup> and CD45<sup>WT</sup> mouse model to gain insight into the role of the CD45 pathway in MPL W515L transformation. This could be achieved by transfecting CD45<sup>null</sup> and CD45<sup>WT</sup> Lin<sup>+</sup> haematopoietic progenitor cells with MPN oncogene MPL W515L to study myelofibrosis disease progression in irradiated recipient mice. The CD45<sup>null</sup> and CD45<sup>WT</sup> mice expressing MPL W515L cells will also be compared in
vitro and we will assess their chemokinesis ability using the transwell assay. This could offer a better understanding on the importance of CD45 in regulating HSC mobility (Williamson et al., 2013). In addition, further analysis of the phosphoproteome and THO complex in CD45+/− and CD45+/+ cells in the presence/absence of MPL W515L oncogene using mass spectrometry will provide useful information on which pathways does CD45 attenuate signalling and whether modulation of these could offer therapeutic benefits in myelofibrosis patients. TGFβ has been reported to be involved in CML pathogenesis, MPN myelofibrosis (Martyré, 1995; Martyré et al., 1997) and motility (Boland et al., 1996; Le Bousse-Kerdilès et al., 1996). Our data have shown that MPL W515L expressing cells display a massive induction of TGFβ secretion and this TGFβ release is involved in the increased chemokinesis observed in MPL W515L cells. Further screening using single or combination therapies based around our novel pathway developed can be exploited by treated the MPL W515L mouse model using MYC inhibitor, JQ1, TGFβi, and SKi and measurement of MPL W515L positive cell viability, cell survival and proliferation to see which pathways are impaired in the myelofibrosis transformation.

Cancer is a genetic disease associated with mutations of tumor suppressor genes or oncogenes. Current research has expanded the mechanistic detail of cancer to include epigenetic dysregulation. Epigenetic modification is characterised by DNA methylation, post translational modification (PTM) of histones and chromatin remodelling which does not involve changes to DNA sequence (Au et al., 2013; Simon and Lange, 2008). Accumulating evidence has shown that EZH2 is a promising drug target and multiple selective inhibitors of EZH2 such as GSK126, EPZ005687, E11, and EPZ6438 target have been developed with some entering clinical trials (Knutson et al., 2012; McCabe et al., 2012; Yamaguchi and Hung, 2014). GSK126, a potent selective S-adenosyl methionine (SAM) competitive inhibitor of EZH2 methyltransferase activity decreases global H3K27me levels and reactivates silenced PRC2 target genes. Given our observations on the increase in c-MYC expression as well TGFβ secretion in MPL W515L cells, there is the possibility that their upregulation contributes to increase in EZH2 expression and chemokinesis observed in MPL W515L expressing cells. Studies have shown that MYC binds to EZH2 promoters and directly
activates their transcription activation, for example, EZH2 expression is correlated with MYC expression in prostate cancer (Koh et al., 2011). In addition, MYC also upregulates EZH2 expression via repression of miRNA 101, miR-26a in AML (Salvatori et al., 2011; Sander et al., 2008). However, in contrast, EZH2 may regulate c-MYC expression in glioblastoma (Suvà et al., 2009; Yamaguchi and Hung, 2014). Therefore, to link the MYC, TGFβ and EZH2 observations in our studies, the MPL W515L expressing Ba/F3 cells or primary MF cells could be treated with JQ1 and TGFβ inhibitors and the level of EZH2 expression in those samples analysed by western blot. In addition, colony forming assay, proliferation and differentiation studies on MPL W515L expressing cells treated with EZH2 inhibitor, GSK126 are of interest to understand the involvement of EZH2 in myelofibrosis. This can include in vivo mouse model studies as well as studies on the motility effects of EZH2 inhibition. The latter may offer a better understanding on the mechanistic detail of motility response in myelofibrosis. Recent studies suggest that aberrant PRC2 gene repression and DNA hypermethylation in Wilm’s Tumor 1 (WT1) mutant induced leukaemogenesis in AML and response to GSK126 (Sinha et al., 2015). Given all our data on pathway analysis we are currently using SWATH mass spectrometry or multiple reaction monitoring (MRM) MS to investigate and link MPL W515L to histone modification and protein level changes as well as THOC5 phosphorylation in primary cells. This will advance our knowledge on which signalling pathway regulates motility in myelofibrosis in humans. Other future work will address the current finding involving EZH2 and its possible role in driving myelofibrosis. This can be done via targeting EZH2 gene or its downstream signalling pathways and may be a promising strategy towards suppression of cancer development particularly in myelofibrosis. Of interest, there are studies that have demonstrated that TGFβ stimulation of SOX4 directly induces the expression of EZH2 mRNA and this is critical for SOX4-mediated epithelial-mesenchymal transition (EMT) in breast cancer cells (Tiwari et al., 2013; Yamaguchi and Hung, 2014).

THOC5, a protein of the TREX complex is a downstream target of many leukaemogenic PTKs (Pierce et al., 2008a) and MPL W515L suggesting that THOC5 is involved in leukaemia development. Given our results in chapter 5, we should therefore ask the question
whether THOC5 phosphorylation plays a role in mRNA metabolism. Therefore, further THOC5 interactome studies in the presence and absence of THOC5 Y225F mutation can be performed to see which proteins bind to THOC5 and (inferred) phospho Y225. These studies will bring new insights into unknown aspects of the role of THOC5 function and other members of the mRNA export complex that bind to it may modulate leukaemic transformation. Further definition of THOC5 protein modification can also be achieved by a mass spectrometry approach using multiple reaction monitoring (MRM) for methylation and acetylation as well as phosphorylation analysis for the effects of THOC5 Y225 phosphorylation on mRNA specific association with the TREX complex. The above studies offer a way to further understand THOC5 function and how the regulation of mRNA metabolism contributes to leukaemic transformation and in addition could identify therapeutic targets in myeloproliferative neoplasms. This could also yield data on how this protein may be involved in normal and abnormal blood cell production.

### 6.3. Conclusion

Figure 6.1 summarises the findings of this thesis and also includes future plan for SOX9 and EZH2 to further understand the biological role of THOC5 on motility with an aim to improve the clinical outcome in patients with MPN. In conclusion I have identified a novel pathway disrupted in MPN and allows to start to understand the mechanisms by which the phosphorylation of THOC5 may contribute to leukaemogenic transformation via TGFβ, MYC, and S1P biology. Our group are in the process of validating these cell line based observations into MPL W515L patient material and investigating whether any of the pathways that I have discovered offer therapeutic targets in the treatment of myelofibrosis.
Chapter 7

Appendices
Chapter 7

Appendices

Appendix 7A- SDS-PAGE and WB reagents and solutions

Separation gel recipes (6 mL per gel)

<table>
<thead>
<tr>
<th>Component</th>
<th>5%</th>
<th>7.5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protogel™ (mL)</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>1.88M Tris-HCl pH 8.8 (mL)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
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<tr>
<td>0.5% (w/v) SDS in distilled H₂O (mL)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
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<tr>
<td>distilled H₂O</td>
<td>2.6</td>
<td>2.1</td>
<td>1.6</td>
</tr>
<tr>
<td>TEMED (µL)</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10% (w/v) APS in distilled H₂O (µL)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Stacking gel recipes (2 mL per gel)

<table>
<thead>
<tr>
<th>Component</th>
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</thead>
<tbody>
<tr>
<td>Protogel™ (mL)</td>
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<td>0.625M Tris-HCl pH 6.8 (mL)</td>
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<td>0.5% (w/v) SDS in distilled H₂O (mL)</td>
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<td>distilled H₂O</td>
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<tr>
<td>TEMED (µL)</td>
<td>5</td>
</tr>
<tr>
<td>10% (w/v) APS in distilled H₂O (µL)</td>
<td>20</td>
</tr>
</tbody>
</table>

4x Laemmli buffer

2 mL 1.25M Tris pH 6.8,
0.4g SDS stock,
6 mL of 100% glycerol,
200 µL bromophenol blue (diluted 1% in ethanol)
1 mL β-2-mercaptoethanol
1.4 mL distilled H₂O

10x SDS-PAGE running buffer

A 10x stock solution of 2M glycine and 0.25M Tris was made using distilled water and stored at room temperature. The buffer was diluted with 9 volumes of distilled H₂O and SDS added to 0.1% just prior to using.

Transfer buffer

10x running buffer was diluted with 9 volumes of distilled water with the addition of methanol (10% v/v)
Appendix 7B- Effects of inhibitors on cell cycle

6 hours treatment of Ba/F3 cells with the inhibitors shown had no effect on cell cycle.

Appendix 7B: Cell cycle analysis of Ba/F3 +/- kinase inhibitors studies on FACS CALIBUR™ analyzer (Becton Dickinson, Oxford, UK). Ba/F3 cells were treated with/out DMSO, TGFβi (50 and 5µM), JQ1 (500nM), SKi (10µM), MEKi (10µM), and ruxolitinib (50µM) for 6 hours, fixed and cellular DNA was stained with propidium iodide (PI). DNA content is represented by PI intensity and cells distinguished by G0/G1, S, and G2/M cycle phase that were analysed by FlowJo 7.6 (Tree Star, USA) software. A G0/G1 peak representing cells with 2N DNA content and G2/M have undergone replication posses 4N DNA content. The area between these two peaks is representing of S phase.
Appendix 7C- Effects of inhibitors on cell cycle

6 hours treatment of Ba/F3 MPL W515L cells with the inhibitors shown had no effect on cell cycle.

Appendix 7C: Cell cycle analysis of Ba/F3 MPL W515L +/- kinase inhibitors studies on FACS CALIBUR™ analyzer (Becton Dickinson, Oxford, UK). Ba/F3 MPL W515L cells were treated with/out DMSO, TGFβi (50 and 5µM), JQ1 (500nM), SKi (10µM), MEKi (10µM), and ruxolitinib (50µM) for 6 hours and cellular DNA, fixed and stained with propidium iodide (PI). DNA content is represented by PI intensity and cells distinguished by G0/G1, S, and G2/M cycle phase that were analysed by FlowJo 7.6 (Tree Star, USA) software. A G0/G1 peak representing cells with 2N DNA content and G2/M have undergone replication possesses 4N DNA content. The area between these two peaks is representing of S phase.
Chapter 8

References
Chapter 8

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Books, website and others


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