Genetic and Chemical Modulators of JAK/STAT Signalling in Cancer

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Abstract

Activation of JAK/STAT signalling is a feature of many haematological malignancies and solid tumours. Better understanding of the molecular events contributing to JAK/STAT pathway activation, and a greater range of therapies acting on the pathway, should lead to improved treatment options for patients with malignancies associated with activation of the pathway. This work builds on screens that identified genes and drugs which modulate JAK/STAT signalling in the fruit fly *Drosophila melanogaster*, to determine whether these effects occur in the conserved human pathway.

The gene *ANKHD1* (Ankyrin Repeat and KH Domain containing 1) has been identified as a positive regulator of JAK/STAT signalling in *Drosophila*, but there has been little investigation of ANKHD1 in human tissues. I show that ANKHD1 protein is expressed in normal blood cells and the malignant clone of cells in acute leukaemias. I also show that ANKHD1 protein is expressed in the skin cancer malignant melanoma, where it is found in the nucleus and cytoplasm in a range of histological sub-types of melanoma.

Methotrexate was identified as a selective suppressor of JAK/STAT signalling in a screen in *Drosophila* cells. No interaction between methotrexate and JAK/STAT signalling has previously been described. I demonstrate that methotrexate suppresses JAK/STAT signalling in human cell lines at drug concentrations equivalent to those measured in patients, suggesting that suppression of JAK/STAT signalling may contribute to the mechanism-of-action of methotrexate in inflammatory conditions. Furthermore, the effect occurs when the pathway is activated by the JAK2V617F mutation found in patients with myeloproliferative neoplasms (MPNs). The potential of methotrexate as a treatment for patients with MPNs is investigated in primary cells obtained from patients with myelofibrosis.

Declaration

I declare that this thesis is the result of my own work except where the work of others is cited, either explicitly or via the list of references. No part of this thesis has been submitted for a degree, diploma or other qualification at any other university.

Sally Thomas, August 2015.

Publications

Thomas S, Fisher KH, Snowden JA, Danson SJ, Brown S, Zeidler MP (2015) Methotrexate is a JAK/STAT pathway inhibitor. *PLoS One*, 10(7) e0130078.

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Abbreviations

ANKHD1 – ankyrin repeat and KH domain containing protein 1

ANKRD17 – ankyrin repeat domain 17

ALL – acute lymphocytic leukaemia

AML – acute myeloid leukaemia

BMMCs – bone marrow mononuclear cells.

BRAF – B Rapidly Accelerating Fibrosarcoma

DAB - diaminobenzidine

dMASK – *Drosophila* multiple ankyrin repeats, single KH domain

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulphoxide

EDTA – ethylenediamenetetraacetic acid

EPO – erythropoietin

ET – essential thrombocythaemia

FBS – foetal bovine serum

GHR – growth hormone receptor

GTAR – gene trap ankyrin repeat

hMASK – human multiple ankyrin repeats, single KH domain

HSCs – haematopoietic stem cells

JAK – janus kinase

Lck – Lymphocyte Specific Protein Tyrosine Kinase

MPNs – myeloproliferative neoplasms

MASK – multiple ankyrin repeats, single KH domain

OSM – oncostatin M

PBMCs – peripheral blood mononuclear cells

PBS – phosphate buffered saline.

PMF – primary myelofibrosis

PV polycythaemia vera

qPCR – quantitative real-time polymerase chain reaction

RA-FLS – rheumatoid arthritis fibroblast-like synoviocytes.

RPMI – Roswell Park Memorial Institute

SOCS – suppressor of cytokine signalling

STAT – signal transducer and activator of transcription.

TBS – Tris buffered saline

TIL – tumour infiltrating lymphocytes

Vpr – HIV-1 viral protein R

VBARP-L, VBARP-S – Vpr-binding ankyrin repeat protein, long and short forms

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Chapter 1: Introduction

1.1 Overview

In metazoans the behaviour of individual cells must be organised and co-ordinated by the whole organism to give rise to functioning organs and tissues. This control is achieved through a network of intracellular signalling pathways activated by transmembrane receptors at the cell-surface. The behaviour of individual cells, therefore, can be controlled by the action of soluble ligands produced locally or distantly, enabling cell behaviour to be regulated according to the needs of the whole organism.

In cancer, a clone of cells escapes this regulation and expands irrespectively of the needs of the whole organism. One of the mechanisms through which cancer cells acquire this property is through inappropriate activation of intracellular signalling pathways. Pathway activation becomes independent of or hyper-responsive to the signals normally promoting cell growth. Understanding this aspect of the cellular biology of cancer has led to improvements in the treatment of patients. Targeted therapies designed to act selectively on inappropriately activated signalling pathways are in clinical use. In some cases our understanding of the mechanisms causing pathway activation have enabled the use of molecular pathology techniques to identify patients who will benefit from these treatments. Further understanding of aberrant activation of intracellular signalling in cancer should lead to benefits for patients.

The JAK/STAT signalling pathway (Janus Kinase/ Signal Transducer and Activator of Transcription) is one of the signalling pathways frequently activated in cancer. It has been particularly associated with myeloproliferative neoplasms, a group of haematological malignancies in which an acquired mutation in *JAK2* that causes a valine to phenylalanine change (V617F) occurs in a large proportion of patients. Aberrant JAK/STAT signalling also plays a role in other haematological malignancies and many solid tumours. In some types of cancer the molecular mechanisms underlying JAK/STAT activation have been determined whereas in others they are incompletely understood and there is little understanding of the factors modulating JAK/STAT activation in cancer. JAK/STAT signalling is a therapeutic target in cancer and there have been great advances in the clinical use of

drugs targeting the pathway. There are, however, ways in which our ability to target the pathway with drugs may be improved.

The aims of this work were to investigate genetic and chemical modulators of the JAK/STAT signalling pathway in cancer. The work is founded on identification of genetic and chemical modulation of JAK/STAT signalling in the fruit fly *Drosophila melanogaster*. *Drosophila* possess a relatively linear JAK/STAT signalling pathway compared to that of vertebrates. In vertebrate evolution duplications of genes encoding pathway components has led to increased complexity and potential redundancy within the pathway. My work has built on data obtained from *Drosophila*, to establish whether effects of genetic and chemical modulators of the pathway are conserved in human cells, and ultimately whether they are relevant to JAK/STAT activation in cancer.

The work focusses on the effects of the gene Ankyrin Repeat and KH domain containing protein 1 (*ANKHD1*) upon the pathway. *ANKHD1* is the human homologue of a gene identified as a novel positive regulator of JAK/STAT signalling in *Drosophila.* The aim of this study was to determine the expression of ANKHD1 protein in JAK/STAT associated cancers (haematological malignancies and the skin cancer malignant melanoma), and examine the mechanism through which ANKHD1 promotes JAK/STAT pathway activation.

A secondary arm of the project examines chemical modulators of JAK/STAT signalling, building on findings from a small-molecule screen performed in *Drosophila* cells that showed that the drug methotrexate suppresses JAK/STAT signalling. The aim of this study was to establish whether methotrexate could suppress JAK/STAT signalling in cancers showing JAK/STAT activation.

Overall this project is founded on the idea that a better understanding of genes and drugs that modulate JAK/STAT signalling will lead to better ways to treat patients with cancers showing JAK/STAT pathway activation, by enabling stratification of patients according to the mechanism of activation and molecular predictors of prognosis, and a creating a wider range of treatment options for patients with cancers showing JAK/STAT activation.

1.2 JAK/STAT signalling

1.2.1 Structure of pathway and pathway components

Activation of the pathway

The JAK/STAT pathway regulates embryonic development and is involved in the control of processes such as stem cell maintenance, haematopoiesis and the inflammatory response. The pathway transduces signals from cytokines, interleukins and growth factors that bind transmembrane receptors, and activate a phosphorylation cascade that ultimately regulates gene transcription. Ligand binding produces conformational changes in receptors that alter the alignment of receptor-associated JAKs, enabling phosphorylation of specific tyrosine residues which converts inactive JAKs into a catalytically active tyrosine kinases. A detailed understanding of the molecular-level events occurring in this process has recently been established for the growth hormone (GH) receptor associated with JAK2 (Brooks, Dai *et al*. 2014).

Active JAKs phosphorylate tyrosine residues in the cytoplasmic region of the receptor creating binding sites that recruit STATs (Signal Transducers and Activators of Transcription). STATs are JAK substrates which form dimers that translocate to the nucleus when phosphorylated on certain tyrosine residues (termed pSTAT). STAT dimers bind specific promoter sequences and modulate transcription of genes controlling cellular processes including proliferation, differentiation and apoptosis (Figure 1-1).

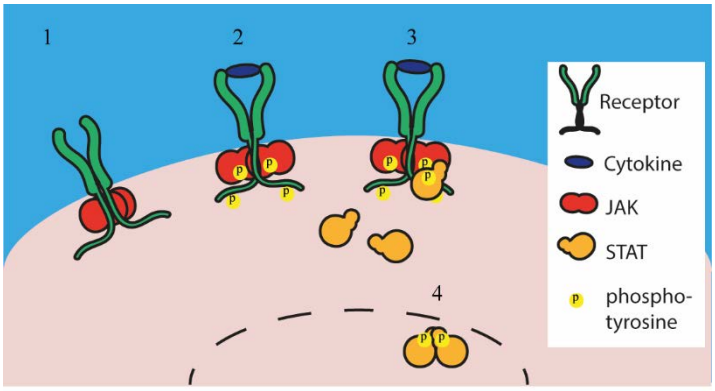


Figure 1-1. Overview of JAK/STAT signalling. JAKs are constitutively associated with transmembrane receptors (1). Ligand binding causes a change in the orientation of receptor sub-units which leads to JAK phosphorylation and activation. Activated JAKs phosphorylate intracellular regions of the receptors (2). This creates binding sites for STATs which are phosphorylated by JAKs (3) enabling them to dimerise and translocate to the nucleus where they modulate gene transcription (4).

Receptors signalling through the JAK/STAT pathway

The JAK/STAT signalling pathway starts with cell surface receptors, which are constitutively associated with JAKs in the absence of ligand (Witthuhn, Quelle *et al*. 1993). These receptors are made up of sub-units which contain an extracellular domain that binds the cytokine, a transmembrane domain and an intracellular cytoplasmic domain, of varying length. The JAK/STAT pathway transduces signals from a number of transmembrane receptor families, which are classified according to their structural similarities. Type I receptors include the erythropoietin (EPO) receptor and the granulocyte colony-stimulating factor (G-CSF) receptor. The granulocyte-macrophage colony-stimulating factor receptor is a type IIa receptor and the type IIb subfamily includes the receptors for interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF) (Watowich, Wu *et al*. 1996).

In order to function as receptors, individual receptor sub-units must be assembled into complexes. Some receptors consist of homodimers of identical subunits, such as the receptors for GH, EPO and thrombopoietin (TPO). For other cytokines, receptors consist of heterodimers, often involving a sub-unit which is shared between the receptors for several different cytokines. For example glycoprotein 130 (gp130) is a component of the receptors for IL-6, oncostatin M (OSM) and LIF (Lupardus, Skiniotis *et al*. 2011), and cytokines signalling through JAK3 act on receptor complexes that contain a common γc subunit (Cornejo, Boggon *et al*. 2009). In some cases the cytokine receptor consists of a higher order complex containing multiple receptor subunits. This has been demonstrated for the receptor for IL-6, where electron microscopy shows that the cytokine-receptor complex is a hexamer consisting of two molecules of IL-6, two IL-6 receptor alpha subunits and two gp130 subunits (Lupardus, Skiniotis *et al*. 2011).

In early models of receptor activation, ligand binding was considered necessary for dimerisation or oligomerisation of receptor subunits. Ligand-mediated dimerisation was considered to be the mechanism that initiated the signalling cascade (Heldin 1995). Although some receptors do appear to require ligand binding to form stable dimers (Russell-Harde, Wagner *et al*. 1999) for other receptors pre-formed dimers are present on the cell surface in the absence of ligand. This has been demonstrated for the EPO receptor by co-localisation of fluorescently tagged receptor subunits (Constantinescu, Keren *et al*. 2001). The regulation of formation of receptor complexes may be a mechanism through which JAK/STAT pathway

activity is controlled. Unpublished data from the laboratory where I carried out my PhD (generated by Dr. Katie Fisher and Dr. Steve Brown) shows that the *Drosophila* JAK/STAT receptor Domeless (Dome) has a widespread tissue distribution in the *Drosophila* embryo, but only exists as a dimer in certain tissues. Conformational changes in relative receptor subunit orientations, rather than a simple dimerisation model, are now thought to be responsible for cytokine-mediated receptor activation in many cases.

JAKs

There are four JAK family members in humans, JAK1, JAK2, JAK3 and TYK2, varying in size from approximately 1120 to 1190 amino acids. The family is defined by the presence of two adjacent kinase domains, resembling the two faces of the Roman god Janus from which their name is derived. JAKs act by forming both homodimers and heterodimers (Koppikar, Bhagwat *et al*. 2012).

JAKs are structurally divided into seven JAK homology regions (JH 1 - 7) based on amino acid sequence similarities across all four JAKs (Figure 1-2). The JH1 domain is the kinase domain (Cornejo, Boggon *et al*. 2009, Ghoreschi, Laurence *et al*. 2009). Phosphorylation of specific tyrosines in the JH1 domain is essential for JAK activation (Ihle and Gilliland 2007). In JAK2 these tyrosines are at position 1007 and 1008 (Feng, Witthuhn *et al*. 1997). JAKs also contain a number of other tyrosine residues whose phosphorylation contributes to positive and negative regulation of JAK activity (Ungureanu, Wu *et al*. 2011). Phosphorylation of serine residues is another mechanism by which JAK activity is modulated (Ishida-Takahashi, Rosario *et al*. 2006, Mazurkiewicz-Munoz, Argetsinger *et al*. 2006).

The JH2 domain, in which the V617F mutation occurs in MPNs, regulates JAK activity. It is described as a ‘pseudokinase’ as it lacks certain amino acids required for catalytic function, but there is evidence that the JH2 domain can act as a kinase despite this. The retained kinase activity is thought to mediate the regulatory functions of JH2, with the JH2 domain phosphorylating sites in JAK2 that negatively regulate its activity (Ungureanu, Wu *et al*. 2011).

The N terminal FERM (4.1 protein Ezrin, Radixin, Moesin) domain, comprising the JH6 and JH7 domains, mediates receptor binding. JAKs are constitutively associated with the intracellular regions of receptor subunits in the

absence of ligand (Witthuhn, Quelle *et al*. 1993). JAK proteins also contain an SH2 (Src homology 2) domain. This is a domain which binds to phosphorylated tyrosine residues, although its function in JAKs is not clear.

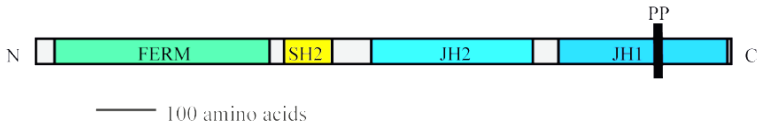


Figure 1-2. Schematic structure of JAK proteins. The FERM domain (4.1 protein, ezrin, radixin, moesin) mediates the interaction with receptor complexes. The SH2 domain is a protein domain that binds to phosphorylated tyrosine residues. The JH2 pseudokinase domain regulates kinase activity of the JH1 kinase domain. PP marks conserved tyrosine residues in JH1 whose phosphorylation is essential for JAK activation. N and C indicate the amino-terminus and carboxy-terminus.

Our understanding of the three-dimensional structures of JAKs is limited. To date, no crystal structures have been determined for the full-length protein of any of the four JAKs. However, crystal structures have been solved for the kinase JH1 and pseudokinase JH2 domains of several JAKs, including JAK2 (Lucet, Fantino *et al.* 2006, Bandaranayake, Ungureanu *et al*. 2012). The structure of JH1 is similar to that of other protein kinases, with a small N-terminal lobe consisting mainly of β sheets, a larger C-terminal lobe composed mainly of α helices, and an activation loop which contains the tyrosine restudies whose phosphorylation regulates activity. The JH2 domain has a similar structure, although the activation loop is shorter and is not regulated by phosphorylation. Despite the availability of these structures, the mechanisms causing JAK activation are not definitively determined.

A recent study of the growth hormone receptor (GHR) in association with JAK2 has given us new insight into the molecular events through which ligand occupation of receptors may cause conversion of inactive JAKs to active kinases. In this model, a receptor that is not occupied by ligand holds the JH1 domain of one JAK is in close association with the JH2 of the other JAK in an orientation that suppresses JAK activation. Ligand binding causes a conformational change that is transmitted through the transmembrane region of the receptor and pulls apart the receptor-associated JAKs in a manner which exposes their kinase domains and allows activation (Brooks, Dai *et al.* 2014). This is consistent with the observation that constitutively active mutant JAKs nonetheless require expression of receptors in

order to produce the changes in cellular behavior that lead to increased colony formation and the MPN phenotype (Sangkhae, Etheridge *et al*. 2014).

STATs

Seven STAT family members are found in humans, STAT 1-4, STAT5A, STAT5B and STAT6. These range in size from 750 to 850 amino acids. STAT proteins contain a number of domains whose function has been defined (Figure 1-3). The C terminal regions of STATs contain the transactivation domain needed for activation of transcription. STAT activation requires phosphorylation of a specific tyrosine residue located near the transactivation domain, at around position 700 (Darnell, Kerr *et al*. 1994). The transactivation domain also contains a number of serine residues which can be phosphorylated, a post-translational modification which increases the ability of STATs to activate transcription (Decker and Kovarik 2000).

Adjacent to the transactivation domain is an SH2 domain, which allows STATs to bind phosphorylated receptors and form dimers with other phosphorylated STATs. A DNA binding domain interacts specifically with particular DNA sequences and the N terminal oligomerisation domain stabilises interactions between DNA-bound STATs (Bowman, Garcia *et al.* 2000).

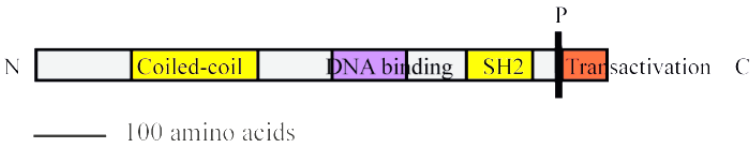


Figure 1-3. Schematic structure of STAT proteins. The SH2 domain binds phosphorylated tyrosines. The carboxy-terminus transactivation domain is required for full transcriptional activation. P marks the conserved tyrosine residue whose phosphorylation is essential for STAT activation.

Alternative splicing and post-translational modifications generate additional diversity amongst STATs. Variants of STAT1 and STAT3 (termed STAT1β and STAT3β) which lack the C terminus required for activation of transcription negatively regulate the activity of the full-length variants (Caldenhoven, van Dijk *et al*. 1996). Short forms of STAT5 missing the C terminus are produced by proteolytic processing and are functionally distinct from the full-length forms (Azam, Lee *et al.* 1997). Complexity in the pathway is also generated by the ability of STATs to form

heterodimers, for example STAT1-STAT3 heterodimers observed in melanoma (Niu, Bowman *et al*. 2002).

Regulation of pathway activation

As in other signalling cascades, activation of the JAK/STAT pathway is tightly controlled by negative regulators acting at multiple levels. Cell membrane regions containing activated receptors are endocytosed. It appears that signalling from receptor-ligand complexes within endosomes may differ from that at the cell surface, but endocytosis also regulates signalling by controlling receptor degradation by lysosomes (Hitchcock, Chen *et al*. 2008).

Several families of phosphatases remove phosphate groups from tyrosine residues in receptors, JAKs and STATs. For example, JAKs may be de-phosphorylated by receptor tyrosine phosphatases such as CD45, cytoplasmic phosphatases including SHP1 and SHP2 and the endoplasmic-reticulum tethered phosphatase PTP1B (Babon, Lucet *et al*. 2014).

PIAS proteins (Protein Inhibitors of Activated STAT) are a protein family involved in negative regulation of JAK/STAT signalling. Co-immunoprecipitation experiments demonstrate that PIAS3 physically associates with STAT3 following pathway activation. Electromobility shift assays show that it inhibits the DNA binding activity of STAT3, and luciferase assays indicate that it reduces transcription from STAT3 responsive promoters (Chung, Liao *et al*. 1997). Examination of the structure of PIAS proteins indicates that they also have domains involved in interactions with the nuclear matrix and in SUMOylation, the post-translational addition of a small ubiquitin-like protein that modifies the biological activity of the proteins to which it is bound. It is not clear, however, to what extent these activities are involved in the regulation of JAK/STAT signalling and other intracellular signalling pathways (Yagil, Nechushtan *et al*. 2010).

SOCS proteins (Suppressor Of Cytokine Signalling) are negative regulators of JAK/STAT pathway activation whose expression is induced by STATs, creating a negative feedback loop that imposes a fine level of control on the pathway. The cytokine-induced expression of SOCS proteins was first demonstrated using northern blotting in the livers of IL-6 treated mice, and confirmed following *in vitro* treatment of bone marrow cells with a range of JAK/STAT activating cytokines (Starr, Willson

et al. 1997). SOCS family proteins have actions at multiple levels of the JAK/STAT signalling cascade, having effects upon receptors, JAKs and STATs. For example a recently solved crystal structure shows that SOCS3 binds to tyrosine phosphorylated receptors via its SH2 domain and simultaneously blocks the substrate-binding region of JAK2 (Kershaw, Murphy *et al*. 2013). SOCS proteins also promote the interaction of pathway components with ubiquitin ligases, leading to proteasomal degradation (Kamizono, Hanada *et al*. 2001).

Tight regulation of pathway activity, mediated by several mechanisms acting at every level of the pathway, enables the JAK/STAT signalling pathway to be employed in processes such as development and immunity, whose activity is spatially and temporally regulated.

Non-canonical signalling

JAK and STAT pathway components have effects on the cell beyond the 'canonical' signalling phosphorylation cascade that positively regulates transcription. These include emerging functions of non-phosphorylated proteins, and effects of the pathway on cellular processes other than transcription.

JAKs have been shown to have epigenetic effects on gene expression. Activated JAK2 can enter the nucleus, where it phosphorylates the core histone H3, altering its binding to the heterochromatin protein HP1 and leading to a chromatin conformation that promotes transcription. This effect of JAK2 upon transcription is thought to be independent of STAT activation, as the JAK/STAT upregulated gene, *lmo*, examined in these experiments lacked a predicted STAT5 binding site (Dawson, Bannister *et al*. 2009). The extent to which JAKs locate to the nucleus *in-vivo*, and the significance this plays in normal JAK/STAT activation and in disease remain controversial (Girodon, Steinkamp *et al*. 2011).

STATs are traditionally thought of as facilitating activation of transcription, but data emerging from large-scale gene expression profiling experiments shows that activated STATs also down regulate expression of a substantial number of genes (Demaria, Giorgi *et al*. 2010). It is also becoming apparent that un-phosphorylated STATs can enter the nucleus and modulate gene expression. For example, in cells transfected with an engineered form of STAT5A in which the conserved tyrosine residue required for canonical activation is replaced with phenylalanine, STAT5 has

been shown to be present in the nucleus using immunofluorescence. Co-immunoprecipitation experiments show that it physically associates with HP1 and microarray studies indicate that this alters gene expression (Hu, Dutta *et al*. 2013). STATs have also been shown to play roles in cellular activities which are independent of transcription, for example STAT3 interacts with stathmin to modify microtubule stability and migration, and serine-phosphorylated STAT3 has effects on mitochondrial respiration (Mohr, Chatain *et al*. 2012).

As roles for JAK/STAT pathway components outside the canonical signalling cascade become apparent, there are suggestions that signalling in JAK/STAT associated diseases may alter these non-canonical activities (Chen, Staudt *et al*. 2012).

Interactions with other signalling pathways

Although usually described as discrete pathways, intracellular signalling cascades are probably more accurately considered as networks made up of multiple interactions between pathways. Physical interactions and functional effects have been described for interactions between JAK/STAT signalling and a number of other signalling pathways known to be involved in oncogenesis, including signalling downstream of the epidermal growth factor receptor (Olayioye, Beuvink *et al*. 1999) and androgen receptor signalling (Tan, Dagvadorj *et al*. 2008). Interactions between JAK/STAT signalling and other pathways include JAKs phosphorylating targets other than STATs and STATs being phosphorylated by kinases other than JAKs.

In addition to its effects on STATs, JAK2 is known to activate signalling via the PI3K-AKT and MAPK/ERK pathways (Vainchenker and Constantinescu 2012). Effects of the mutant JAK2V617F upon DNA repair are thought to be mediated via the PI3K-AKT pathway, as they can be rescued by shRNA-mediated knockdown of the catalytic subunit of PI3K (Chen, Ahn *et al*. 2014). The Ras/MAPK signalling pathway appears to interact with JAK/STAT signalling in melanoma in in-vitro models. Expression of the mutant pathway intermediate BRAF in melanocytes leads to serine any tyrosine phosphorylation of STAT3 which is suppressed by treatment with the BRAF inhibitor vemurafenib (Liu, Cao *et al*. 2013).

The Src family of kinases can activate STATs independently of JAK activation. This was first established for v-Src, a viral oncoprotein, which was

demonstrated to bind to and phosphorylate STAT3, leading to nuclear localisation and conferring DNA binding activity (Cao, Tay *et al*. 1996). Endogenous Src kinases have subsequently been demonstrated to phosphorylate STATs, for example c-src acting downstream of epidermal growth factor receptor (EGFR)(Olayioye, Beuvink *et al*. 1999).

The JAK/STAT pathway interacts with the NfkB pathway. This pathway is involved in the innate and adaptive branches of the immune system, and is often activated in cancer. Activators of the pathway include toll-like receptors and TNFalpha. Phosphorylated STAT3 promotes activity in the pathway by enhancing nuclear retention of RelA, a transcriptional activator in the pathway (Lee, Herrmann *et al*. 2009).

An understanding of the complex interactions between JAK/STAT signalling and other intracellular signalling pathways is of particular relevance when considering aberrant signalling in cancer. Combination therapies targeting several signalling pathways may be more effective than targeting a single pathway, and in future may be used to overcome resistance to individual targeted therapies (Liu, Cao *et al*. 2013, Gallipoli, Cook *et al*. 2014).

1.2.2 JAK/STAT signalling in cancer

Aberrant activation of the JAK/STAT signalling pathway is seen in a broad range of haematological malignancies and solid tumours. Our knowledge of the mechanisms causing this activation and the role it can play in the malignant phenotype of cancer cells is growing. In many cases, however, our understanding of the degree to which JAK/STAT activation is driving disease is limited.

Mechanisms causing JAK/STAT activation in cancer

In many cancers where JAK/STAT activation is a feature, the mechanism underlying inappropriate pathway activation is not known. However, examining the instances in which a mechanism has been identified shows that cancer cells employ a diverse range of strategies to activate the pathway.

In head and neck squamous cell carcinoma, phosphorylation of STAT3 is a consequence of increased production of IL-6 by tumour cells (Sriuranpong, Park *et al*. 2003). Increased expression of the G-CSF receptor is observed in high-grade

ovarian epithelial tumours, and experiments in cell culture suggest that G-CSF contributes to JAK/STAT activation in this disease (Kumar, Fraser *et al*. 2014).

Gain-of-function mutations in JAKs have been observed to cause pathway activation in haematological malignancies including *JAK2* mutations in MPNs and *JAK1* mutations in T cell acute lymphocytic leukaemia (T-ALL) (Flex, Petrangeli *et al*. 2008). More recently large-scale sequencing efforts have identified genetic changes affecting JAKs in certain solid tumours. Protein-altering mutations in *JAK1* have been identified in tumours in 9% of patients with hepatitis B associated hepatocellular carcinoma, and validation in cell culture shows that these mutations increase phosphorylation of JAK1 and STAT3 and enable cytokine-independent growth (Kan, Zheng *et al*. 2013). In gastric adenocarcinoma, a comprehensive molecular characterization project has revealed frequent amplification of the chromosomal locus containing *JAK2*. Corresponding increases in JAK2 messenger RNA suggest this may increase JAK2 protein levels and pathway activity (The Cancer Genome Atlas Research 2014).

Activating mutations in STATs, although generally rare, have been described in cancer. In large granular lymphocytic leukaemia 40% of patients have mutations affecting the SH2 domain of STAT3. The mutations cluster in the SH2 domain, and modelling the amino-acid changes indicated an increase in hydrophobic residues, suggesting that the mutations might increase affinity of STAT3 for phosphorylated receptors, increasing their availability for phosphorylation by JAKs. Western blotting from primary cells indicated that there was increased STAT3 phosphorylation compared to leucocytes from normal healthy individuals. Gene expression profiling showed an increase in mRNA for STAT3 target genes (Jerez, Clemente *et al*. 2012, Koskela, Eldfors *et al*. 2012). Amplification of the *STAT5A/B* locus has been described in prostate cancer and is associated with increased expression and nuclear localization of STAT5 in tumour samples. These amplifications increase cell survival in culture and promote tumour growth in a xenograft model (Haddad, Gu *et al*. 2013).

Reduced expression of negative regulators can cause increased pathway activation. In non-small cell lung cancer (NSCLC) tumour samples, expression of SOCS-3 is lost due to promoter hypermethylation. The impact of this on pathway activation was validated using a NSCLC cell line, where restoration of SOCS3 expression reduced constitutive STAT3 phosphorylation (He, You *et al*. 2003).

PIAS3 protein levels have been shown to be reduced in glioblastoma, possibly due to increased protein degradation. In glioblastoma tissue samples low levels of PIAS3 are associated with increased pSTAT3 and increased expression of proteins produced from STAT target genes (Brantley, Nabors *et al*. 2008).

Role of JAK/STAT signalling in the malignant phenotype of cancer cells

In order for solid tumours to enlarge, cancer cells must not only increase in number but also adapt to and alter their microenvironment. Genes controlled by STATs play roles in both of these aspects of the malignant phenotype.

Pre-clinically, therapies that inhibit STAT activity decrease proliferation and increase apoptosis in cell culture studies and tumour xenograft models (Kim, Lee *et al*. 2014). STAT3 facilitates cell cycle progression by promoting activation of cyclin dependent kinases (CDKs). It increases transcription of positive regulators such as cyclin D2 and downregulates transcription of CDK inhibitors such as p21 (Fukada, Ohtani *et al*. 1998). STAT5 confers protection from apoptosis, by activating transcription of Bcl-x, to produce the anti-apoptotic protein Bcl-x_L (Dumon, Santos *et al*. 1999).

Cancer cells undergo changes in energy metabolism, switching from mitochondrial oxidative phosphorylation to glycolysis for ATP production. STAT3 reduces expression of genes encoding mitochondrial proteins and increases expression of genes involved in glycolysis, such as pyruvate dehydrogenase kinase 1. These effects are dependent upon transcription of the hypoxia-inducible factor HIF-1α, which is induced by STAT3 (Demaria, Giorgi *et al*. 2010). STAT3 plays a global role in the adaptation of tumour cells to a hypoxic environment. It physically associates with HIF1α and is required for the full transcriptional activation of HIF1α regulated genes in hypoxia (Pawlus, Wang *et al*. 2014). Angiogenesis is required for tumour growth, with a key role played by vascular endothelial growth factor (VEGF). STAT3 binds to the *VEGF* promoter and induces VEGF expression. In tumour allograft models expression of a constitutively active STAT3 leads to increased VEGF expression and increased vasculogenesis (Niu, Wright *et al*. 2002).

JAK/STAT activation contributes to acquisition of properties required for tumour invasion and metastasis. This is in part mediated by activation of the programme of epithelial to mesenchymal transition (EMT), usually involved in

embryonic development. The transcription factor TWIST1 is a key regulator of the induction of EMT. STAT3 is required for TWIST1 expression, as abrogation of STAT3 activity by siRNA knockdown, pharmacological inhibition of STAT3 or mutation of the STAT binding site in the promoter of *TWIST1* reduces its expression (Cho, Jeong *et al*. 2013). STAT3 can activate transcription of matrix-degrading enzymes such as matrix metalloproteinase-2. Inhibiting STAT3 reduces invasion through an *in-vitro* basement membrane model, and prevents the establishment of metastatic tumours in a mouse model (Xie, Wei *et al*. 2004).

A key feature in the interaction of malignant cells with the tumour microenvironment is their ability to suppress anti-tumour immune responses. This is illustrated by the importance of the graft-versus tumour effect in allogeneic stem-cell transplantation in haematological malignancies (Andersen 2014), and by the effects of immune checkpoint inhibitors in certain solid tumours (Victor, Rech *et al*. 2015). As JAK/STAT signalling is involved in inflammation and immunity, there is therefore interest in the role of JAK/STAT signalling in the immune response in the tumour microenvironment (Yu, Pardoll *et al*. 2009). Activation of STAT1 by interferons promotes immune surveillance and anti-tumour immunity, partly by upregulating MHC class I mediated antigen presentation by tumour cells. In contrast, STAT 3 and 5 signalling in immune cells appears to suppress anti-tumour immunity. Anti-tumour immune responses in mice are enhanced by ablation of *STAT3* from haematopoietic cells and by drugs that block STAT3 (Kortylewski, Kujawski *et al*. 2005). The enhanced anti-tumour immunity observed in these mice is in part due to a reduction in tumour-infiltrating regulatory T cells (Treg cells), a cell type whose development is dependent on the STAT3/5 target gene *FOXP3* (Zorn, Nelson *et al*. 2006).

Myeloproliferative neoplasms

Historically, JAK/STAT signalling has been associated with myeloproliferative neoplasms, due to the high proportion of patients with this category of diseases possessing acquired activating mutations of *JAK2*. The myeloproliferative neoplasms (MPNs) are a group of haematological malignancies characterised by over-production of mature blood cells of the myeloid lineage. The group comprises polycythaemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis

(PMF). PV and ET result in elevated red cell and platelet counts respectively, both of which cause a tendency to thrombosis. In PMF the marrow is effaced by fibrous tissue which prevents adequate haematopoiesis. PV and ET can progress to myelofibrosis, and all three disorders are associated with a risk of transformation to acute myeloid leukaemia (AML)(Campbell and Green 2006).

Pathological activation of JAK/STAT signalling is a feature of MPNs. 95% of patients with PV and 40 – 60% of patient with ET have an acquired point mutation in exon 12 of *JAK2*, a G to T change in the malignant clone. This causes a valine to phenylalanine change, V617F, in the JAK2 protein, which causes constitutive pathway activation (Baxter, Scott *et al*. 2005, James, Ugo *et al*. 2005, Kralovics, Passamonti *et al*. 2005, Levine, Wadleigh *et al*. 2005). The demonstration that JAK2V617F caused a PV phenotype in a mouse model, and that *STAT5a/b* is necessary for development of PV in these mice provided evidence for a causative role for JAK2-STAT5 activation in MPNs (Walz, Ahmed *et al*. 2012).

There is evidence that individuals in whom the malignant clone lacks the V617F mutation nonetheless show increased JAK/STAT activation in haematopoietic stem cells (HSCs), the compartment in which the malignant clone arises. Flow cytometry to assess STAT5 phosphorylation in HSCs and progenitor cells identified by expression of the cell surface glycoprotein CD34+ shows comparable STAT5 phosphorylation in patients with JAK2V617F and wild-type JAK2 (Anand, Stedham *et al*. 2011). Comparison of gene expression profiles in granulocytes from patients with MPNs with mutant and wild-type JAK2 also show similar patterns of expression of genes considered to be JAK/STAT targets (Rampal, Al-Shahrour *et al*. 2014). There have been calls for this data to be interpreted cautiously, however, as it depends upon the methods used to define the initial set of JAK/STAT target genes (Lau, Hannah *et al*. 2015).

In a proportion of patients with MPNs, acquired mutations in other regions of *JAK2* or other genes involved in JAK/STAT signalling have been identified and account for the observed increase in JAK/STAT signalling. Alternative mutations in exon 12 of *JAK2*, affecting an evolutionarily conserved protein region between lysine 537 and glutamic acid 543, occur in around 3% of patients with PV (Scott, Beer *et al*. 2007). These mutations have been demonstrated to activate the signalling pathway in cell-culture models and one shown to cause erythrocytosis in a mouse model (Scott, Tong *et al*. 2007). A mutation in the thrombopoietin receptor *MPL*

(originally named myeloproliferative leukaemia protein) occur in a small proportion of patients with PMF and ET (Pardanani, Levine *et al*. 2006). *In-vitro* models show that this mutation confers cytokine-independent growth and constitutive phosphorylation of JAK2 and downstream pathway proteins. In a mouse model it causes a myeloproliferative disorder characterised by thrombocytosis (Pikman, Lee *et al*. 2006).

Mutations have also been described in modulators of JAK/STAT signalling which are not directly involved in the phosphorylation cascade. Around 2% of patients with MPNs have an acquired loss-of-function mutation in *SH2B3*, a scaffolding protein which negatively regulates signalling. Primary CD34+ cells from peripheral blood of patients with the mutation show increased STAT3 and STAT5 phosphorylation in response to ligand stimulation. Experiments in cell culture indicate that the mutation prevents SH2B3 from performing its normal inhibitory function as overexpression of the wild-type protein inhibits cytokine-dependent growth, whereas overexpression of the mutant protein does not (Oh, Simonds *et al*. 2010).

MPNs were the subject of a further interesting development at the end of 2013, with whole exome sequencing studies revealing that a substantial proportion of JAK2 negative patients with ET and PMF have mutations in *CALR*, the gene encoding calreticulin (Klampfl, Gisslinger *et al*. 2013, Nangalia, Massie *et al*. 2013). A diverse range of mutations have been observed all of which cause a frameshift that encodes an identical novel c-terminal peptide. The mechanism through which mutant CALR contributes to disease pathogenesis is not yet clear. One of the initial studies suggested that activation of JAK/STAT signalling plays a role, as expression of the mutant *CALR* was able to confer cytokine independent growth *in-vitro* that was sensitive to JAK inhibition, and led to constitutive STAT5 phosphorylation (Klampfl, Gisslinger *et al*. 2013). However, a study on a cell line, MARIMO, with endogenous mutant *CALR*, derived from a patient with acute myeloid leukaemia secondary to ET, suggests mutant CALR does not affect JAK/STAT signalling. MARIMO cells showed reduced expression and phosphorylation of JAK2 and STATs 1,3 and 5 compared to JAK2V617F positive cell lines, and were resistant to concentrations of JAK inhibitors that profoundly affected these lines (Kollmann, Nangalia *et al*. 2015).

In addition to mutations in JAK/STAT pathway components and *CALR*, which are considered 'driver' mutations in MPNs, recurrent mutations in other genes should also be addressed. These are not unique to MPNs, occurring in other haematological malignancies. Loss-of-function mutations in *TET2* (Ten-Eleven Translocation-2) occur in around 12% of patients with MPNs (Delhommeau, Dupont *et al*. 2009). *TET2* mutations are of particular interest in light of a study which showed that the order in which *TET2* and *JAK2* mutations are acquired in the malignant clone affects disease phenotype (Ortmann, Kent *et al*. 2015). TET2 is thought to be involved in synthesis of intermediates involved in DNA methylation. Mutations in other genes affecting epigenetic modifications such as *EZH2* (Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit), *DNMT3A* (DNA Cytosine-5-methyltransferase 5A) and *ASXL1* (Additional Sex Combs-Like 1) are also relatively common in MPNs. Mutations affecting splicing factors, which are more commonly associated with the myelodysplastic syndromes, also occur (Nangalia and Green 2014). These mutations are of particular relevance as it has been shown that profiling of mutations can be used to predict prognosis amongst patient with myelofibrosis (Vannucchi, Lasho *et al*. 2013).

Other Haematological malignancies

Mutations in JAK/STAT signalling components and increased pathway activity are seen in a diverse group of lymphoid and myeloid malignancies. This is covered comprehensively in recent reviews (Chen, Staudt *et al*. 2012, Vainchenker and Constantinescu 2012). Examples of genetic changes leading to JAK activation in haematological malignancies include translocations that promote JAK oligomerisation, such as *TEL-JAK2* fusion (Translocation ETS Leukaemia) in childhood T-ALL (acute lymphocytic leukaemia) (Lacronique, Boureux *et al*. 1997), amplification of *JAK2* in Hodgkin lymphoma (Hao, Chapuy *et al*. 2014), and activating point mutations in JAKs. Other events that lead to pathway activation include loss-of-function of negative regulators such as the protein-receptor-tyrosine phosphatase CD45 in T-ALL (Raponi, Gianfelici *et al*. 2015) and activation of STATs by other kinases, including FLT-3 (FMS-related tyrosine kinase 3) and BCR-ABL (Breakpoint Cluster Region-Abelson Tyrosine Protein Kinase 1).

In recent years the use of whole-genome and whole exome sequencing (WES) has led to identification of genetic changes affecting JAK/STAT signalling in an increasing list of haematological malignancies. Particularly notable in the recent findings from WES is the identification of *STAT* mutations in several haematological malignancies. Until recently, *STAT* mutations were surprisingly rare as a mechanism of JAK/STAT activation in cancer. In 2012, it was found that around 30-40% of patients with T cell large granular lymphocytic leukaemia (LGL) and natural killer cell large granular lymphocytic leukaemia possess mutations in *STAT3* (Jerez, Clemente *et al*. 2012, Koskela, Eldfors *et al*. 2012). The presence of such STAT3 mutations immediately offered potential new diagnostic approaches for these disorders, which can be difficult to distinguish from reactive clonal proliferations of lymphocytes. Interestingly, no clear difference was observed in disease characteristics in the groups of patients with or without mutations. Subsequently, mutations affecting STAT5 have been identified in small groups of LGL patients with wild-type STAT3 (Rajala, Eldfors *et al*. 2013).

WES approaches have also led to the identification of STAT6 mutations in around 11% of patients with follicular lymphoma (Yildiz, Li *et al*. 2015). These mutations cluster in the DNA-binding region of STAT6. Immunofluorescence showed increased nuclear localisation of STAT6 and increased level of STAT6-regulated transcripts in flow sorted primary cells from these patients. Functional assays in HEK and HeLa cells showed that the mutations increased STAT-6 responsive transcription and promotes proliferation in the presence of IL-4, a cytokine whose levels are elevated in the microenvironment in follicular lymphoma.

In patients with T cell prolymphocytic leukaemia a WES study identified mutations in STAT5B and JAK3 in around 30% of patients each, and rarer mutations in JAK1 and the common gamma chain of the IL-2 receptor, which signals via the JAK/STAT pathway (Kiel, Velusamy *et al*. 2014). In some patients with JAK1 mutations, the mutations cause amino acid changes in the JH2 domain which are analogous to the JAK2V617F mutation. Functional experiments in cell culture showed that these lead to increased STAT activation. Notably, inhibition of STAT5 using the antipsychotic drug pimozide led to decreased cell viability in cell lines expressing the mutant pathway components, suggesting STAT inhibition as a future therapeutic strategy in these patients.

Solid tumours

Early evidence that JAK/STAT signalling is activated in solid tumours was derived from cancer cell lines. There is now substantial data demonstrating tyrosine phosphorylation and nuclear localization of STATs, indicative of STAT activation, in tumour tissue derived from a large number of patients across a range of tumour types. A relationship between JAK/STAT activation and prognosis has been observed in many of these tumour types (Table 1-1). In general activation of STAT3 or STAT5 is associated with a worse prognosis, although in breast cancer and in some studies in colorectal cancer and head and neck squamous cell carcinoma it appears to be associated with more favourable outcomes. In breast cancer this relationship is consistent with the role of pSTAT5 in normal physiology – constitutive phosphorylation of STAT5 is a feature of normal breast epithelial cells, where it is thought to promote differentiation (Peck, Witkiewicz *et al*. 2011). For other tumour types, differences in the strategies used to quantify STAT phosphorylation, which vary across all the studies described below, may account for the apparently conflicting associations between STAT phosphorylation and outcome (Monnien, Zaki *et al*. 2010). Activation of STAT1, in contrast, is generally associated with better outcomes across all tumour types. Although STAT activation has now been observed in a wide range of tumour types, in many cases the mechanisms causing STAT activation have not been well defined.

Table 1-1. Summary of studies describing STAT activation and its clinical implications in solid tumours

Cancer type	**STAT activation, tissue sample**	**Number of patients**	**Clinical implications of STAT activation**
NSCLC (Xu and Lu 2014)	STAT3 and pSTAT3 detection with immunohistochemistry	1793 (meta-analysis of 17 studies)	Positivity for STAT3 or pSTAT3 associated with reduced overall survival (Hazard ratio (HR) 0.67, p<0.0001)
Prostate (Mirtti, Leiby *et al*. 2013)	Nuclear STAT5A/B, immunohistochemistry on tissue microarrays from prostatectomy or transurethral resection of prostate	562 radical prostatectomy 106 palliative treatment	Presence of nuclear STAT5 associated with early recurrence (HR 1.6, p 0.012) Presence of nuclear STAT5 associated with prostate cancer specific death (HR 1.59, p 0.034)

Cancer type	STAT activation, tissue sample	Number of patients	Clinical implications of STAT activation
Breast (Sonnenblick, Uziely *et al*. 2013)	Immunohistochemistry for pSTAT3 on tissue microarrays	137/375 positive (36%)	Presence of pSTAT3 associated with improved overall survival in patients receiving adjuvant chemotherapy (10 year survival 79% for
(Peck, Witkiewicz *et al*. 2011)	Immunohistochemistry and immunofluorescence for nuclear pSTAT5 on tissue microarrays	208/421 positive (49%) Node negative, with no adjuvant therapy	pSTAT3 positive, vs 61.5% for pSTAT3 negative, HR 0.48, p 0.01). Absence of activated STAT5 associated with decreased cancer specific survival (HR 2.39, p 0.023)
Rectal/ Colorectal (Monnien, Zaki *et al*. 2010)	Immunohistochemistry for nuclear pSTAT3	39/104 (37.5%) positive. 104 rectal , T3 or resectable T4M0	Presence of activated STAT3 associated with better overall survival (HR 0.3, p 0.01)
(Kusaba, Nakayama *et al*. 2006)	Immunohistochemistry for pSTAT3	62/108 (57%) positive Colorectal adenocarcinoma	Presence of activated STAT3 associated with worse overall survival (p<0.001)
Oral squamous cell carcinoma (Macha, Matta *et al*. 2011)	Immunohistochemistry for nuclear pSTAT3	63/94 (67%) positive. (Follow-up data for 71)	Nuclear pSTAT3 associated with shorter median disease free survival (13months vs 64 months, p 0.019).
(Pectasides, Egloff *et al*. 2010)	Automated quantitative analysis immunohistochemistry for nuclear STAT3		High nuclear STAT3 associated with improved overall survival (Mean 119 months vs 57.3 months, p 0.009)
Cervical squamous cell carcinoma (Takemoto, Ushijima *et al*. 2009)	Immunohistochemistry for nuclear pSTAT3	71/125 (56.8%) positive	Nuclear pSTAT3 associated with reduced overall survival (5 year survival 79.2 months vs 95.3 months, p 0.006)
Malignant	Immunohistochemistry	6/14 primary	In patients with lymph

Cancer type	STAT activation, tissue sample	Number of patients	Clinical implications of STAT activation
melanoma (Messina, Yu *et al*. 2008)	for pSTAT1 and pSTAT3	tumours positive for nuclear pSTAT3, 16/26 lymph node metastases positive for pSTAT3, 6/23 positive for STAT1	node metastases higher rates of recurrence with high pSTAT3 staining compared to low-grade staining (9/16 vs 3/10). Lower rates of recurrence with high pSTAT1 staining 8/23 vs 2/6) activation in lymph node and brain metastases
Renal cell carcinoma (Horiguchi, Oya *et al*. 2002)	Immunohistochemistry for nuclear pSTAT3	24/48 (50%) positive	Nuclear pSTAT3 associated with shortened cancer-specific survival (p 0.0439)
Glioblastoma (Birner, Toumangelova-Uzeir *et al*. 2010)	Immunohistochemistry for pSTAT3 on tissue microarrays	58.8% of 111 positive	High or very high numbers of cells positive for pSTAT3 associated with reduced overall survival (p 0.001)

Melanoma

Activation of intracellular signaling pathways plays a role in melanoma, and an understanding of signaling in melanoma has become particularly relevant in recent years, with the clinical success of BRAF (B Rapidly Accelerated Fibrosarcoma) inhibitors.

40-60% of patients with melanoma have an acquired mutation in the serine/threonine kinase *BRAF*, most commonly causing a valine to glutamic acid change, V600E, that leads to constitutive activation of the Ras/Raf/MEK/ERK signaling cascade (Davies, Bignell *et al*. 2002). This signaling pathway is activated by several families of cell surface receptors and regulates gene expression through a cascade of intracellular kinases, ultimately controlling cell behaviours such as proliferation, differentiation and migration (Holderfield, Deuker *et al*. 2014). Inhibitors of BRAF have shown benefit in patients with metastatic melanoma (Chapman, Hauschild *et al*. 2011), although tumours develop resistance to inhibitors by re-activation of the pathway, or activation of alternative signaling pathways (Fedorenko, Gibney *et al*. 2015). *N-RAS* mutations are also a relatively common

event in melanoma leading to altered intracellular signalling, although these have so far proven more difficult to target than *BRAF* mutations.

Activation of STAT3 is seen in melanoma cell lines and primary tumour tissues (Niu, Bowman *et al*. 2002). Cell and animal studies demonstrate that STAT3 can contribute to the malignant phenotype in melanoma by promoting metastasis (Xie, Wei *et al*. 2004, Xie, Huang *et al*. 2006) and angiogenesis (Niu, Wright *et al*. 2002), and preventing apoptosis (Niu, Bowman *et al*. 2002). Inhibition of STAT3 signalling causes apoptosis in melanoma cells (Bill, Fuchs *et al*. 2010). In patients with melanoma, STAT3 phosphorylation is associated with increased risk of recurrence and death (Messina, Yu *et al*. 2008, Wu, Cheng *et al*. 2011).

STAT1 phosphorylation is seen in tumour tissue from a proportion of patients with melanoma, and the presence of phosphorylated STAT1 appears to be associated with better outcomes, although the patient numbers in the study showing this were small (Messina, Yu *et al*. 2008). It is also notable that treatment with interferon has been shown to lead to a change in the ratio of STAT1 and STAT3 phosphorylation, to the apparently beneficial pattern of high STAT1 phosphorylation and low STAT3 phosphorylation. This small study did not show, however, whether this led to better outcomes (Wang, Edington *et al*. 2007).

JAK/STAT signaling also plays a role in the immune response to tumour cells in melanoma. This is highly relevant, as immune 'checkpoint inhibitors' such as ipilimumab are showing success as a treatment for melanoma (Victor, Rech *et al*. 2015). Previous immunotherapies for melanoma include interferon and IL-2, both of which are JAK/STAT pathway ligands. Cell surface expression of MHC class II is positively regulated by JAK/STAT signaling in response to interferons, suggesting that loss of STAT1 activity facilitates immune evasion by melanoma (Osborn and Greer 2015). Immune evasion by tumours is in part mediated by induction of regulatory T cells (Tregs) which suppress anti-tumour immunity. STAT3 and STAT5 modulate expression of the transcription factor FoxP3, a key regulator in the differentiation of Tregs that suppress anti-tumour immunity (Zorn, Nelson *et al*. 2006). In a mouse model employing melanoma cell lines, deletion of STAT3 in the haematopoietic cells or treatment with an inhibitor of STAT3 lead to a reduction in the number of FoxP3 Tregs in the tumour (Kortylewski, Kujawski *et al*. 2005).

Together, this data from patient samples and experimental models suggests that STATs play a role in the malignant behavior of melanoma cells and their

interaction with the microenvironment. Better understanding is needed, however, of the mechanisms contributing to STAT activation in melanoma. In particular, there is conflicting evidence regarding the kinase which phosphorylates STATs in melanoma, with one study suggesting that src is responsible (Niu, Bowman *et al*. 2002) and another indicating JAKs are responsible (Liu, Cao *et al*. 2013).

1.2.3 JAK/STAT signalling in immunity and inflammation

The JAK/STAT signalling pathway is responsible for transducing signals from several families of cytokines, including interferons, colony-stimulating growth factors and many interleukins. It therefore has a role in controlling behaviour of cells involved in immune function, including proliferation, survival, differentiation and response to inflammatory stimuli (Ghoreschi, Laurence *et al*. 2009). Pathological alterations of JAK/STAT signalling are involved in autoimmune diseases and immunodeficiencies, indeed much of our understanding of the roles of JAK/STAT signalling components in normal immune function originates from examination of individuals with immunodeficiencies caused by mutations in signalling components.

JAK/STAT signalling in normal immune function and immunodeficiencies

JAK1 is essential for transduction of signals from receptors for interferons, making it important in innate immunity and the response to viruses (Muller, Briscoe *et al*. 1993). Consideration of the role of JAK3 in immunity is significant because its expression is predominantly limited to haematological cells (Cornejo, Boggon *et al*. 2009). JAK3 is particularly important in T cell activation (Kawamura, McVicar *et al*. 1994), as well as being found in Natural killer cells (Kawamura, McVicar *et al*. 1994) and dendritic cells (Yamaoka, Min *et al*. 2005). Furthermore JAK3 binds only to cytokine receptors containing the common gamma chain (γ_c) which are also mostly restricted to blood cells (Cornejo, Boggon *et al*. 2009). Mutations in *JAK3* cause a severe combined immunodeficiency (SCID) involving a defect in activity of T and NK cells, and lesser effects on B cells (Macchi, Villa *et al*. 1995). Mutations in γ_c cytokine receptors cause a similar SCID phenotype to *JAK3* mutations (Noguchi, Yi *et al*. 1993). Evidence for the role of TYK2 in immune function also comes from examination of patients with immune deficiencies. An individual with a homozygous *TYK2* loss-of-function mutation suffered from recurrent bacterial, viral

and fungal infections, including infections from organisms which are not normally highly pathogenic. In addition TYK2 deficiency produced allergic symptoms due to alterations in the balance of helper T lymphocyte subgroups (Minegishi, Saito *et al*. 2006).

STATs play many roles in the innate and adaptive branches of the immune system (O'Shea and Plenge 2012). In particular, STAT1 and STAT2 are required for the response to interferons. STAT 4 and 6 have also recently been identified to act as key regulators of helper T cell differentiation (Vahedi, Takahashi *et al*. 2012). STAT3 is involved in neutrophil migration in acute inflammation (Panopoulos, Zhang *et al*. 2006). STAT3 and STAT5 interact to control T cell differentiation, and loss of function mutations in STAT3 cause an immunodeficiency syndrome due to absence of differentiation of the Th17 subset of helper T lymphocytes (Holland, DeLeo *et al*. 2007).

The use of whole exome sequencing has led to the recent identification of germline STAT mutations in individuals with autoimmune syndromes. Three papers published at the beginning of 2015 describe gain-of-function STAT3 mutations in autoimmune syndromes. The initial group of patients was identified due to extremely early onset diabetes (Flanagan, Haapaniemi *et al*. 2014). Examination of further individuals with germline STAT3 mutations show that they are in fact associated with a diverse spectrum of autoimmune features, and that the mutations show incomplete penetrance as some family members carrying the STAT3 mutations are unaffected (Haddad 2015). The functional effects of some of the STAT3 mutations was examined in cell culture in the interleukin dependent mouse BAF-3 cell line, where they do not affect basal STAT3 phosphorylation, but lead to increased STAT3 phosphorylation and STAT3 dependent transcription following stimulation with IL-6 (Milner, Vogel *et al*. 2015). Slight increases in STAT3 phosphorylation were observed in peripheral blood lymphocytes and bone-marrow cells from affected individuals (Haapaniemi, Kaustio *et al*. 2015). Interestingly patients also had immune deficiencies, attributed to decreased STAT1 and STAT5 activity. These STATs are affected by the increased levels of SOCS3 that result from STAT3 activation, illustrating the complexities of JAK/STAT signalling that arise from interactions between pathway components.

JAK/STAT signalling in Rheumatoid Arthritis and autoimmune diseases

Rheumatoid arthritis is a chronic inflammatory disorder characterised by swelling, deformity and bone erosion in the small joints of the hands. In addition other joints may be affected, and systemic complications and early mortality are associated with the disease. Rheumatoid arthritis is caused by a combination of genetic and environmental factors. Pathogenesis is incompletely understood but appears to be mediated by auto-immunity. Affected joints have an infiltrate of macrophages, neutrophils and lymphocytes in the synovial membrane and synovial fluid. The synovial membrane is thickened by proliferation of activated fibroblast-like-synoviocytes (RA-FLS). Cartilage erosion is caused by production of matrix metalloproteases by RA-FLS, and bone is eroded by osteoclasts stimulated by cytokines from the inflammatory environment of the synovium (McInnes and Schett 2011).

It is recognised that the JAK/STAT signalling pathway plays a role in rheumatoid arthritis (Walker and Smith 2005, Malemud 2010). Single-nucleotide polymorphisms (SNPs) in STAT4 are associated with increased risk of rheumatoid arthritis (Remmers, Plenge *et al*. 2007). Increased expression of STAT1 and STAT1 responsive genes is seen in synovial tissues in a subgroup of patients with rheumatoid arthritis (van der Pouw Kraan, van Gaalen *et al*. 2003), with phosphorylated STAT1 observed in lymphocytes and fibroblast-like synoviocytes (Kasperkovitz, Verbeet *et al*. 2004). Experimental manipulation of JAK/STAT pathway components in rheumatoid arthritis mouse-models shows increased inflammation following knockout of STAT1 (de Hooge, van de Loo *et al*. 2004) and STAT6 (Finnegan, Grusby *et al*. 2002), and reduced inflammation in when STAT4 is knocked out (Finnegan, Grusby *et al*. 2002). Although these studies indicate that the JAK/STAT pathway is activated in rheumatoid arthritis, it is not entirely clear which of the cell types involved in pathogenesis this activation is important in. Neither is it completely clear to what extent JAK/STAT activation is promoting inflammation and to what extent it is exerting anti-inflammatory effects (Walker and Smith 2005).

Independent of these details, the significance of JAK/STAT signalling in rheumatoid arthritis is underlined by the effect of JAK inhibition upon the disease. An inhibitor of JAK3 has shown efficacy in clinical trials in rheumatoid arthritis (van Vollenhoven, Fleischmann *et al*. 2012). Other anti-inflammatory drugs used in

rheumatoid arthritis have also been demonstrated to have effects on JAK/STAT signalling (Siemasko, Chong *et al*. 1998, Choe, Park *et al*. 2013).

JAK/STAT signalling is implicated in a number of other diseases mediated by activation of the immune system. STAT3 activation is observed in epithelial cells (Li, de Haar *et al*. 2010) and immune cells (Mudter, Weigmann *et al*. 2005) from patients with Ulcerative colitis and Crohn's disease. Increased STAT1 activation is also seen in mucosa of patients with these diseases, and STAT1 activation decreases following remission (Schreiber, Rosenstiel *et al*. 2002). A mutation has been identified in TYK2 which increases risk of multiple schlerosis (Dyment, Cader *et al*. 2012), whereas a polymorphism in a separate region of TYK2 appears to protect from multiple sclerosis by decreasing TYK2 activation and altering the balance of T lymphocyte subsets (Couturier, Bucciarelli *et al*. 2011). Polymorphisms in STAT3 and two JAK-associated receptor components, IL-23R and IL-12B, affect risk of ankylosing spondylitis and Crohn's disease (Danoy, Pryce *et al*. 2010). Polymorphisms of STAT4 affect risk of systemic lupus erythematous (Sigurdsson, Nordmark *et al*. 2008). Although caution is needed when considering disease associations with genetic changes, these studies do suggest that the JAK/STAT signalling pathway plays a role in a broad group of inflammatory and immune-mediated conditions.

1.2.4 The JAK/STAT pathway as a therapeutic target

The JAK/STAT pathway is a promising target for the development of new therapies for cancer and inflammatory diseases, and JAK inhibitors have shown benefit in clinical studies in these conditions.

Haematological malignancies

The effectiveness of targeting JAK/STAT signalling has been demonstrated in phase 3 trials in patients with PMF and myelofibrosis secondary to PV and ET. The JAK1/2 inhibitor ruxolitinib improves symptoms and prolongs survival (Harrison, Kiladjian *et al*. 2012). More recently, ruxolitinib has been shown to improve haematocrit, reduce spleen volume and improve symptoms in patients with PV (Vannucchi, Kiladjian *et al*. 2015). There have been calls for JAK2 inhibitors to be

examined in the treatment of patients with other haematological malignancies with genetic changes affecting JAK2 (Bain and Ahmad 2014).

Solid tumours

In solid tumours, where the cause of JAK/STAT activation and its role in driving disease is less clear than MPNs, JAK inhibition has been less successful. In a phase 1 trial of a JAK1/2 inhibitor in patients with solid tumours no response was seen, when assessed according to RECIST criteria (Response Evaluation Criteria in Solid Tumours). There was, however, a reduction in pSTAT3 in granulocytes and in tumour tissue in one patient who had pre and post-treatment biopsies. Furthermore, as phase 1 trials are primarily for dose finding they are not powered to detect a response (Plimack, Lorusso *et al*. 2013). It may be that JAK inhibitors show greater success in solid tumours when examined in stratified trials, where tumour JAK mutational status has been defined.

Immune-mediated conditions

Tofacitinib, a JAK3 inhibitor, has been shown to improve joint signs and symptoms and reduce joint damage in patients with rheumatoid arthritis in phase 3 trials (Fleischmann, Kremer *et al*. 2012, van Vollenhoven, Fleischmann *et al*. 2012, Lee, Fleischmann *et al*. 2014). Ruxolitinib has shown benefit in alopecia, an autoimmune condition affecting hair growth (Xing, Dai *et al*. 2014) and dermatomyositis, an inflammatory condition affecting skin and muscle (Hornung, Janzen *et al*. 2014). Tofacitinib is being examined in a number of other conditions involving activation of the immune system, including psoriasis, inflammatory bowel disease (Sandborn, Ghosh *et al*. 2012) and renal transplantation (Vincenti, Tedesco Silva *et al*. 2012).

Therapies under development

Approaches to develop therapies have also been directed towards suppressing the activity of STATs. The beneficial effect of reducing STAT3 and STAT5 activity in cancer has been demonstrated in cell culture and animal models using overexpression of dominant negative STATs and knockdown of STAT expression with siRNAs. Translating findings based on experimental genetic techniques into therapies that can be administered to patients has been challenging, however.

Approaches that disrupt protein–protein interactions needed for STAT phosphorylation or inhibit STAT-DNA binding have shown promise in pre-clinical studies. Both strategies have been shown to affect cancer cell proliferation and apoptosis, and decrease tumour growth in mouse models.

Drugs that act by blocking protein-protein interactions include the natural product withacnistin, which inhibits the binding of STAT3 and STAT5 to the cytoplasmic region of receptors, preventing the recruitment of STATs for phosphorylation (Zhang, Blaskovich *et al*. 2014). High throughput methods to develop peptide drugs with the capacity to enter cells have been employed to create an inhibitor of STAT3 activation, which acts by binding the SH2 domain (Kim, Lee *et al*. 2014). A small-molecule inhibitor of STAT3 which is thought to interact with the SH2 domain has been examined in a phase 1 trial in patients with solid tumours. No further investigation of this molecule is planned, however, as pharmacokinetic studies showed that plasma concentrations in patients were extremely variable and substantially lower than those observed in pre-clinical models (Bendell, Hong *et al*. 2014).

A synthetic inhibitor of STAT-DNA binding has shown promise in cell and animal models, including *in-vitro* effects on tumour cells from the ascites of patients with ovarian cancer (Rath, Naidu *et al*. 2014). The STAT-DNA interaction has also been disrupted by ‘decoy oligonucleotides’ which contain STAT binding sequences and competitively inhibit STAT binding to genomic DNA. A decoy oligonucleotide targeting STAT3 has been shown to decrease expression of STAT target genes in head and neck squamous cell carcinoma when injected into tumours intra-operatively (Sen, Thomas *et al*. 2012).

It is hoped that some of the JAK/STAT targeted therapies shown to be beneficial in pre-clinical models will soon enter clinical trials. Combining strategies to target STAT activation with those affecting other signalling pathways, such as B-RAF and BCR-ABL may be particularly effective, as has already been demonstrated in pre-clinical models of melanoma and chronic myeloid leukaemia (Liu, Cao *et al*. 2013, Gallipoli, Cook *et al*. 2014).

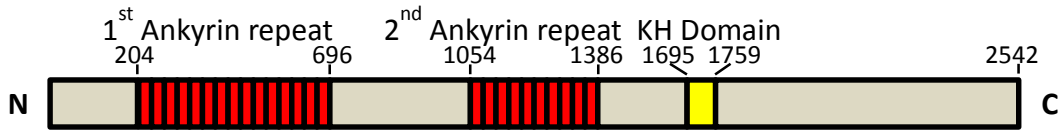
1.3 ANKHD1

ANKHD1 (Ankyrin Repeat and KH domain containing protein 1, also called hMASK, human Multiple Ankyrin Repeats Single KH domain) is a large protein encoded by a gene on chromosome 5q. It plays roles in regulation of proliferation, apoptosis and migration, and has been identified as a modulator of a number of signalling pathways.

1.3.1 ANKHD1 protein

Structure

Full-length ANKHD1containins 2542 amino acids and has a predicted molecular



weight of 269kDa (Figure 1-4). The protein contains two blocks of ankyrin repeats and a KH domain.

Figure 1-4. Schematic representation of the primary structure of ANKHD1. The principal features of the protein are two blocks of ankyrin repeats and a KH domain.

The protein also contains two predicted coiled-coil domains, at amino acids 775 to 852 between blocks of ankyrin repeats, and at 1415 to 1485, between the second block of ankyrin repeats and the KH domain. Coiled-coil domains consist of alpha helices that are arranged into bundles, a feature which creates a relatively inflexible region within the protein.

Post-translational modifications

Information on post-translational modifications in Uniprot (Consortium 2015) suggests that the methionine residue at position 1 is acetylated in ANKHD1, and that there is phosphorylation of a serine residue at position 101 and a threonine residue at position 1653. This is based on work on large-scale experiments examining thousands of proteins however, and has not been replicated in experiments specifically examining ANKHD1. Although there is no suggestion of phosphorylated tyrosine resides in proteomics databases, ANKHD1 has been

examined for phosphorylated tyrosine residues by immunoprecipitating the protein and blotting with anti-phosphotyrosine antibodies. It was not found to contain phosphorylated tyrosine in this experiment (Traina, Favaro *et al*. 2006).

The ankyrin repeat domain

Ankyrin repeats are a protein domain which mediate protein-protein interactions (Sedgwick and Smerdon 1999, Mosavi, Cammett *et al*. 2004, Li, Mahajan *et al*. 2006). They are named due to their presence in the cytoskeletal erythrocyte protein ankyrin, but occur widely in the human proteome, and are conserved in bacteria and archaea. The domain consists of 33 amino acids which fold into two alpha helices and a loop or beta hairpin. High resolution structures have been determined for the ankyrin repeat regions of several proteins, and show that the domains have an L shaped structure. Binding to other proteins occurs on the concave surface of this L.

The ankyrin repeat domain appears to bind to a wide range of substrates of different structures. Variation in the amino acid residues exposed on the concave surface mediates substrate binding specificity. This property is being exploited to generate novel peptide drugs consisting of ankyrin repeats with high binding affinities for different molecules (Eggel, Baumann *et al*. 2009). Ankyrin repeats tend to be found in multiple copies in the proteins in which they occur, but numbers of repeats as high as 25, as found in ANKHD1, appear to be relatively unusual.

The KH domain

KH domains (hnRNP K homology domains, named due to their identification in human heterogeneous nuclear ribonucleoprotein K) bind single stranded nucleic acids – RNA and single stranded DNA (Valverde, Edwards *et al*. 2008). They are a widespread protein domain showing evolutionary conservation from prokaryotes and archaea, and play roles in transcription control and RNA processing.

The domain consists of around 70 amino acids, which are arranged to create a binding cleft that spans four DNA or RNA bases. The RNA binding function of KH domains has been studied in detail in FMRP (fragile X mental retardation protein), a KH domain containing protein involved in the pathogenesis of fragile x syndrome. The RNA targets and the recognition elements that are common to RNAs bound by this protein have been identified (Ascano, Mukherjee *et al*. 2012). ANKHD1 is

unusual in having only a single KH domain, usually proteins contain more than one KH domain, with the multiple copies thought to bind nucleic acids cooperatively.

1.3.2 *ANKHD1* gene

Genomic location

The gene encoding ANKHD1 is located on chromosome 5q 31.3. Of relevance to haematological malignancies, deletions of large regions of 5q give rise to a sub-type of myelodysplastic syndrome (MDS), a clonal disorder of haematopoietic stem cells that leads to the generation of abnormal mature blood cells. *ANKHD1* lies within the genomic regions frequently deleted in 5q- syndrome (Boultwood, Pellagatti *et al*. 2010). Altered ribosomal function due to haploinsufficciency of *RPS14* (Ribosomal Protein S14) is known to play a large role in disease pathogenesis. Although *ANKHD1* lies outside the critical deleted region, 5q32-33, haploinsufficiency for ANKHD1 could co-operate in disease pathogenesis.

Deletion of 5q31.3 – q32 has been described in colorectal cancer and a correlating change in ANKHD1 mRNA observed. *ANKHD1* may merely be a passenger in this region of genomic loss, but it is interesting that the deletion is associated with reduced progression-free survival in patients treated with certain chemotherapy regimens (Haan, Labots *et al*. 2014).

Homologues

In humans, *ANKHD1* has a paralogue on chromosome 4, named *ANKRD17* (ankyrin repeat domain 17) or *GTAR* (gene trap ankyrin repeat), and more recently referred to as ‘hMASK2’ (human multiple ankyrin repeats single KH domain 2) (Sansores-Garcia, Atkins *et al*. 2013, Sidor, Brain *et al*. 2013). The two proteins share 71% identity, with greater sequence similarity in the regions of ankyrin repeats and the KH domain (Poulin, Brueschke *et al*. 2003).

As *ANKHD1* and *ANKRD17* are thought to have arisen from a duplication event, it might be expected that they would have similar biological roles, and an understanding of ANKRD17 function might provide insight into ANKHD1 function. ANKRD17 has been demonstrated to physically interact with intracellular receptors involved in the innate immune response, and plays a role in the release of cytokines (Menning and Kufer 2013) and interferons (Wang, Tong *et al*. 2012). These include

IL-6 and IFN-β, known ligands of the JAK/STAT pathway. Curated databases which include ANKHD1 do not show any nuclear localisation sequence (NLS) or nuclear export sequence (NES), whereas these have been defined in ANKRD17, between the second ankyrin repeat cluster and the KH domain. ANKRD17 is a substrate of cyclin E, which phosphorylates several serine residues within the protein (Deng, Li *et al*. 2009). Of particular relevance increased expression of p21 is seen following ANKRD17 knockdown, an effect also described following ANKHD1 knockdown (Dhyani, Machado-Neto *et al*. 2015). These results based on siRNA knockdown should be interpreted cautiously, however, as sequence similarities between the two genes might cause both to be knocked down by the same siRNA.

1.3.3 ANKHD1 transcripts

Splice variants

Ensembl lists 14 splice variants of ANKHD1 that appear to be protein coding, as they contain an open reading frame, although evidence for the existence of these variants at the protein level is lacking for most. A few ANKHD1 splice variants have been examined in some detail. One variant encodes a short 627 amino acid form of the protein, named by different groups as 'splice variant 4'(Duarte, Traina *et al*. 2005) and VBARP-L (HIV-1 viral protein R – binding ankyrin repeat protein, long form) (Miles, Janket *et al*. 2005). In most tissues and cell lines examined mRNA for this variant is expressed at a comparable level to full-length ANKHD1. However, in an in-vitro model of erythroid differentiation in which the Hl-60 cells (a line derived from a patient with acute promyelocytic leukaemia) are induced to differentiate by the addition of erythropoietin, levels of the mRNA encoding splice variant 4 show a much greater increase than levels of mRNA encoding the full-length protein (Duarte, Traina *et al*. 2005). This suggests differential regulation or function, although it is not known to what extent changes in mRNA levels are indicative of changes in protein levels.

Fusion transcript

In addition to variants generated through alternative splicing, ANKHD1 also encodes two variants of a fusion transcript produced by read-through into the adjacent gene, *EIF4EBP3* (eukaryotic translation initiation factor 4E binding protein 3).

The EIF4EBPs are a family of three proteins involved in the regulation of translation. Ribosome assembly on mRNA requires the assembly of a complex on the mRNA cap that includes the protein EIF4E. The assembly of this complex is in part modulated by 4EBPs, which sequester EIF4E and prevent complex assembly. EIF4EBP3 is the least well understood member of the family. It has been shown to bind to EIF4 (Kleijn, Scheper *et al*. 2002), and appears to also function in the regulation of nuclear export of mRNA (Chen, Lee *et al*. 2012).

The ANKHD1 transcripts that include read-through into *EIF4EBP3* are named ANKHD1-EIF4EBP3 or hMASK-BP3^{ARF}. The variants are 636 amino acids and 2617 amino acids in length. The larger of the two variants generates a form of ANKHD1 with a novel c-terminus consisting of 94 amino acids encoded by an alternative reading frame of *EIF4EBP3*. This alternative reading frame-encoded sequence does not display similarity to any known proteins. Although the function of the ANKHD1-EIF4EBP3 transcript is not clear, it's conservation in mouse and human suggests it may have a biological function (Poulin, Brueschke *et al*. 2003). The formation of read-through transcripts has been said to in some cases indicate proteins that act within the same biological pathways, in the case where the reading frames are preserved. Fusion transcripts that use different reading frames are rare (Akiva, Toporik *et al*. 2006). Interestingly, levels of ANKHD1-EIF4EBP3 mRNA transcripts in human lymph node are about five-fold higher than levels of ANKHD1 mRNA (Duarte, Traina *et al*. 2005).

1.3.4 ANKHD1 functions

Relatively little is understood about the functions of ANKHD1. It appears to be a widely-expressed protein that acts as a modulator of intracellular signalling pathways, promotes cell cycle progression and protects cells from apoptosis. These functions are interrelated since cell cycle progression and apoptosis are regulated by signalling pathways.

ANKHD1 knockdown

Evidence that ANKHD1 performs essential functions comes from considering the effects of knocking down or knocking out its activity in animal models. In *Drosophila,* mutations in dMASK cause lethality in most homozygous flies early in

larval development (Smith, Carroll *et al*. 2002). These mutations introduce premature stop codons, presumably leading to nonsense-mediated-decay of the mRNA, Attempts to generate an ANKHD1 knockout mouse have thus far been unsuccessful as it appears to cause early embryonic lethality, thought to be due to effects on gastrulation (S.Constantinescu, unpublished observations).

Tissue distribution

ANKHD1 appears to be expressed ubiquitously in human tissues. This is based on mRNA expression (Duarte, Traina *et al*. 2005, Miles, Janket *et al*. 2005), and corroborated to some extent by publically available data on immunohistochemistry in several types of normal and malignant human tissue (Uhlen, Oksvold *et al*. 2010).

Intracellular signalling

It has been proposed that ANKHD1 acts as a scaffolding protein in intracellular signalling pathways, with the multiple ankyrin repeats creating a platform for protein binding that brings together proteins involved in the same signalling cascade (Traina, Favaro *et al*. 2006). This is consistent with published work indicating interactions between ANKHD1 and three signalling cascades, the EGFR/RAS/MAPK pathway, JAK/STAT pathway and Hippo pathway.

Evidence for an interaction between ANKHD1 and EGFR/RAS/MAPK comes from a study in *Drosophila.* Expression of a dominant-negative form of the phosphatase corkscrew in the eye gives flies a roughened eye phenotype as it reduces EGFR signalling leading to a decreased number of photoreceptors. The phenotype was worsened following the removal of one genomic copy of *dMASK,* a *Drosophila* homologue of *ANKHD1*. This suggests that dMASK is a positive regulator of the pathway. Interestingly, levels of diphosphorylated MAPK, an effector of pathway activation, were not altered, suggesting that dMASK acts in parallel to or downstream of MAPK (Smith, Carroll *et al*. 2002). Some evidence that this interaction is conserved to humans comes from co-immunoprecipitation experiments in leukaemia cell lines. SHP2, the human homologue of corkscrew, has been shown to bind to ANKHD1 (Traina, Favaro *et al*. 2006) (and D. Pugazhendhi, M Fragiadaki, M Zeidler unpublished). The roles of SHP2 do extend to other signalling

pathways, however, so this does not necessarily indicate a function in EGFR/RAS/MAPK signalling.

Evidence for the role of ANKHD1 in JAK/STAT signalling also comes from work in *Drosophila.* dMASK was identified as a positive regulator of JAK/STAT signalling in a genome-wide RNAi screen in *Drosophila* cells, where a luciferare based transcriptional reporter was used to assess the effect of an RNAi library upon STAT responsive transcription (Müller, Kuttenkeuler *et al*. 2005). Subsequent examination of the human homologues of the genes identified in the screen in flies suggested that ANKHD1 had a similar positive effect on JAK/STAT signalling in the more complex JAK/STAT pathway of humans. RNAi knockdown of ANKHD1 in HeLa cells led to a reduction in phosphorylation of STAT3 following stimulation with OSM, demonstrated using western blotting (Müller 2012).

ANKHD1 interacts with the Hippo signalling pathway. This pathway is involved in regulation of cell proliferation, in particular in relation to control of organ size. Although upstream activators of the pathway in vertebrates are incompletely characterised, the pathway is known to modulate transcription. In vertebrates the effectors of the pathway are the transcriptional activator YAP (yes-associated protein) and TEAD, and in *Drosophila* the homologous protein is Yki (yorkie). Phosphorylation of YAP leads to its export from the nucleus, and cessation of transcriptional activation (Pan 2010). In both *Drosophila* and human cells, ANKHD1 has been shown to physically associate with Yki/YAP, translocate to the nucleus, and modulate expression of yki/YAP regulated genes (Sansores-Garcia, Atkins *et al*. 2013, Sidor, Brain *et al*. 2013).

Cell cycle progression

Evidence from experiments in *Drosophila* and human cell lines indicates that ANKHD1 promotes progression through the cell cycle. In *Drosophila* the heat-shock-FLP system allows the effect of homozygous *dMASK* mutation to be examined despite its lethality, by enabling stochastic genomic rearrangement in larvae following heat shock. This system was used to generate larvae with eye imaginal discs containing adjacent clones of homozygous *mask* loss-of-function and homozygous *mask* wild-type cells. Antibody staining for phosphohistone H3, which is found in the nuclei of cells undergoing mitosis, was used to examine the effect of

mask loss-of function upon stereotyped waves of cell proliferation that normally occur in eye development. Mitosis was rare in cells lacking dMASK. However, this effect was not seen in larger clones, suggesting that the effect of loss of dMASK was related to competition with surrounding cells (Smith, Carroll *et al*. 2002). It is not clear from these experiments whether the effects of dMASK on the cell cycle are indirect, a consequence of its effect on intracellular signalling.

In human cell lines originally derived from patients with prostate cancer and multiple myeloma, knockdown of ANKHD1 with siRNA led to a reduction in cell proliferation assessed using a methylthiazoletetrazolium (MTT) assay. Flow cytometry of cells stained with propidium iodide indicated that ANKHD1 loss led to accumulation of cells in S phase of the cell cycle (Dhyani, Duarte *et al*. 2012, Machado-Neto, Lazarini *et al*. 2014). A mechanistic link is suggested by increased levels of the cyclin-dependent kinase inhibitor p21, and it was subsequently found that ANKHD1 suppresses transcription from the p21 promoter (Dhyani, Machado-Neto *et al*. 2015).

Apoptosis

Current evidence for the effect of ANKHD1 on apoptosis is conflicting. The suggestion that ANKHD1 protects cells from apoptosis came from the *Drosophila* eye development experiment described above. In this study there were substantially more apoptotic cells in the clones lacking dMASK compared to clones with wild-type dMASK, when apoptotic cells containing fragmented DNA were identified using TUNEL staining (Terminal deoxynucleotidyl transferase dUTP Nick End Labelling) (Smith, Carroll *et al*. 2002).

Evidence that ANKHD1 protects human cells from apoptosis comes from examination of two short isoforms of ANKHD1 that contain some of the ankyrin repeats but lack the KH domain. These variants were originally identified in a yeast-two-hybrid screen for proteins that bind to HIV-1 viral protein R (Vpr), and named VBARP-L and VBARP-S (Vpr-binding ankyrin repeat protein, long and short forms). Knockdown of these ANKHD1 isoforms in cells from the teratocarcinoma cell line NT2 led to increased cell death, assessed using trypan blue exclusion, and increased Caspase 3 activation indicated that this was due to increased apoptosis (Miles, Janket *et al*. 2005).

Several other experiments in human cell lines, however, suggest that ANKHD1 has little effect on apoptosis. Knockdown of ANKHD1 in prostate cancer and multiple myeloma cell lines does not lead to increased apoptosis, when assessed using TUNEL staining or staining with propidium iodide and labelled Annexin-V (Machado-Neto, Lazarini *et al*. 2014). Furthermore, experiments in myeloma and acute leukaemia cell lines indicate that ANKHD1 knockdown does not affect apoptosis induced by UV exposure or cytotoxic drugs (Machado-Neto, Lazarini *et al*. 2014).

The conflicting evidence for the role of ANKHD1 in protecting cells from apoptosis may be due to species and cell-type specific effects, or due to differential effects of ANKHD1 splice variants. As all the experiments in human cells depend upon siRNA-mediated knockdown of ANKHD1 it is possible that off-target effects also contribute to these differences.

Other functions

There is some evidence that ANKHD1 may have a role in regulation of transcription. Chromatin immunoprecipitation assays (ChIP) show that ANKHD1 is associated with the gene encoding p21, and knockdown of ANKHD1 leads to decreased transcription from the p21 promoter (Dhyani, Machado-Neto *et al*. 2015). The same group have also shown that ANKHD1 associates with the promoters of several histone genes using ChIP (A. Dhyani, P. Favro, S. Saad, presented at American Society of Haematology Annual Meeting, December 2014).

ANKHD1 appears to affect cell migration, since ANKHD1 knockdown leads to reduced migration of multiple myeloma cell line cells towards foetal bovine serum (FBS) or the chemokine CXCL12 (Dhyani, Machado-Neto *et al*. 2015). A mechanism has recently been described for this, whereby ANKHD1 promotes microtubule stability via indirect inactivation of the microtubule-stabilising protein Stathmin 1, mediated by a physical interaction with the protein SIVA (Machado-Neto, Lazarini *et al*. 2014).

A role for dMASK in regulating mitochondrial morphology and turnover by mitophagy has recently been described in *Drosophila,* but effects of ANKHD1 on mitochondria in vertebrates have yet to be described (Zhu, Li *et al*. 2015).

1.3.5 ANKHD1 in cancer

The roles of ANKHD1 in positive regulation of intracellular signalling pathways activated in cancer, promoting proliferation and migration, and possibly protecting cells from apoptosis suggest it may contribute to the phenotype of cancer cells. There is some data from patient tissues to suggest that ANKHD1 expression may be upregulated in cancer and affect cancer cell behaviour in haematological malignancies and solid tumours.

ANKHD1 mRNA levels are elevated in peripheral blood mononuclear cells in patients with acute myeloid leukaemia and acute lymphocytic leukaemia, compared to healthy individuals (Traina, Favaro *et al*. 2006). ANKHD1 mRNA is also increased in the malignant cells in patients with multiple myeloma, compared to plasma cells from individuals without myeloma. Furthermore, knockdown of ANKHD1 reduces tumour size in a myeloma xenograft mouse model (Dhyani, Machado-Neto *et al*. 2015).

ANKHD1 protein expression has been examined in a small number of tumour samples from epithelial cancers, and compared to the corresponding normal tissue, using immunohistochemistry. In these samples ANKHD1 protein was seen in the tumour tissue, and in the basal layers of the normal epithelia (Sidor, Brain *et al*. 2013). The only published data that examines ANKHD1 expression in a large number of patients comes from examination of microarray data from a cohort of patients with breast cancer. Compellingly, increased expression of ANKHD1 was associated with reduced survival (Sansores-Garcia, Atkins *et al*. 2013).

1.3.6 Rationale behind the investigation of ANKHD1 as a modulator of JAK/STAT signalling.

Unpublished data from the Zeidler lab adds to the published data, showing that ANKHD1 promotes STAT dependent transcription, physically interacts with JAK/STAT pathway proteins and affects JAK/STAT dependent phenotypes (this data was produced by Katie Fisher, Maria Fragiadaki, Kirsty Johnstone and Dhamayanthi Pugazhendhi).

Transient transfection with a plasmid encoding ANKHD1 leads to increased STAT transcriptional activity in HeLa cells, as assessed by SOCS3 mRNA levels. In addition, STAT5 responsive transcription in γ2A cells is preferentially increased by

overexpression of ANKHD1 in the presence of JAK2V617F, compared to wild-type JAK2 (Constantinescu lab, unpublished).

Co-immunoprecipitation experiments in HeLa cells indicate that ANKHD1 physically interacts with JAK2 and with the cytokine receptor subunit gp130.

Effects of ANKHD1 on JAK/STAT signalling have also been demonstrated *in-vivo*. The hop^{Tuml} fly strain has a mutation in Hopscotch, the fly JAK, which causes constitutive activation and leads to the formation of melanised tumours due to accumulation of haemocytes, a macrophage-like blood cell. When dMASK expression is reduced, the number and size of these tumours is reduced. dMASK knock down also alters development of structures associated with the wing, causing an 'outstretched' wing phenotype which is characteristic of reduced JAK/STAT activation (Johnstone, Wells *et al*. 2013).

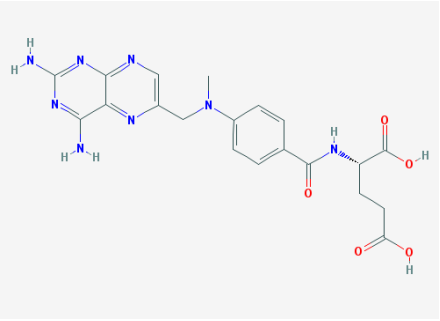
1.4 Methotrexate

Methotrexate is a drug which has been widely used for many years, given intravenously at high doses in chemotherapy regimens and given orally at lower doses for the treatment of inflammatory conditions such as rheumatoid arthritis. Methotrexate is a folate analogue which inhibits dihydrofolate reductase, although not all of its biological effects are accounted for by effects on folate metabolism.

1.4.1 Structure

The chemical structure of methotrexate is similar to that of folic acid (Figure 1-5). Methotrexate has a molecular weight of 454g/mol.

A



B

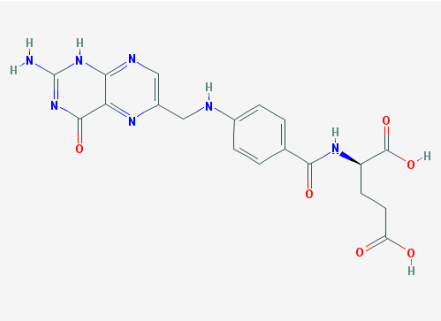


Figure 1-5. The structure of methotrexate (A) and folic acid (B) (from the PubChem database (NCBI))

1.4.2 Pharmacokinetics

Oral methotrexate is absorbed in the small intestine via active transport, through reduced folate carrier 1 (RFC1). When the transporter is not saturated, bioavailability is around 70%. At concentrations at which the folate carriers are saturated, there is little further increase in absorption with increased doses. Serum concentration peaks about two hours after administration. Methotrexate distributes rapidly into interstitial fluid, and is excreted predominantly by the kidneys – serum half-life is 6-8 hours. Around 10% of absorbed methotrexate is converted to 7-hydroxymethotrexate in the liver, a metabolite which is also renally excreted (NCBI , Tian and Cronstein 2007).

Methotrexate is actively transported into cells, by reduced folate carriers. Increased expression of folate transporters has been described in cancer cells, an adaptation which facilitates cell proliferation (Chen, Ke *et al*. 2013). At serum concentrations above 100µM, passive diffusion also makes a major contribution to cellular uptake (NCBI) (PubChem database). In the cell, methotrexate, like physiological folates, is a substrate for the enzyme folylpolyglutamate synthetase which attaches glutamate molecules to create a peptide chain up to eight residues long. Unlike methotrexate, methotrexate polyglutamates are not exported from the cell by multidrug resistance proteins, so they accumulate in cells and can be retained. The inhibitory effect of methotrexate polyglutamates on dihydrofolate reductase is similar to the effect of methotrexate (Visentin, Zhao *et al*. 2012).

1.4.3 Methotrexate as a chemotherapy drug

The use of methotrexate in chemotherapy originates from development of aminopterin, a structurally similar molecule in which the methyl group is replaced by an amino group. Aminopterin was the first drug shown to slow the progression of a malignant disease, heralding the development of chemotherapy. It was designed as an antagonist to folate and employed in children with acute leukaemia, following the observation that treatment with folates led to an acceleration of disease progression. Aminopterin treatment produced temporary remission, although it was associated with significant toxicity and side-effects (Farber and Diamond 1948). Methotrexate replaced aminopterin due to an improved side-effect profile, although aminopterin is still being examined in clinical trials (Cole, Drachtman *et al*. 2008).

Doses

Methotrexate is now used in chemotherapy regimens in some solid malignancies and in regimens for several haematological malignancies. In these situations it is given intravenously, at high doses (e.g 5000mg/m^2) and high plasma concentrations (over 50μM) are achieved (Radtke, Zolk *et al*. 2013).

Mechanism of action

Methotrexate exerts its effects as a chemotherapy drug through impairment of folate metabolism, via competitive inhibition of dihydrofolate reductase (DHFR) (Visentin, Zhao *et al*. 2012). This inhibition reduces intracellular levels of intermediate compounds required for nucleotide synthesis, resulting in impaired DNA replication and repair that ultimately causes cell death (Li and Kaminskas 1984).

1.4.4 Methotrexate in the treatment of inflammatory conditions

Methotrexate is also used to treat autoimmune and inflammatory diseases. It is particularly associated with rheumatoid arthritis, but is also used in other situations where immune activation is harmful, such as in the suppression of graft-versus-host disease following allogeneic haematopoietic stem-cell transplant.

Doses

In rheumatoid arthritis methotrexate is given orally, at much lower doses than are used for chemotherapy, typically 5-20mg once-weekly.

Mechanism of action

The mechanism of action of methotrexate in this context is not well understood. It is thought to be at least partially independent of effects on folate metabolism since folate supplementation has little effect on efficacy (Whittle and Hughes 2004). It has been suggested that methotrexate has a greater effect on lymphocytes proliferating in active inflammation, compared to less proliferative cells (Nakajima, Hakoda *et al*. 1996). Other proposed mechanisms include effects on cytokine release by leucocytes, effects on the expression of adhesion molecules on vascular endothelium, and increased release of adenosine (Wessels, Huizinga *et al*. 2008). Members of the adenosine receptor family have anti-inflammatory effects (Saze, Schuler *et al*. 2013)

and methotrexate causes adenosine release indirectly via inhibition of the enzyme 5-aminoimidizole 4-carboxamideribonucleotide (AICAR) (Tian and Cronstein 2007). Despite these hypotheses, the mechanism mediating the clinical effects of methotrexate is uncertain.

1.4.5 Rationale behind the investigation of methotrexate as a suppressor of JAK/STAT signalling

Prior to the start of my PhD, members of the laboratory carried out a luciferase based screen to identify compounds that suppress JAK/STAT pathway activity in *Drosophila* cell culture. This system has been used previously to identify JAK/STAT pathway inhibitors (Kim, Oh *et al*. 2010). *Drosophila* KC_{167} cells were transfected with a plasmid encoding firefly luciferase under the control of a STAT responsive promoter. In addition cells were transfected with a plasmid encoding *Renilla* luciferase under the control of a constitutively active promoter, and a plasmid expressing the *Drosophila* JAK/STAT pathway ligand Unpaired (Upd). Autocrine and paracrine effects of Upd triggered pathway activation, and the luminescence from the firefly luciferase gives a measure of the degree of pathway activation. *Renilla* luminescence acts as a control for transfection efficiency.

This system was used to screen a small-molecule library containing 2000 compounds – 60% FDA approved drugs, 25% natural products and 15% other bioactive compounds (Spectrum Collection)(Kocisko, Baron *et al*. 2003) . Methotrexate and aminopterin were independently identified to produce a substantial reduction in pathway activation. Their chemical structures are similar, which reinforces their interest by suggesting that they are true hits produced by a common mechanism of action on the pathway.

The independent identification of chemically similar compounds in the screen, the use of methotrexate in inflammatory conditions where the JAK/STAT pathway is activated, and the unanswered question of the mechanism of action of low-dose methotrexate suggested further investigation of the effect of methotrexate on JAK/STAT signalling would be worthwhile.

1.5 Synopsis

The aims of this study are to investigate the modulation of JAK/STAT signalling by the gene *ANKHD1* and the drug methotrexate, with particular emphasis on the implications these have for JAK/STAT signalling in cancer. The clinical relevance of ANKHD1 is addressed in Chapters 3 and 4, which describe the expression of ANKHD1 in blood and haematological malignancies and malignant melanoma. The effect of methotrexate on JAK/STAT signalling in cancer cell lines and *Drosophila* is examined in Chapter 5, and in Chapter 6 these findings are built upon by an examination of methotrexate effects in primary cells from patients with diseases associated with JAK/STAT activation. Chapter 7 draws conclusions from the work and contains a discussion of implications for patients and potential future study.

Chapter 2: Materials and methods

2.1 Cell culture

2.1.1 Cell lines and culture conditions

Hodgkin's Lymphoma cell lines

Hodgkin's Lymphoma cell lines HDLM-2 and L-540 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Leibniz Institute, Germany). The HDLM2 line was derived from a pleural effusion in a 74 year old man with stage IV Nodular Sclerosing Hodgkin's Lymphoma. The L-540 cell line was established from bone marrow from a 20 year old woman with stage IVB Nodular Sclerosing Hodgkin's lymphoma.

Cells were grown in RPMI 1640 + GlutaMAX medium (Gibco) supplemented with 20% heat-inactivated Foetal Bovine Serum (FBS) (Sigma) and penicillin (100 units/ml)/ streptomycin (100μg/ml) (Gibco), in a 37ºC humidified incubator with 5% CO_2. HDLM2 cells were seeded at a density of 1 x 10^6 cells per ml and L-540 cells seeded at 0.5 x 10^6cells per ml. Cells were centrifuged at 12,000 rpm for five minutes and resuspended in new media, splitting at a ratio of 1:2 Every 2 – 3 days.

HeLa cells

HeLa cells were a gift from Dr. V. James and Dr. M. Fragiadaki. Cells were grown in DMEM+GlutaMAX medium (Gibco), supplemented with 10% FBS and penicillin/ streptomycin in a 37ºC humidified incubator with 5% CO_2. Cells were released from culture vessels with Trypsin/ EDTA (Gibco) and split 1:7 every 2 – 3 days.

Rheumatoid Arthritis fibroblast-like-synoviocytes

Primary rheumatoid arthritis fibroblast-like-synoviocytes were a gift from Professor G. Wilson (University of Sheffield, Department of Infection and Immunity). Cells were obtained from patients who gave informed consent for ethically approved research to study Rheumatoid Arthritis (REC reference SSREC/03/106). Cells were used between passages 6 and 8. Cells were grown in DMEM/F-12 (1:1) + GlutaMAX medium (Gibco) supplemented with 5% FBS and penicillin/

streptomycin, in a 37ºC humidified incubator with 5% CO_2. When confluent, cells were released from culture vessels using trypsin and split 1:5.

Myeloma cell lines and primary cells

Myeloma cell lines JJN-3 and U-266 and the primary cells CB-1 were a gift from Dr. A. Chantry (University of Sheffield, Department of Oncology). The JJN-3 line is a sub-clone of JJN-1, originally derived from the bone marrow of a 57 year old woman with plasma cell leukaemia (IgA1kappa). The U-266 line was derived from the peripheral blood of a 53 year old man with IgE secreting multiple myeloma refractory to treatment. CB-1 primary cells were derived from the peripheral blood of a 68 year old man diagnosed with plasma cell leukaemia. The patient gave informed consent for ethically approved research to study myeloma (Rec reference 05/Q2305/96).

Cells were grown in a 37ºC humidified incubator with 5% CO_2, in RPMI 1640 + GlutaMAX medium (Gibco) supplemented with 10% heat-inactivated Foetal Bovine Serum (Sigma), penicillin (100 units/ml)/ streptomycin (100μg/ml) (Gibco), non-essential amino acids (Gibco) and sodium pyruvate (1mM) (Gibco). Cells were split every 2 – 3 days, the U-266 cells at a ratio of 1:3 and the JJN3 cells at a ratio of 1:5. For this project, CB-1 cells were used for analysis after a few days in culture and were not passaged.

HEL cells

Cells from the HEL cell line were a gift from Dr. M. Fragiadaki. The cell line was originally derived from peripheral blood from a 30 year old male patient who had relapsed acute myeloid leukaemia (WHO AML classification acute erythroid leukaemia). The patient had previously been treated for Hodgkins Lymphoma. Sequence analysis has demonstrated that cells of this line are homozygous for the JAK2 V617F mutation, and they show constitutive phosphorylation of JAK2 and STAT5 (Levine, Wadleigh *et al*. 2005). Cells were grown in the same media and conditions as the myeloma cell lines described above, and were split 1:3 every 2 – 3 days.

Melanoma Cell lines

Melanoma cell lines were a gift from Prof. Sheila MacNeil in the Faculty of Engineering at the University of Sheffield. The cell line HBL was originally established in the laboratory of Prof. Ghanem, from a lymph node metastasis of a nodular melanoma (Ghanem, Comunale *et al*. 1988). The C8161 cell line was donated to Prof. MacNeil by Professor F Meyskens (USA) via M. Edwards (University of Glasgow). The cell line was established from an abdominal wall metastasis from a woman with recurrent malignant melanoma. Cells from the C8161 cell line are highly invasive and are used to model metastatic melanoma (Welch, Bisi *et al*. 1991). Cells were grown in DMEM+GlutaMAX medium (Gibco), supplemented with 10% FBS and penicillin/ streptomycin in a 37ºC humidified incubator with 5% CO_2. HBL cells were split 1:5 every 3-4 days, and C8161 cells were split 1:5 every 2-3 days.

Tissue engineered skin to model melanoma invasion

Formalin fixed paraffin embedded specimens of engineered skin containing invading melanoma cells from the C8161 cell line were a kind gift from Ceyla Yorocu in the Faculty of Engineering at the University of Sheffield. The tissue was grown in culture as previously described (Eves, Layton *et al*. 2000).

Primary CD34+ haematopoietic stem cells

Cells were obtained from stored apheresis samples, from patients who gave informed consent (see patient samples materials and methods section for details. Cells were grown in StemMACS HSC Expansion Media Xeno Free (Miltenyi) supplemented with StemMACS HSC Expansion Cocktail (Miltenyi) containing recombinant human Flt3-ligand, recombinant human Stem Cell Factor and recombinant human TPO. No serum or antibiotics were added to media. Cells were grown in a 37ºC humidified incubator with 5% CO_2. Cells were seeded at 0.5 x 10^6/ml.

***Drosophila* Kc_{167} cells**

Drosophila Kc_{167} cells were a gift from the Sheffield RNAi Screening Facility. Cells were maintained in Schneider's *Drosophila* media supplemented by 10% foetal calf

serum and 100units/ml penicillin and 100µg/ml streptomycin, and grown at 25ºC in a humidified incubator.

2.1.2 Drug and ligand treatments

Estimation of drug doses

The range of concentrations of drugs used for cell culture experiments was selected based on existing data and literature. A dose corresponding to the value measured in the serum of individuals who had taken the drug was included, with a range to cover at least on order of magnitude above and below this. For methotrexate, this is 0.4µM for oral dosing for rheumatoid arthritis (Hobl, Mader *et al*. 2012), although peak serum concentrations in individuals receiving methotrexate intravenously as a chemotherapy drug may be over 50µM (Radtke, Zolk *et al*. 2013). In the pharmacokinetic studies of ruxolitinib a peak plasma concentration of around 1µM was recorded (Shilling, Nedza *et al*. 2010). For folinic acid, concentrations corresponding to those advised in the British National Formulary for methotrexate overdose were examined as a high concentration. To represent a clinically relevant lower concentration, a concentration equivalent to that measured in the plasma of individuals taking an oral dose corresponding to that recommended for methotrexate toxicity in rheumatoid arthritis was used (Whittle and Hughes 2004, McEvoy 2005).

To calculate appropriate EPO concentrations a conversion was made from international units to nanograms (Jelkmann 2009). Physiological levels of EPO in normoxic individuals are low, but EPO concentrations rise exponentially when stimulated by hypoxia. To estimate an EPO concentration equivalent to that triggered by hypoxia we used EPO values measured in individuals with secondary polycythaemia (Spivak 2002).

Treatment with methotrexate, aminopterin and ruxolitinib

Methotrexate and Aminopterin (Sigma) and ruxolitinib (Cayman Chemicals) were dissolved in 100% dimethyl sulphoxide (DMSO). Stock solutions were stored at –20ºC and diluted in media prior to use. All treated cells experienced a final concentration of DMSO 1:1000 volume: volume in addition to the dissolved drugs.

Suspension cells were seeded in 12 well plates (1 x 10^6 cells/ml for HDLM2, 0.5 x 10^6 cells/ml for HEL, up to 0.5 x 10^6 cells/ml for primary cultures) and grown

for 24 hours prior to application of drugs. Cells continued to grow for a further 48 hours before being pelleted at 6000rpm for 5 minutes and washed with TBS prior to protein or RNA extraction.

RA-FLS were seeded in t25 flasks in 5ml media and grown to confluence. Media was exchanged for 5ml of fresh pre-warmed media and drugs applied. Cells were exposed to drug-containing media for 48 hours. After removal of media cells were washed in 1.5ml TBS, detached from the flask using a cell scraper, then pelleted at 8000rpm for 5 minutes prior to protein extraction.

Treatment with folinic acid

Leucovorin (calcium folinate, Refolinon®) was obtained from Pharmacia as a 3mg/ml solution for injection and stored in the dark at 4^{o}C. HEL cells were seeded into 3ml wells and grown for 24 hours. Methotrexate was added to these wells and the cells grown for a further 24 hours. For each methotrexate concentration paired 1ml samples of cells were transferred to new wells, and folinic acid added to one of these wells. Final folinic acid concentrations of 21µg/ml, 3µg/ml and 0.3µg/ml were used. Cells were treated with folinic acid for 48 hours before protein extraction.

Stimulation with OSM, IL-2 and EPO

Recombinant Human Oncostatin M (R&D systems), recombinant human IL-2 (Cell Signalling Technology) and recombinant human EPO (Sigma) were dissolved in sterile TBS to generate a 10µg/ml stock solution, stored at -20^{o}C. Cells were stimulated with OSM and IL-2 at a final concentration of 10ng/ml. Cells were stimulated for 30 minutes prior to RNA extraction and 6 hours prior to protein extraction. For stimulation with EPO cells were grown for 48 hours in the presence of methotrexate, split into separate wells, and one set of wells stimulated with 15 ng/ml EPO for 20 minutes prior to protein extraction.

2.1.3 Transfection of HeLa cells

Forward transfection

HeLa cells were seeded in 12 well plates at a density of 1 – 2 x 10^5 cells per well, in DMEM GlutaMAX media (Gibco) supplemented with 10% FBS and penicillin/ streptomycin. Cells were grown for 24 hours then media was replaced with fresh

media free of antibiotics. Transfection using Fugene HD (Promega) transfection reagent was carried out according to the manufacturer's instructions. Cells were transfected with 2μg of plasmid DNA per well, using 6μg of Fugene transfection reagent per well. Cells continued to grow for a further 48 hours before ligand stimulation or extraction of protein or RNA.

Drosophila Kc_{167} cells were transfected with Effectene (Qiagen) according to the manufacturer's instructions.

Reverse transfection

HeLa cells were seeded into 12 well plates at a density of 1 x 10^5 cells/ml, 1ml per well in DMEM GlutaMAX media (Gibco) supplemented with 10% FBS. At the time of plating cells were transfected with siGENOME SMARTpool siRNA ANKHD1 or siGENOME Non-Targeting siRNA (Thermo Scientific) at a final concentration of 10nM. Target sequences of the SMARTpool siRNAs are listed in table 2-1. Transfection was performed with Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. Cells were grown for 72 hours before protein or RNA extraction.

Table 2-1 Target sequences of siRNA to knock down ANKHD expression.

SMARTpool siRNA	**Target sequence**
D-014405-01	GUAAAUUGCUAGAUGAAGG
D-014405-02	UGGCAGCUCUACUUAUUGA
D-014405-03	GCGCUAAUGUGCAUGCUAC
D-014405-04	ACACUGCGCUAACUUAUGC

2.2 Protein techniques

2.2.1 Protein extraction

Cell pellets were lysed on ice in a buffer containing 50mM Tris HCL pH 7.4, 250mM NaCl, 5mM EDTA, 0.3% Triton X-100 and protease inhibitor (Complete mini EDTA free protease inhibitor cocktail, Roche). For adherent cells, wells were washed twice with TBS and cells lysed in their wells using the buffer described above. Large samples were sonicated at 10 microns for two ten second bursts then cooled on ice.

2.2.2 Western Blotting

Samples were boiled for 5 minutes in an equal volume of Laemmli sample buffer (125mM Tris HCL pH 6.8, 20% glycerol, 4% SDS, 0.005% Bromophenol blue and 5% Beta Mercaptoethanol). Samples were separated on 4-15% polyacrylamide pre-cast gels (Mini-Protean TGX, Bio-Rad) run at 75mV for 10 minutes and 90mV for 1hour 20 minutes. Separated samples were transferred to a nitrocellulose membrane (Amersham Hybond ECL, GE Healthcare) at 85mV for 1 hour 20 minutes. Membranes were blocked for thirty minutes in 5% skimmed milk in Tris buffered saline (pH 7.4) with 0.1% Tween-20 then probed with appropriate antibodies in 5% bovine serum albumen (BSA)/TBST overnight at 4^{o}C. Primary antibodies are listed in table 2-2 (antibodies binding both phosphorylated and non-phosphorylated forms of the specified protein) and table 2-3 (antibodies binding only the phosphorylated form of the specified protein).

Table 2-2. Primary antibodies used in western blotting, antibodies binding both phosphorylated and non-phosphorylated forms of the specified protein

Protein	**Antibody source**	**Species**	**Dilution**
JAK1	Cell Signalling Technology	Rabbit	1:1000
JAK2	Cell Signalling Technology	Rabbit	1:1000
JAK3	Cell Signalling Technology	Rabbit	1:1000
STAT1	Cell Signalling Technology	Rabbit	1:1000
STAT3	Cell Signalling Technology	Rabbit	1:1000
STAT5	Cell Signalling Technology	Rabbit	1:1000
Beta Actin	Abcam	Mouse	1:2000
c-myc (9E10)	Santa-Cruz Biotechnology	Mouse	1:400
Flag (clone M2)	Sigma	Mouse	1:1000
HA (clone 3F10)	Roche	Rat	

ANKHD1 (HPA008718)	Sigma	Rabbit	1:1000
ANKHD1 (N-terminal) (SAB270098)	Sigma	Rabbit	1:1000
ANKHD1 (N-TERM) (SAB1302423)	Sigma	Rabbit	1:250
ANKHD1	Abcam	Rabbit	1:2000
ANKHD1 (clone D-12)	Santa-Cruz	Goat	1:200
ANKHD1 (clone N-14)	Santa-Cruz	Goat	1:200
YAP	Cell Signalling Technology	Rabbit	1:200
p21	Cell Signalling Technology	Rabbit	1:1000

Table 2-3. Primary antibodies used in western blotting, antibodies specific for protein phosphorylated on the residues stated.

Protein	**Phosphorylated residues**	**Antibody source**	**Dilution**
phospho-JAK1	Tyr 1022/1023	Millipore	1:1000
phospho-JAK2	Tyr 1007/1008	Cell Signalling Technology	1:1000
phospho-JAK3	Tyr 980/981	Cell Signalling Technology	1:1000
phospho-STAT1	Tyr 701	Cell Signalling Technology	1:1000
phospho-STAT3	Tyr 705	Cell Signalling Technology	1:1000
phospho-STAT5	Tyr 694	Cell Signalling Technology	1:1000
phospho-c-Jun	Ser 73	Cell Signalling	1:1000

		Technology	
phospho-MAPK (Erk1/2)	Erk1 – Thr 202 and Tyr 204 Erk2 – Thr 185 and Tyr 187	Cell Signalling Technology	1:2000
phospho-AKT	Thr 308	Cell Signalling Technology	1:1000

Membranes were incubated in horseradish peroxidase conjugated secondary antibodies (Dako) at a dilution of 1:10,000 for 45 minutes and imaged using ECL reagent or ECL select reagent (GE healthcare). All blots using cell lines were repeated on three independent samples.

2.3 DNA techniques

2.3.1 Bacterial culture and starting plasmids

E-coli transformation with plasmid DNA

Plasmids containing full-length ANKHD1 (RC223067) and empty vector (PCMV6-entry PS100001) were obtained from Origene.

50 µl of 10-beta competent E. coli cells (New England Biolabs) were defrosted on ice. 1µ1 of plasmid DNA was added to cells and the mixture incubated for 10 minutes at 37ºC. Cells were then incubated with 1ml of Luria-Bertani (LB) medium (10g/l tryptone, 5g/l yeast extract, 5g/l NaCl) for 1 hour at 37ºC shaking at 250rpm. Cells were concentrated by centrifuging at 13000rpm for 10s and removing the supernatant, before plating on LB agar containing 50µg/ml kanamycin.

Bacterial culture

E-coli were grown in Luria-Bertani medium containing 50µg/ml kanamycin or 50µg/ml ampicillin. Cultures were incubated at 37ºC and shaken at 250rpm. For minipreps a single colony from a selective agar plate was grown overnight in 1.5 to 2ml selective LB. For midipreps a single colony from a selective agar plate was used to set up a starter-culture in 2ml selective LB. After 8 hours incubation 250µl of this starter culture was inoculated into 150ml of selective LB, and grown overnight.

Plasmid DNA extraction

Plasmid DNA was extracted from bacterial cultures using a QIAprep Spin miniprep kit (Qiagen) or a Hi-speed plasmid midi kit (Qiagen) according to the manufacturer's instructions.

2.3.2 Generation of plasmid encoding fragment of ANKHD1 containing first and second ankyrin repeats and the intervening region

Cloning strategy

The plasmid was generated with the PCMV6-entry vector backbone, containing the fragment of ANKHD1 in frame with the sequences for the myc and DKK tags, between Sgf-1 and Mlu-1 restriction sites. The plasmid RC223067, containing the full-length ANKHKD1 sequence was used as a template for PCR using primers containing Sgf-1 and Mlu-1 restriction sites. The PCR product had deoxyadenosine overhangs added and was ligated into TA vectors. This plasmid was then subject to restriction digest with Sgf-1 and Mlu-1, separated by agarose gel electrophoresis, and ligated into the PCMV6-entry vector backbone.

Restriction digests

Restriction enzymes Sgf-1 and Mlu-1 were obtained from Promega. BglII was obtained from New England Biolabs. Digests were carried out for 1 hour at 37^{o}C using the buffers and bovine serum albumen supplied and recommended by the manufacturer.

Agarose gel electrophoresis

Digested DNA fragments were separated on agarose gels made with TAE (40mM Tris, 20mM Acetic Acid, 1mM EDTA), in TAE at 100mV for 1 – 1½ hours. 1%, 1.5% or 2% agarose gels were chosen depending on expected fragment sizes. Agarose gels contained 0.1µl/ml SYBRSafe DNA gel stain (Invitrogen) and DNA was visualised using an Invitrogen Safe Imager.

PCR

Primers used to generate fragments of the ANKHD1 coding sequence flanked by appropriate sgf-1 or mlu-1 restriction sites are listed in Table 2-4. Primers were obtained from Integrated DNA Technologies.

Table 2-4. Primers used to generate ANKHD1 fragments.

Primer	**Sequence (5' to 3')**
1st Ankyrin repeat domain forward	GCG ATC GCC ATG AAA GCA GAA AAC AGC CAC AAT G
2nd Ankyrin repeat domain reverse	ACG CGT GGT TTC GAC ACA TTG ATG ACA TTT TTT C

The forward and reverse primers were combined to produce sequences encoding regions of the ANKHD1 protein described in table 2-5.

Table 2-5. Primer combinations to generate ANKHD1 coding fragments, and sizes of PCR products.

Protein domains encoded	**Forward primer**	**Reverse primer**	**Product size**
1st and 2nd ankyrin repeat domains and intervening sequence	1st ankyrin repeat domain forward	2nd ankyrin repeat domain reverse	3.6kb

Primers were diluted to a concentration of 100µM in DNAse/RNAse free distilled water (Gibco) to make a stock solution. This stock was used to make working dilutions of primers at a concentration of 10µM. PCR was performed in a 50µl reaction volume, containing 2.5µl of 10µM primer and 10 ng template (RC223067 Origene plasmid containing full-length ANKHD1 cDNA). PCR was carried out using Q5 High-Fidelity DNA Polymerase (M0491) (New England Biolabs). Primer annealing temperatures were calculated using the NEB T_m calculator (www.neb.com/tools-and-resources/interactive-tools/tm-calculator) and the instruction in the product literature used to create the following thermocycling conditions:

1) Initial denaturation – 98^{o}C, 30s.

2) Denaturation – 98°C, 7s.
3) Annealing – variable temperature (table 2-6), 20s.
4) Extension – 72°C, variable time (table 2-6).
5) Repeat steps 2-4 for total of 25 or 30 cycles.
6) Final extension – 72°C, 2 minutes.

Table 2-6 Annealing temperatures and extension times for primer pairs.

Forward primer	**Reverse primer**	**Annealing temp**	**Extension time**
1st ankyrin repeat domain forward	2nd ankyrin repeat domain reverse	67°C	100s

Addition of deoxyadenosine overhangs

Q5 High-Fidelity DNA Polymerase produces PCR products with blunt ends. To add 'A' overhangs 6.5µl of PCR product in a was incubated in a 10µl reaction (or 13µl PCR product in a 20µl reaction) with Taq polymerase (New England Biolabs), thermopol buffer (New England Biolabs), dATP (Fermentas) at a final concentration of 5mM and $MgCl_2$ (Fermentas) at a final concentration of 2.5mM, at 72°C for 30 minutes.

Purification of PCR products

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

TA cloning

A TOPO TA Cloning Kit (Invitrogen) was used to ligate PCR products into the pCR 2.1-TOPO vector, according to the manufacturer's instructions.

E.coli transformation with ligated plasmids

10-beta competent E. coli cells (New England Biolabs) were transformed with the product – 50µl of cells were thawed on ice then mixed with 4µl of the cloning reaction product and left to stand on ice for 15 minutes. Cells were then heat-shocked at 42°C for 30 seconds, chilled on ice, then incubated in 250µl SOC or LB

medium for 1 hour at 37°C at 250 rpm. 10µl or 50µ of this cell suspension was spread onto LB agar plates containing 50µg/ml ampicillin, previously spread with 50µl of 33mg/ml X-gal in dimethylformamide.

To identify positive transformants DNA was extracted from cultures of white colonies, and an Sgf-1 and Mlu-1 double-digest performed. Digested DNA was separated by agarose gel electrophoresis as described above. Digests from positive transformants produced two bands, corresponding to the size of the vector and insert.

Gel extraction

RC222623 (Origene plasmid containing Myc and DKK tagged ANKHD1 Splice Variant 3) and pCR 2.1 TOPO containing PCR products were digested with Sgf-1 and Mlu-1 and separated by agarose gel electrophoresis as described above. Gel blocks containing the appropriate vector and insert bands were excised and DNA extracted using a QIAquick Gel Extraction Kit (QIAgen) according to the manufacturer's instructions.

Ligation

Vector and insert fragments were ligated using T4 DNA Ligase (Roche) at 16°C overnight. The molar ratio of insert: vector DNA in the ligation reaction was approximately 3:1. A vector-only control ligation reaction was also set up. 10-beta competent E.coli cells were transformed as described above. After the 1hr incubation in SOC, cells were centrifuged for 10s at 13,000rpm, the supernatant removed and all the possible transformants plated on selective LB agar containing 50µg/ml kanamycin.

Identification of positive transformants and sequencing

To identify positive transformants DNA was extracted from cultures of colonies, and an Sgf-1 and Mlu-1 double-digest performed. Digested DNA was separated by agarose gel electrophoresis as described above. Digests from positive transformants produced two bands, corresponding to the size of the vector and insert. To confirm that the selected plasmids contained the correct insert and to check that no mutations had been introduced during PCR inserts were sequenced at the Core Genomic Facility at the University of Sheffield.

2.4 RNA techniques

2.4.1 RNA extraction

RNA was extracted from cells using an RNeasy Mini Kit and Qiashredder columns (QIagen) according to the manufacturer's instructions. RNA was eluted in DNAse/RNAse free distilled water (Gibco) and stored at -80^{o}C.

2.4.2 cDNA synthesis

1µg of RNA was reverse transcribed to cDNA in a 20µl reaction using a High Capacity RNA-to-cDNA Kit (Applied Biosystems). Control reactions lacking the reverse transcriptase enzyme were run in parallel. The 20µl cDNA was diluted 1:1 in DNAse/RNAse free distilled water (Gibco) for use as a template in quantitative real-time PCR.

2.4.3 Quantitative real-time PCR

Quantitative real-time PCR was carried out in 96 well plates on a Bio-RAD CFX96 Real-Time System C1000 touch thermal cycler.

Primers used for real-time PCR are described in table 2-7.

Table 2-7. Sequences of primers used for real-time PCR.

Target transcript	**Forward primer (5' to 3')**	**Reverse primer(5' to 3')**
Full-length ANKHD1	CCT GCT TGG AAC CCT CTG ATA AA	CGT GCC AGG CCA AAT CTG
Actin	ATC ATT GCT CCT CCT GAG CG	GAC AGC GAG GCC AGG ATG

Each primer was diluted in DNAse/RNAse free water to make a 100 µM stock solution. To make working dilutions of primers, forward and reverse primers for each target gene were mixed together in DNAse/RNAse free water at a concentration of 2.5µM for each primer.

For quantitative real-time PCR each well contained 5µl SYBR green (BioRAD), 1µl cDNA template (diluted as described above) and 4µl primers (diluted as described above).

Melt curves were examined to ensure these primers were producing a single product. For both primer pairs standard curves were generated using serial twofold dilutions of template, performed in duplicate. The slopes of these curves (ANKHD1 -3.07±0.13, efficiency 112%, actin -3.01±0.07, efficiency 115%) met the requirements for the ΔΔCt method to be used for quantification.

2.5 Drosophila techniques

2.5.1 Luciferase assays

Media was removed form *Drosophila* Kc_{167} cells in 96 well plates by tipping and cells were lysed before adding the substrate for firefly luciferase (D-luciferin, Apollo Scientific) and read on a Varioskan plate reader (Thermo-Fisher). Buffer containing substrate for *Renilla* luciferase (Coelenterazine, Apollo Scientific) was then added and measured using a 500nM long pass filter.

2.5.3 Preparation of fly media containing drugs

Freshly prepared molasses agar was obtained whilst molten. A 50mM or 0.5mM stock solution of methotrexate was diluted in DMSO to create the following working concentrations: 750µM, 500µM and 100µM. 100µl of DMSO, or 100µl of each of the working solutions was thoroughly mixed into 100ml of media, to give final concentrations of 1:1000 DMSO, 0.75µM, 0.5µM and 0.1µM methotrexate. Approximately 10ml of drug-containing media was aliquoted per tube. Media was prepared weekly and stored at 4ºC. Dried yeast was added to the surface of the media before use.

2.5.4 Crosses

Hop^{T42}/FM7, Act-GFP virgins were crossed with w1118 males, with approximately eight females and two males per vial.

Adults were maintained at room temperature on standard molasses agar with yeast for 48 hours before being transferred to tubes of drug-containing media. Adults were left to lay eggs for approximately 24 hours before being transferred to a fresh tube. Tubes containing eggs were maintained at room temperature for approximately 24 hours before being transferred to 25ºC. This was repeated for ten days.

2.5.5 Tumour assay

Hop^{T42} heterozygous females were scored for tumour formation after hatching. Each fly was examined for tumours, which were scored as follows: tumour the diameter of one abdominal segment = 1, diameter of two abdominal segments = 2, larger than two abdominal segments = 4, half an abdominal segment = 0.5, a quarter of an abdominal segment = 0.25. For each fly the size of each tumour was multiplied by the number of tumours of that size, and the values summed. At least 150 flies were assessed for each drug concentration. The tumour index for each drug treatment was the mean value for all the flies at that drug concentration.

2.6 Kinase profiling

In-vitro kinase profiling was carried out by the International Centre for Kinase Profiling, at the University of Dundee. The effect of 10µM methotrexate upon the activity of 50 kinases was measured with a radioactive filter binding assay using 33P ATP (Hastie, McLauchlan *et al*. 2006). The incubation time with 33P is not uniform across all kinases examined, but varies according to the optimal incubation time for each kinase. Each assay is performed in duplicate, and the plate on which the assay is performed includes reference compounds and blank wells for quality control.

2.7 Immunofluorescence

2.7.1 Preparation of slides

Cultured cells were grown on glass coverslips. After removal of media, coverslips were washed three times in ice-cold TBS before fixing with 100% methanol at -20^{o}C for 30 minutes. Coverslips of cultured cells or slides of fixed blood or marrow films were washed in TBST then incubated in blocking solution (5% dried skimmed milk in TBST) at room temperature for 30 – 60 minutes. Coverslips or films were then incubated overnight at 4^{o}C in primary antibody diluted in 1%BSA/TBST. Primary antibodies and the concentrations at which they were used are listed in table 2-8.

Table 2-8 primary antibodies used in immunofluorescence

Antibody	**Source**	**Final concentration**
Rabbit anti-ANKHD1 (0.2mg/ml) (polyclonal)	Sigma	1µg/ml
Mouse anti-FLAG, clone	Sigma	5µg/ml

M2 (1mg/ml) (monoclonal		
Rabbit anti-pSTAT3 (Y705)	Cell Signalling Technology	Diluted 1:100

The slides were washed in TBST then incubated for one hour in the dark at room temperature in secondary antibody diluted 1:1000 in 1% BSA/TBST. Details of secondary antibodies are listed in table 2-9.

Table 2-9 secondary antibodies used in immunofluorescence.

Antibody	**Source**
Mouse anti-rabbit IgG heavy and light F(ab')2 fragment Alexa Fluor (R) 488 conjugate	Cell signalling technology
Donkey anti-rabbit IgG Alexa Fluor 568 conjugate	Invitrogen, Molecular Probes
Goat anti-mouse IgG Alexa Fluor 488 conjugate	Life Technologies

Coverslips or slides were then washed in TBST and mounted in Fluoroshield with DAPI (Sigma).

2.7.2 Fluorescence Microscopy

Images were gathered using an Olympus BX61 epifluorescence microscope with a Hamatsu orca monochrome camera and Volocity imaging software, in the Wolfson Light Microscopy Facility in the Department of Biomedical Science at the University of Sheffield.

2.7.3 Image analysis

Images were prepared and analysed using ImageJ software (v1.48).

2.8 Source and initial processing of patient samples

2.8.1 Ethical approval

The study on ANKHD1 was reviewed by the Yorkshire and the Humber-Bradford Research Ethics Committee (13/YH/0018). All participants for whom personal data was accessed in the study gave written informed consent. A substantial amendment

to the study that allowed the use of un-linked de-identified tissue that was surplus to requirements for clinical care without specific patient consent was approved by the Research Ethics Committee.

The study on the effect of methotrexate on primary cells from patients with MPNs was reviewed under the proportionate review scheme by the North West-Greater Manchester South Research Ethics Committee (14/NW/1524). Participants gave written informed consent for the use of stored material surplus to requirements for their clinical care, and access to their medical records.

2.8.2 Whole blood and bone marrow aspirate

Origin of samples

3-4 ml of whole blood was drawn from peripheral sites into an EDTA tube. For individuals with a Hickman line, blood was drawn from the line at the time of routine sampling for clinical management, and transferred to an EDTA tube. During bone marrow biopsies performed for clinical reasons an additional 1 – 4 ml of aspirate was drawn and transferred to an EDTA tube. For de-identified samples supplied by the diagnostic haematology laboratory blood or bone marrow aspirate was supplied as a 100-200µl aliquot from EDTA samples that would otherwise be disposed of as waste following clinical testing or were surplus to requirements for clinical care. Samples of blood to be used as normal controls were selected as samples with a normal full blood count obtained from individuals attending as an out-patient at a clinic for a specialty other than haematology or oncology.

Preparation of blood and bone marrow films

Fresh blood or bone marrow in EDTA was mixed and approximately 2µl spread on a glass slide. Films were air dried, fixed in room temperature 100% methanol for 30s then air dried again. Fixed films were stored at room temperature.

Films which were stained were stained in Wright's stain (Sigma) for 15 seconds before washing in deionised water for 30s and rinsing in deionised water.

Films which were examined with immunofluorescence were prepared using the protocol used for cultured cells (section 2.7.1) starting at the blocking step, and with increased antibody volumes to cover the larger slide area.

Isolation of peripheral blood and bone marrow mononuclear cells

Mononuclear cells were separated from whole blood or bone marrow using Histopaque-1077 (Sigma-Aldrich) according to the manufacturer's instructions. Following fractionation mononuclear cells were counted on a haemocytometer and processed to obtain protein (see section 2.2.1) and RNA (see section 2.4.1).

2.8.3 Apheresis material

Fresh apheresis waste for ANKHD1 study

50ml of blood/ saline mixture (waste remaining in machine tubing following harvest) was centrifuged at 1200rpm for 5 minutes) to concentrate the blood cells. Mononuclear cells were obtained from 3ml of the blood-rich fraction using Histopaque-1077 (Sigma-Aldrich) according to the manufacturer's instructions. Mononuclear cells were separated into CD34+ and CD34- fractions using a magnetic bead kit (CD34 MicroBead Kit human, Miltenyi Biotec) according to the manufacturer's instructions. Cells in each fraction were counted on a haemocytometer and processed to obtain protein as described in the section 2.2.1.

Stored apheresis material for methotrexate study

0.5ml aliquots of apheresis material in 10% DMSO (samples stored for quality assurance testing) were retrieved from liquid nitrogen storage at NHS blood and transplant, transported on dry ice, and immediately transferred to liquid nitrogen. Cells were thawed in a 37^{o}C water bath until only a small ice crystal remained and transferred to a 15ml tube. 1ml of pre-warmed HSC expansion media (without cytokines) was used to rinse the cryotube, and added drop by drop (approx. one drop every 5s) down the side of the 15ml tube. Cells were centrifuged at 1200 rpm at room temperature for 5 minutes, supernatant removed, the washing step repeated with fresh media, and cells resuspended. The cell suspension was passed through a 40µm nylon mesh filter, and suspended in 15ml chilled separation buffer (PBS Ph 7.2, 0.5% BSA, 2mM EDTA, de-gassed). Cells were centrifuged at 1200rpm at 4^{o}C and resuspended in 300µM separation buffer. Isolation of CD34+ cells was carried out using a CD34 MicroBead Kit UltraPure (Miltenyi Biotec), following the manufacturer's instructions. The isolated cells were suspended in 5ml HSC expansion media supplemented with cytokines (as described in section 2.1.1).

Seeding densities varied between patients, but were around 0.3 x 10^5 cells/ml. Cells were analysed for CD34+ enrichment and viability 24-48 hours after isolation. Cells were expanded for 3-4 days before seeding for drug treatments.

2.8.4 Formalin fixed paraffin embedded melanoma samples

Slides were prepared from tissue blocks by NHS laboratory staff. 4µM sections were cut onto coated slides from formalin-fixed paraffin embedded (FFPE) tissue samples and dried overnight at 55ºC. Slides labelled with an un-linked identifier, and supplied with the age and gender of the patient and the skin site of the melanoma.

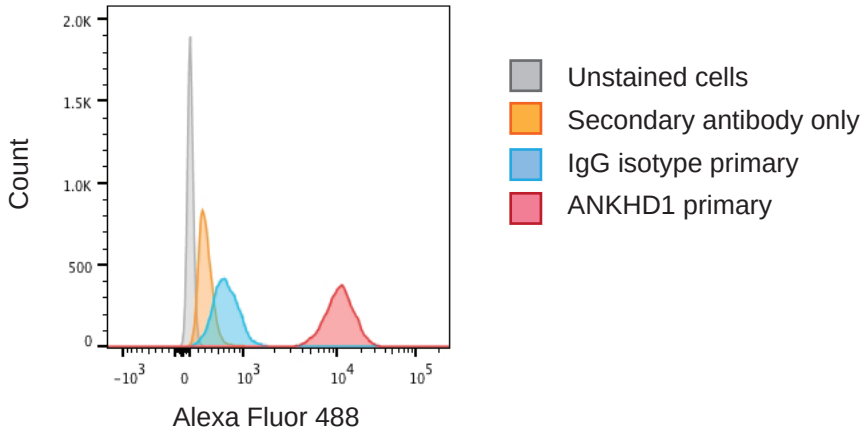
2.9 Flow cytometry

2.9.1 Development of flow cytometry protocol

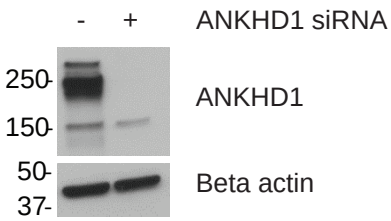
A protocol to detect ANKHD1 using flow cytometry was developed using the myeloma cell line JJN-3 and HeLa cells, both of which express ANKHD1. None of the large antibody suppliers produce fluorophore-conjugated anti-ANKD1 antibodies, so two approaches were investigated to label cells for flow cytometry. A commercial kit was used to label the ANKHD1 antibody, but when JJN-3 cells stained with conjugated anti-ANKHD1 were compared to cells stained with polyclonal IgG labelled using the same kit there was no difference in the median fluorescence intensity of the two populations (data not shown). A second approach, using an unlabelled primary antibody and a fluorescently-conjugated anti-rabbit secondary FAB fragment was more successful. The median fluorescence intensity of the cell population stained with anti-ANKHD1 and anti-rabbit Alexa Fluor 488 was seventeen-fold higher than in cells from the same passage stained with the same concentration of polyclonal rabbit IgG and anti-rabbit secondary (Figure 2-1A).

To determine the specificity of the flow cytometry method to detect ANKHD1 a cell population that does not express ANKHD1 was required. siRNA was used to knock down expression of ANKHD1 in HeLa cells, and western blotting showed the ANKHD1 level was reduced to 3% of the level seen in untreated cells (Figure 2-1B). Flow cytometry on cells from the same population showed that the median fluorescence intensity of the cells in which ANKHD1 was knocked down was about 60% of that of the untreated cells (Figure 2-1C). Other published studies

A



B



C

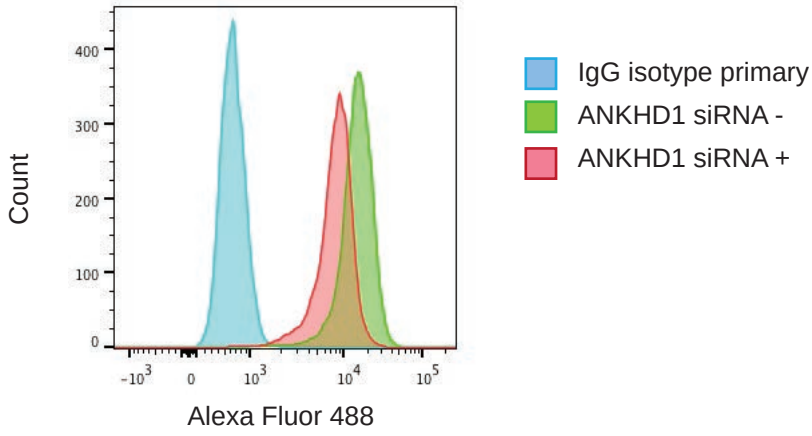


Figure 2-1 . Flow cytometry using an unlabelled primary antibody and a labelled secondary antibody can be used to detect ANKHD1. A In the myeloma cell line JJN-3, fluorescence in the presence of ANKHD1 primary antibody is seventeen fold higher than that seen when a polyclonal rabbit IgG is used as the primary antibody. B Western blotting shows that ANKHD1 protein levels are substantially reduced by siRNA treatment. C Flow cytometry of cells from the same population as shown in the western blot. The median fluorescence intensity of the ANKHD1 siRNA treated cells is 60% of that of the untreated cells.

comparing western blotting and flow cytometry for measurement of protein have described a similar disparity between the two methods (Anand, Stedham *et al*. 2011).

The flow cytometry method was considered adequate to proceed to examining whole blood. Further optimisation was carried out using whole blood to optimise secondary antibody concentrations and to combine staining for ANKHD1 with other antibodies and fluorophores.

2.9.2 Fixation and permeabilisation

Cell lines and primary cells in culture

Prior to fixation and permeabilisation cell grown in suspension were centrifuged at 12,000 rpm for five minutes, the supernatant was discarded and the pellet resuspended in 1ml phosphate buffered saline (PBS) (138 mM NaCl, 2.7 mM KCl, 1.1 mM KH_2PO_4, 8.1 mM $Na_2HPO_4.2H_2O$, pH 7.4). For experiments in which flow cytometry and western blotting were being compared, cells grown in a single well were separated into aliquots for each experimental procedure at this stage. HeLa cells were released from cell culture flasks by digestion with trypsin and resuspended in media containing 5% or 10% FBS before centrifugation and resuspension in PBS as described above.

Fixation was performed by adding formaldehyde to the cell suspensions to a final concentration of approximately 2.5 – 3% and incubating at 37^{o}C for 10 minutes. Cells were then cooled on ice for one minute before permeabilising by adding ice-cold 100% methanol to a final concentration of 90% methanol, and incubating on ice for 30 minutes. Cells were either stained immediately, or stored in 90% methanol at -20^{o}C.

Whole blood or bone marrow aspirate

100µl of blood was prepared from each sample. When larger volumes of peripheral blood were available volumes were scaled up accordingly. Cells were fixed by adding 65µl of 10% formaldehyde/PBS to each tube, vortexing briefly, and incubating at room temperature for 15 minutes. 1ml of 0.1% Triton X-100 was then added to each tube, which was vortexed and incubated at room temperature for 30 minutes. Cells were washed by adding 2ml of incubation buffer (0.5% BSA in PBS) to each tube and pelleting cells at 1250rpm for 5 minutes. The wash step was

repeated, before resuspending cells in 1ml of 50% methanol/PBS. Cells were incubated on ice for 10 minutes and stained immediately or stored at -20°C.

2.9.3 Immunostaining

Fixed and permeabilised cells

Cells suspended in methanol were vortexed to thoroughly resuspend them. Approximately 1 x 10^6 cells were aliquotted per condition for cultured cell lines, determined by counting cells suspended in methanol on a haemocytometer or by counting cells growing in suspension prior to fixation and permeabilisation. For primary cells in culture initial cell numbers were lower. For blood or bone marrow cells, 0.5ml of methanol-suspended cells, corresponding to 50µl whole blood or bone marrow, was used for each condition. Where the white blood cell count was elevated the volume of methanol-suspended cells was reduced .

All wash steps were carried out by adding 2ml incubation buffer (0.5% BSA in PBS) and centrifuging at 1250rpm for 5 minutes. Cells were washed twice, resuspended in 100µl incubation buffer and blocked by a 10 minute incubation in this buffer at room temperature. Primary antibodies were then added, and cells incubated for 1 - 2 hours at room temperature. For fluorescently conjugated antibodies this incubation was done in the dark. Fluorescently conjugated primary antibodies and the concentrations are listed in table 2-10, unlabelled primary antibodies in table 2-11.

Table 2-10. Fluorescently conjugated primary antibodies used in flow cytometry. Dilution factors are those recommended by the manufacturer.

Antibody	**Antibody source**	**Dilution factor/ final concentration**
PerCP anti-CD45	BD Biosciences	1.25µg/ml
PE anti-CD34	Miltenyi Biotec	1:11
Alexa Fluor 488 anti-STAT5 (pY694), clone 47, mouse IgG1κ	BD Biosciences	1:20
Alexa Fluor 488 mouse IgG1κ isotype control	BD Biosciences	1:5

Table 2-11. Un-labelled primary antibodies used in flow cytometry.

Antibody	**Antibody source**	**Final concentration**
Normal Rabbit IgG (1mg/ml) (polyclonal)	Cell Signalling Technology	1µg/ml
Rabbit anti-ANKHD1 (0.2mg/ml) (polyclonal)	Sigma	1µg/ml

Cells were washed twice, and resuspended in PBS or incubated in incubation buffer with a 1:1000 dilution of fluorescently conjugated secondary antibody (Anti-rabbit IgG heavy and light F(ab')2 fragment Alexa Fluor (R) 488 conjugate, Cell Signalling Technology). Cells were incubated at room temperature in the dark for 30 minutes. Cells were washed twice in incubation buffer as above and resuspended in PBS.

Un-fixed cells

24-48 hours after isolation of CD34+ HSCs from stored apheresis material an assessment of CD34+ cell enrichment was performed. 1 x 10^5 cells were washed and resuspended in 95µl incubation buffer. Cells incubated with labelled antibodies at 4^{o}C in the dark for 15 minutes, washed, resuspended in PBS and analysed immediately.

2.9.4 Assessment of cell viability

Flow cytometry to assess cell viability was performed on fresh cells, using an Annexin V-FITC Early Apoptosis Detection Kit (Cell Signalling Technology) according to the manufacturer's instructions.

2.9.5 Analysis on flow cytometer

Cells were analysed on a BD LSRII or BDFACSCanto II. Data was analysed using BD FACSDiva software or Flowjo. Compensation for spectral overlap between fluorophores was based on values used for clinical tests in the diagnostic laboratory, and applied as follows:

Table 2-12. Compensation matrix for flow cytometry

Fluorochrome	% Fluorochrome	Spectral Overlap
PerCP	FITC	3.32
PE	FITC	16.65
FITC	PerCP	0.00
PE	PerCP	0.00
FITC	PE	1.07
PerCP	PE	22.15

2.9.6 Gating strategies

Cell populations were identified with flow cytometry using side scatter and staining with PerCP-conjugated anti-CD45, a method routinely used in diagnostic flow cytometry. CD45, also known as the leucocyte common antigen, is a receptor tyrosine phosphatase expressed on the surface of white blood cells whose expression levels vary in a manner that is useful to separate cell populations. Gating strategies used to identify cell populations are shown as follows:

Normal blood cell populations Figure 2-2

CD34+ cells from stem cell donors Figure 2-3

Blasts from patients with acute leukaemia Figure 2-4

CD34+ cells from a patient with MF and a patient with myelodysplasia with high circulating CD34+ counts Figure 2-5.

2.9.7 Flow cytometry calculations

Staining index was calculated using the formula (MFI positive – MFI negative)/ 2 x SD negative, where MFI is median fluorescence intensity and SD is the robust standard deviation. Fold change in MFI was calculated as MFI positive/ MFI negative. Percentage inhibition was calculated as (1-[treated cells MFI – isotype MFI]) / (untreated cells MFI – isotype MFI), where the treatment was siRNA or drug.

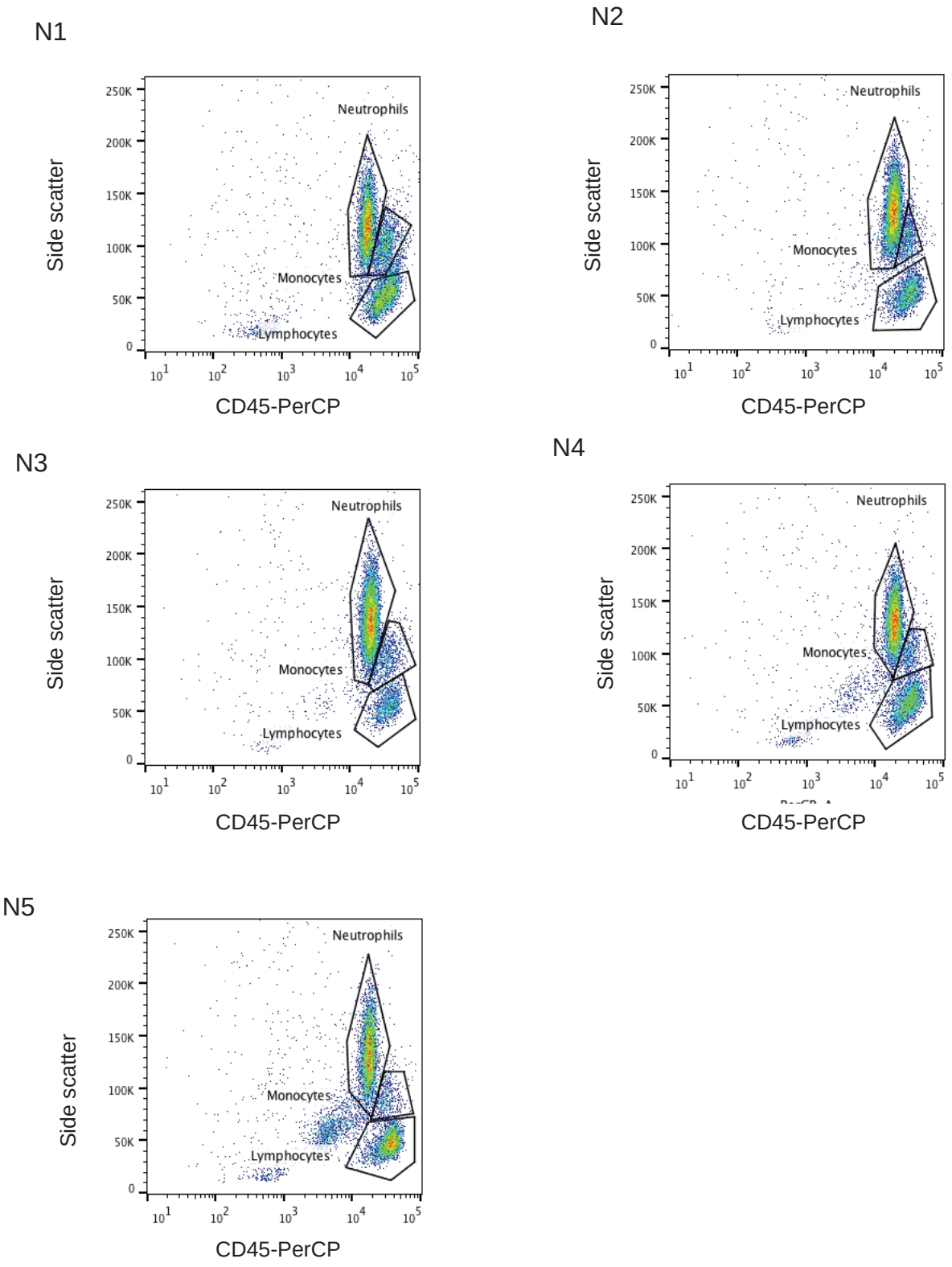


Figure 2-2 Gating strategy used to define populations of leucocyte types in normal blood using flow cytometry . The positions of the populations varied slightly between patients.

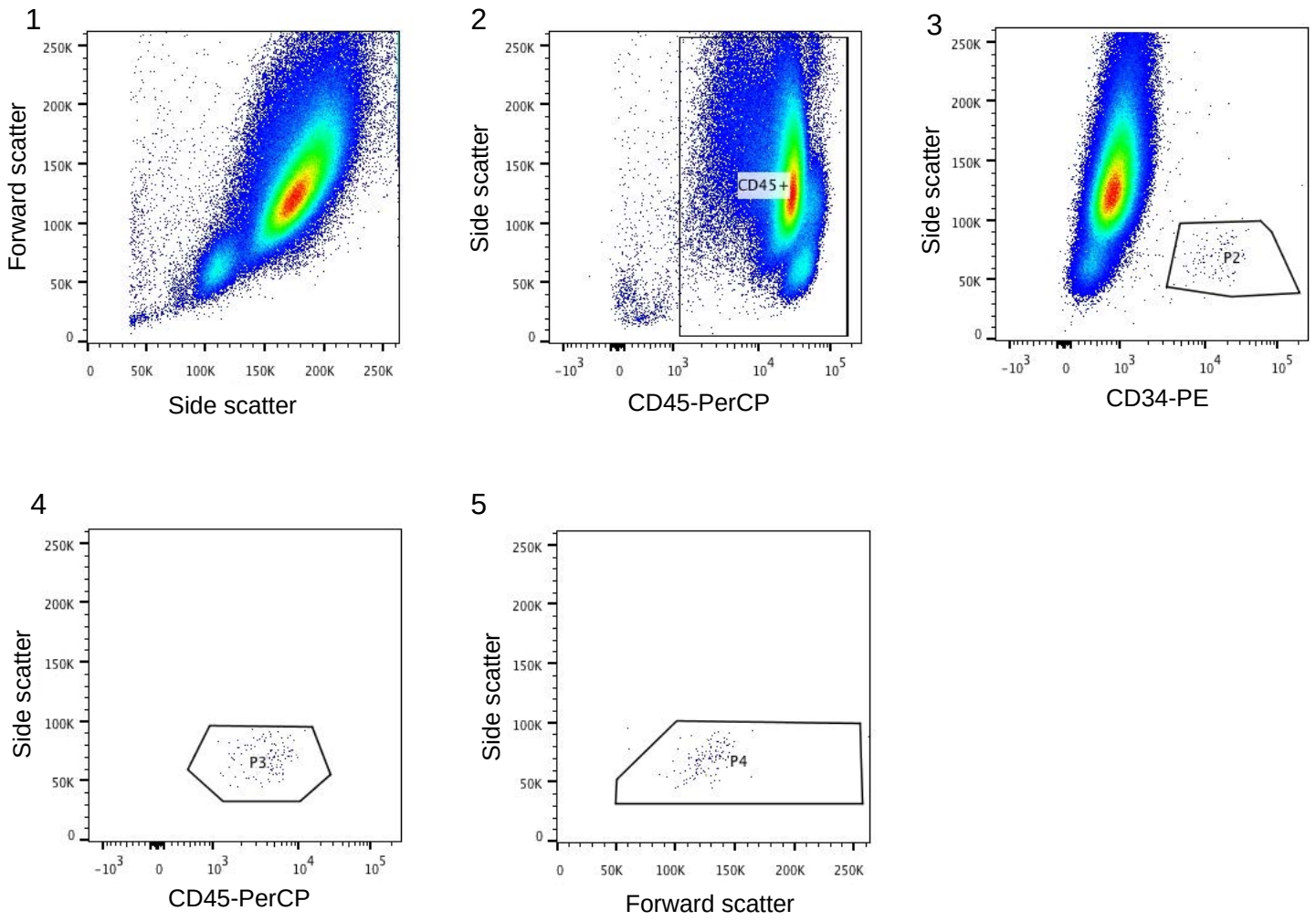


Figure 2-3 The gating strategy to identify CD34+ cells in peripheral blood is adapted from the approach used by the diagnostic haematology laboratory:
Step 1: Collect data for 300,000 events
Step 2: Gate to identify CD45+ cells
Step 3: Gate to isolate CD34+ cells
Step 4: Re-examine and re-gate CD34+ population using CD45
Step 5: Re-examine and re-gate based on side scatter and forward scatter

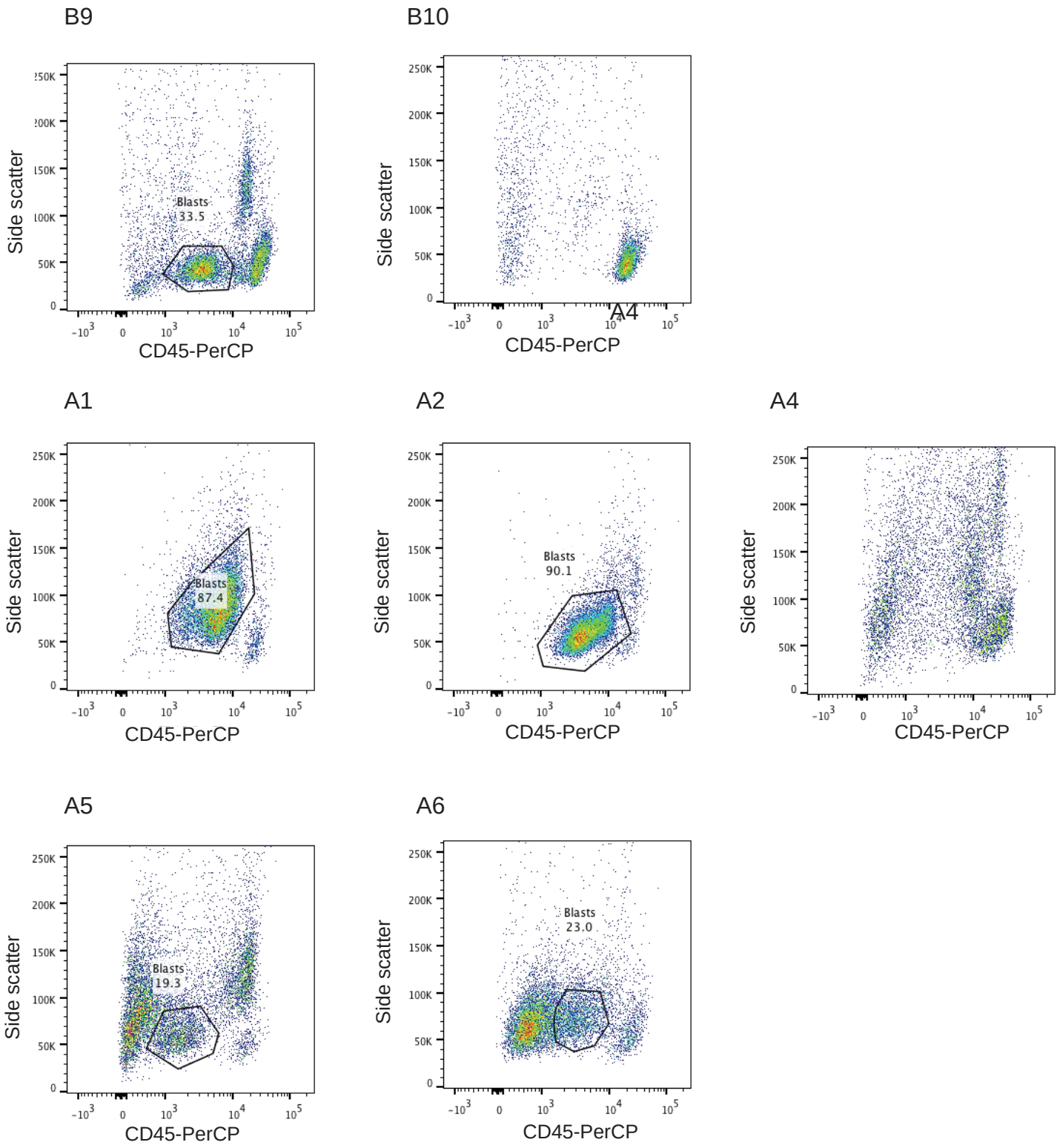


Figure 2-4 Gating strategy to identify blasts in samples from patients with acute leukaemia.The blast population appears in slightly different areas of the CD45-PerCP/Side scatter plot for each patient. For some patients, a blast cell population could not be identified. This may be where the sample was taken after treatment had been started, or due to degradation of the sample.

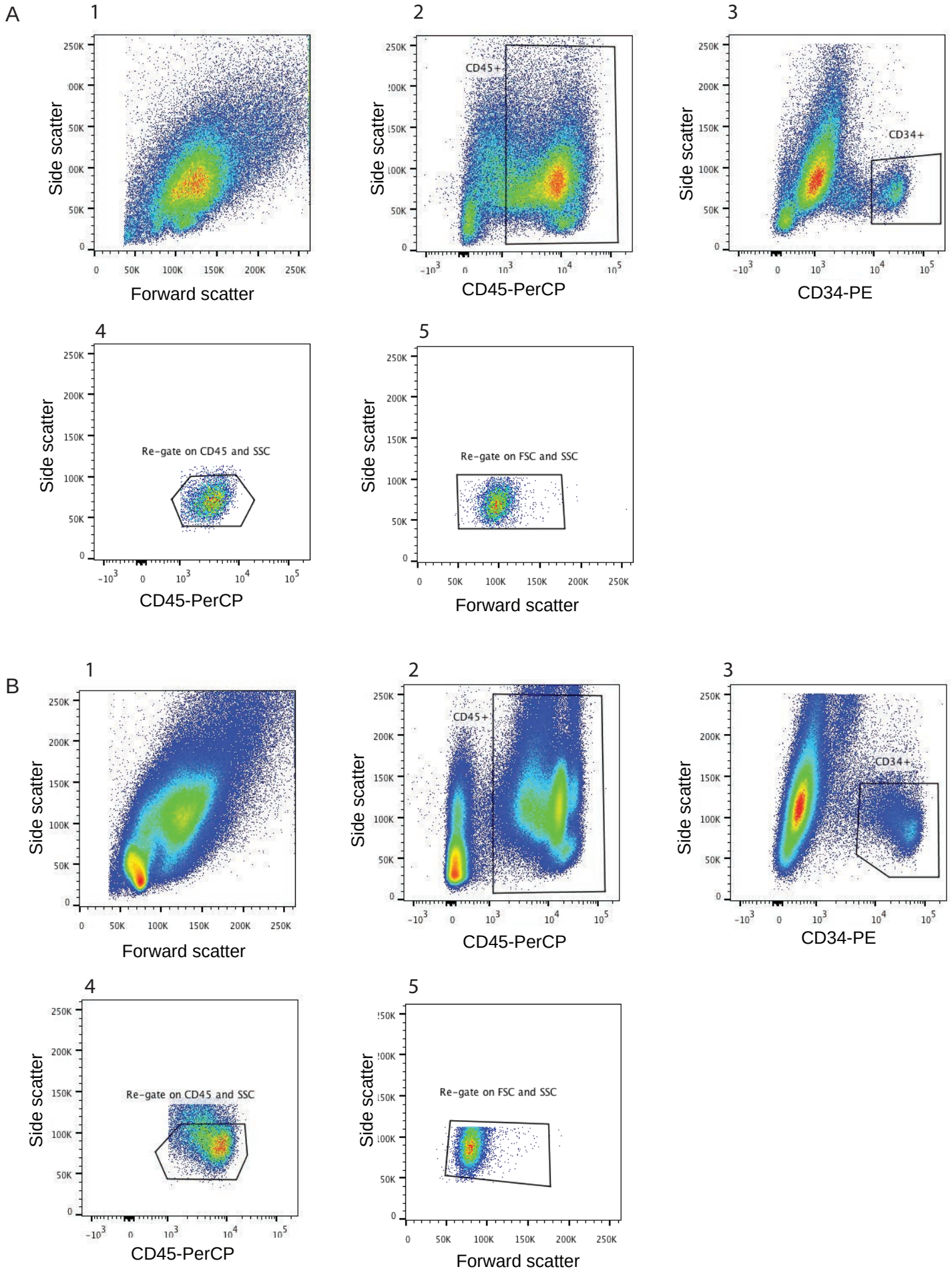


Figure 2-5 Gating strategy to identify CD34+ cells in two patients with haematological malignancies with high circulating CD34+ cells in peripheral blood. A Patient A7, an individual with MDS. B Patient A8, an individual with MF. The gating strategy is the same as that used in Figure 2-3 , but the positions of the cell populations are slightly different.

2.10 Immunohistochemistry

2.10.1 Preparation of slides

Samples were dewaxed in xylene twice for five minutes each, then washed twice in absolute ethanol and once in 95% ethanol each for two minutes. Endogenous peroxidase activity was blocked by incubating slides in 3% hydrogen peroxide in methanol for 20 minutes. Slides were then washed in tap water for one minute.

Heat-induced epitope retrieval was performed by heating slides in a pressure-cooker in a 10mM solution of trisodium citrate with 1mM EDTA at ph 6.5. The pressure-cooker cycle consisted of 4 minutes at 125^{o}C, followed by 45 minutes of cooling to approximately 90^{o}C. After the pressure-cooker cycle was complete, slides were washed in tap water for 1 minute.

Immunostaining was carried out employing the streptavidin-biotin-peroxidase method, using a Vectastain ABC Universal kit (Vector Laboratories, Peterborough) according to the manufacturer's instructions. Slides were incubated in normal horse serum diluted in PBS for 30 minutes, before incubating in primary antibody for 30 minutes. Anti-ANKHD1 antibody HPA008718 by Atlas Antibodies, obtained from Sigma was used. All slides were stained with antibody from the same lot. Optimal primary antibody dilutions were established on sections of tonsil. A final antibody concentration of 8µg/ml (a 1:25 dilution of the stock antibody) was used. A rabbit polyclonal IgG antibody was used at the same concentration to stain an adjacent tissue section, as a control for non-specific antibody binding.

Slides were then washed with PBS and incubated in biotinylated secondary antibody, blocked in horse serum, for 30 minutes. After washing with PBS, slides were incubated with a mixture of streptavidin and biotin-conjugated horseradish peroxidase for 30 minutes. Regions of antibody binding were visualised by 10 minutes incubation with a solution of diaminobenzidine and horseradish peroxidase (Vector DAB kit). After washing, a nuclear counterstain was performed by immersing slides in Gill's haematoxylin for 30 seconds, washing in tap water and blueing in Scotts's tap water for 10 seconds. Slides were dehydrated through 70% ethanol, 95% ethanol and absolute ethanol before transferring to xylene and mounting.

2.10.2 Microscopy

Slides were photographed on a Leica DMI4000B inverted widefield microscope.

2.11 Antibody validation

A number of anti-ANKHD1 antibodies are available from the major antibody suppliers. These are raised against different epitopes in the protein, shown in Figure 2-6. All antibodies are polyclonal, purified by affinity purification. With the exception of the Santa Cruz antibodies, which are raised in goat, they are raised in rabbit.

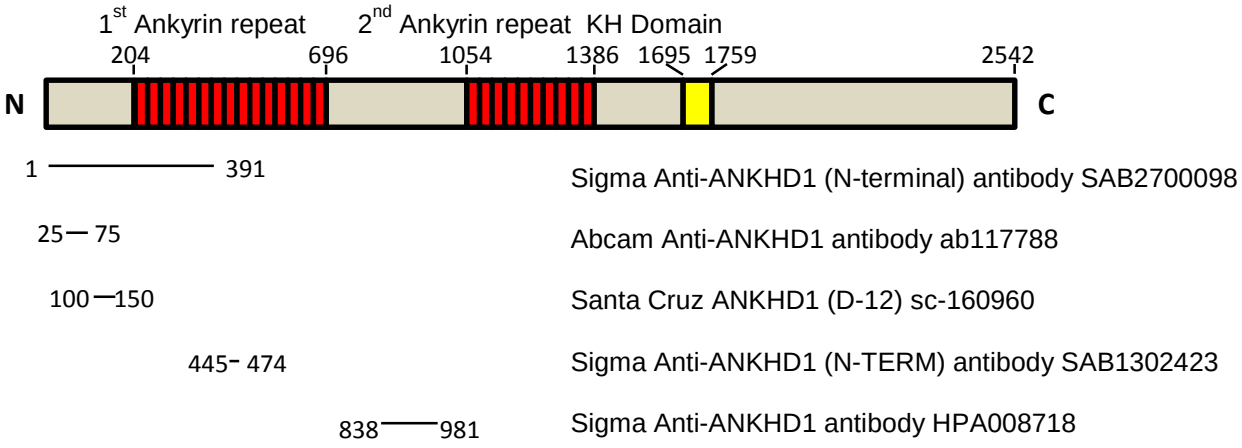


Figure 2-6. Regions of ANKHD1 containing the sequences of the epitopes to which anti-ANKHD1 antibodies investigated for use in this study were raised.

These antibodies were examined with western blotting in two myeloma cell lines (U-226 and JJN-3) and HeLa cells. To examine for possible nonspecific binding, HeLa cells in which ANKHD1 expression had been knocked down with siRNA were also examined (Figure 2-7 and 2-8). Sigma antibody HPA008718 gave the best combination of sensitivity and specificity based on western blots, although it is notable that some prominent bands were still seen with this antibody when ANKHD1 was knocked down.

Several of the antibodies were also compared using immunofluorescence in Hela cells (Figure 2-9). Sigma antibody HPA008718 was further examined in Hela cells in which a plasmid encoding the region of ANKHD1 containing the epitope the antibody was raised against was expressed, and in which ANKHD1 expression was knocked down with siRNA (Figure 2-10).

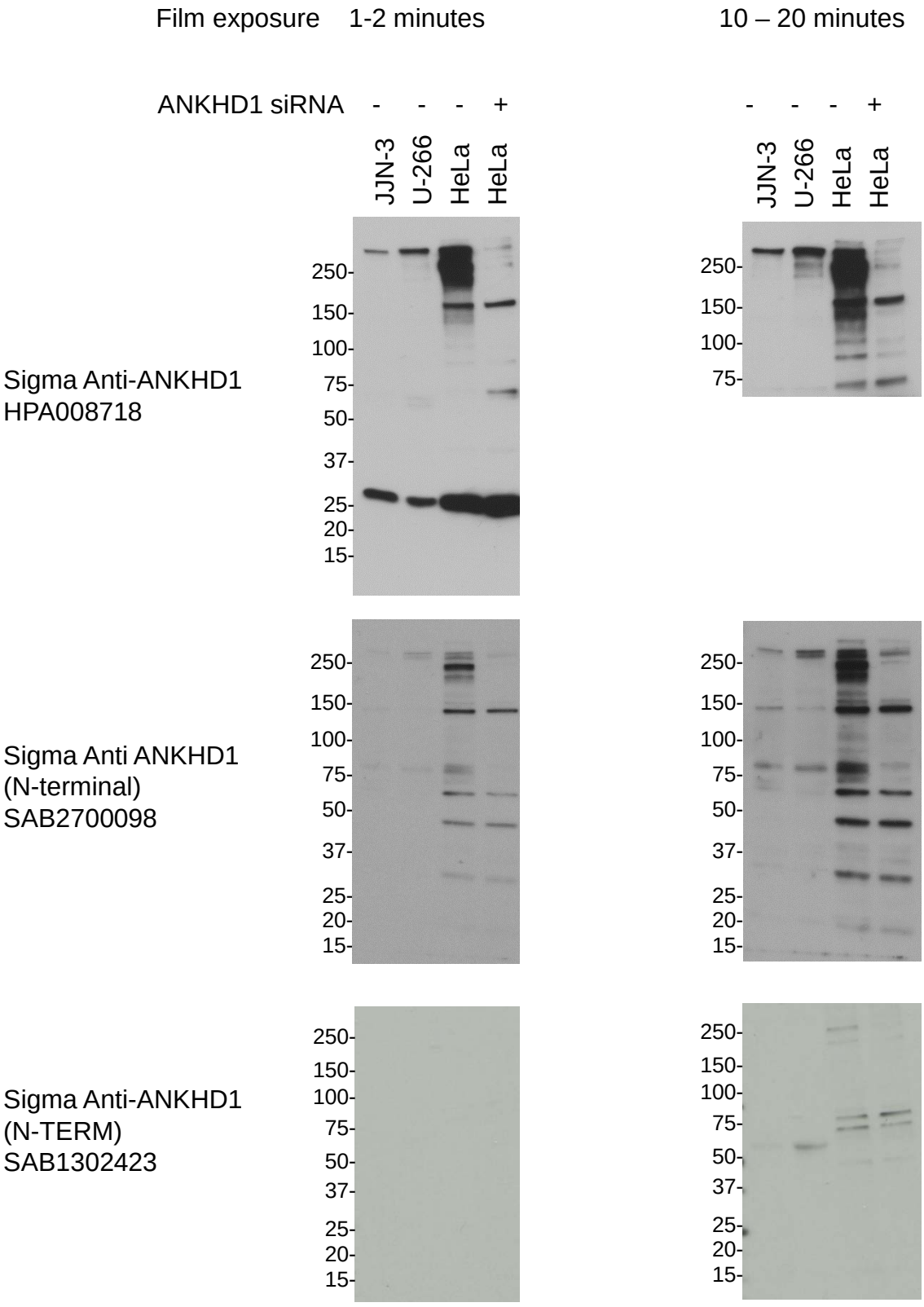


Figure 2-7..Western blotting comparing sigma anti-ANKHD1 antibodies. Molecular weight of full-length ANKHD1 is 269kDa.

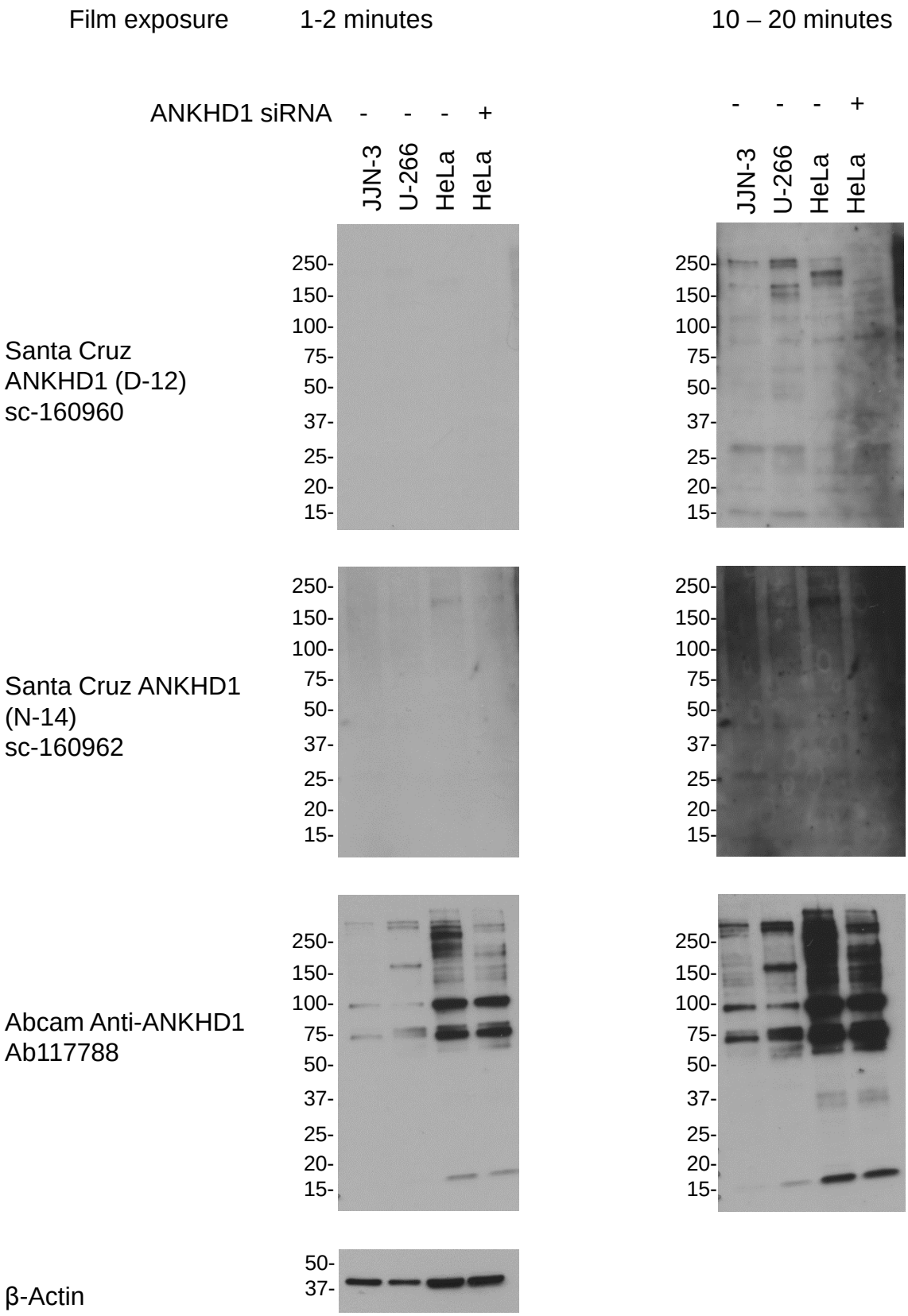


Figure 2-8. Western blotting comparing Santa Cruz and Abcam anti-ANKHD1 antibodies. Molecular weight of full-length ANKHD1 is 269kDa.

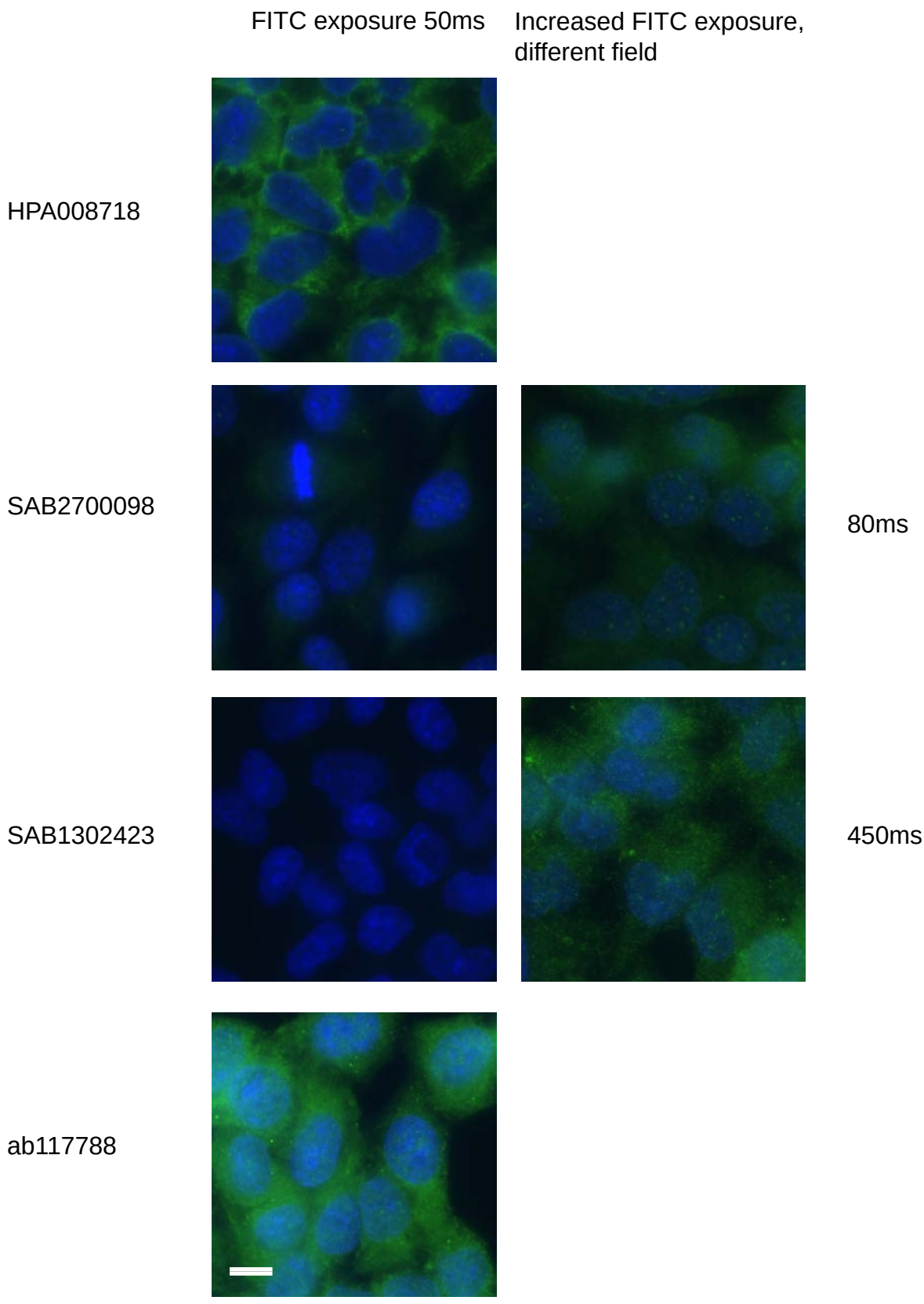


Figure 2-9. Comparison of different anti-ANKHD1 antibodies using immunofluorescence in HeLa cells (sc-160960 D-12 and sc-160962 N-14 were not examined using immunofluorescence). Green anti-rabbit FITC, rabbit primary antibodies indicated on left. Blue DAPI nuclear counterstain.

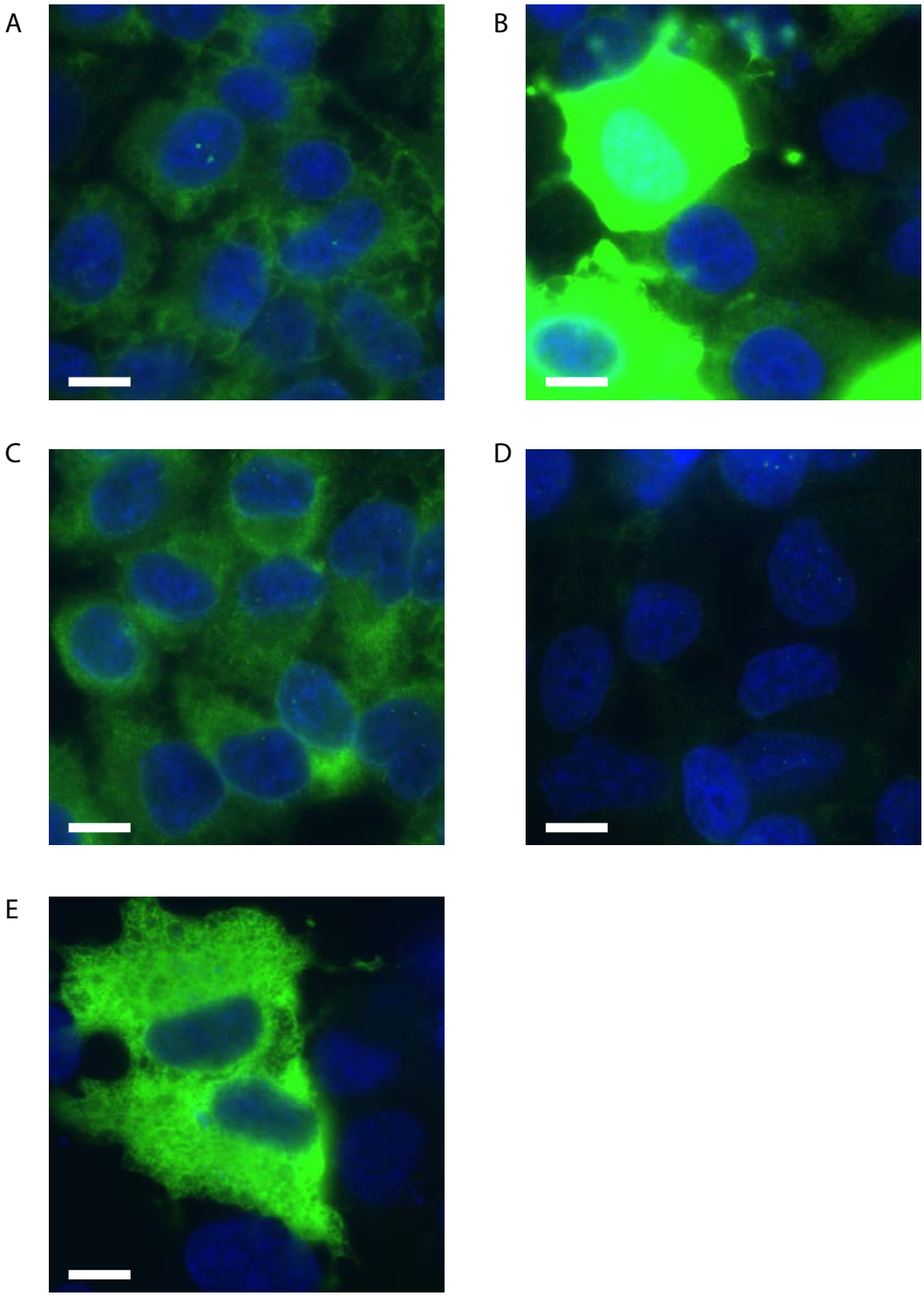


Figure 2-10.Validation of anti-ANKKHD1 antibody HPA008718 (Sigma) using immunofluorescence in HeLa cells. In all images green = Alexa Fluor 488 conjugated anti-rabbit secondary antibody, blue = DAPI nuclear counterstain, scale bar = 10uM. Images A-D taken with identical exposure conditions. (A) Cells transfected with empty vector pCMV6. (B) Cells transfected with a construct encoding the first and second ankyrin repeat domains and intervening region, including the epitope the antibody was raised against. (C) Cells transfected with non-target siRNA. (D) Cells transfected with siRNA targetting ANKHD1. (E) Different field of view of cells from experiment shown in A, taken with a shorter exposure.

2.12 Statistical analysis

Statistical analysis was carried out using Prism (Graphpad). Mean and standard deviation were used to describe continuous data. One-way ANOVA with Dunnett's multiple comparisons test or Tukey's multiple comparisons test was used to make comparisons between multiple groups. Paired *t* tests were used to make comparisons between paired samples. Correlation was examined with Pearson's correlation coefficient.

Chapter 3: ANKHD1 in normal blood and haematological malignancies

3.1 Introduction

Given the evidence that ANKHD1 positively regulates JAK/STAT signalling in laboratory models, I hypothesised that ANKHD1 may be involved in JAK/STAT activation in haematological malignancies. I wished to examine ANKHD1 expression in haematological malignancies and the corresponding blood cells from healthy individuals.

ANKHD1 in normal blood cells

There has been some examination of ANKHD1 mRNA expression in blood cells, but little description of its expression at the protein level. ANKHD1 mRNA, as well as ANKHD1-EIF4EBP3 and a short isoform termed splice variant 4 have been detected in peripheral blood leucocytes, lymph node, spleen and bone marrow mononuclear cells using RT-PCR. In lymph node, expression of ANKHD1-EIF4EBP3 is higher than that of the other splice variants (Duarte, Traina *et al*. 2005). A second group has performed a similar examination of mRNA expression for these transcripts, although differences in the approaches used to normalise mRNA expression levels make comparisons between the two studies difficult (Miles, Janket *et al*. 2005). This group also examined mRNA expression in macrophages and dendritic cells differentiated *in-vitro* from monocytes extracted from PBMCs. Both expressed all three splice variants, and expression of the short isoform was particularly high in dendritic cells.

In an *in vitro* model of myeloid differentiation in which the acute promyelocytic leukaemia cell line HL-60 is induced to differentiate by the addition of retinoic acid, expression of these transcripts is upregulated, with the greatest increase seen in ANKHD1-EIF4EBP3. This contrasts with the changes seen in a model of erythroid differentiation, in which non-adherent sub-cultures of PBMCs are treated with erythropoietin, where the greatest increase is see in ANKHD1 splice variant 4. These results suggest ANKHD1 may play a role in differentiation in haematopoiesis (Duarte, Traina *et al*. 2005).

ANKHD1 protein expression has been detected in lymph node but not peripheral blood mononuclear cells using western blotting. Using immunofluorescence, however, ANKHD1 has been detected in PBMCs. PBMCs consist of a heterogeneous group of blood cells, so this tells us little about which sub-types of blood cells ANKHD1 is expressed in (Traina, Favaro *et al*. 2006).

ANKHD1 in haematological malignancies

ANKHD1 mRNA expression has been shown to be higher in bone marrow from individuals with AML and ALL than bone marrow or PBMCs from healthy donors (Traina, Favaro *et al*. 2006). ANKHD1 mRNA expression is also higher in myeloma cells from patient bone marrow than it is in plasma cells purified from bone marrow or tonsils of healthy individuals (Dhyani, Duarte *et al*. 2012). ANKHD1 protein expression has not been examined in haematological malignancies.

I designed a study protocol to allow examination of ANKHD1 expression in blood cells in individuals with haematological malignancies and healthy individuals.

3.2 Results

3.2.1 Patient characteristics

Five groups of patients were included in the study:

- Individuals under the care of haematology for a bone marrow biopsy or care for a haematological malignancy, who provided consent for an additional sample to be taken for the study (denoted by prefix B, summarised in Table 3-1).
- A 55 year old lady undergoing apheresis as a sibling donor (patient S1) who gave permission for the use of apheresis waste.
- De-identified samples from presumed healthy individuals (denoted by prefix N). These samples were supplied by the diagnostic haematology laboratory from material that would otherwise be discarded as waste. Samples were selected from individuals attending an outpatient clinic which was not haematology or oncology, who had a normal full blood count. Five samples were examined. Three individuals were female, and two were male. Mean age was 52, range 28 – 65.

- De-identified samples from individuals undergoing apheresis (denoted by prefix CD34, summarised in Table 3-2)
- De-identified samples from individuals with a haematological malignancy (denoted by prefix A, summarised in Table 3-3).

Clinical information for the de-identified samples is limited by the ethics permission granted to access these samples.

Table 3-1. Individuals who consented to provide an additional sample for research (details removed from library copy of thesis to protect patient anonymity).

Patient number	**Gender**	**Age**	**Diagnosis**	**Reason for sample**	**Marrow (M) or blood (B)**
B2					M
B3					M
B4					M
B5					M
B6					M
B8					B
B9					B
B10					B

Table 3-2. Individuals whose samples were analysed for ANKHD1 expression in CD34+ cells.

Patient number	**Gender**	**Age**	**Indication**
CD34 1	F	70	Autologous
CD34 2	F	67	Autologous
CD34 3	F	30	Unknown
CD34 4	F	30	Unknown
CD34 5	F	36	Unknown
CD34 6	F	50	Unknown

Table 3-3. Patient characteristics of the individuals whose de-identified blood or marrow samples were analysed for ANKHD1 expression in haematological malignancies.

Patient number	**Gender**	**Age**	**Diagnosis**	**Marrow (M) or blood (B)**
A1	F	37	AML	M
A2	F	66	Acute leukaemia	B
A3	M	81	Acute leukaemia	B
A4	65	65	AML, relapsed	M
A5	M	90	AML	M
A6	M	50	ALL (B lymphocytic)	M
A7	M	76	Myelodysplastic syndrome with CD34+ cells in blood	M
A8	M	63	MF with circulating CD34+ cells in blood	B

3.2.2 Quantitative real time PCR

mRNA of full-length ANKHD1 can be detected in bone marrow mononuclear cells (BMMCs). BMMCs for this stage of the study were extracted from bone-marrow aspirate from patients with a range of haematological malignancies undergoing bone marrow biopsy for remission monitoring or prior to stem cell transplantation. The level of mRNA expression (relative to β actin mRNA) is about one third to two thirds of that found in HeLa cells (Figure 3-1 A). This is consistent with a previous study showing higher ANKHD1 mRNA expression in cell lines than PBMCs (Traina, Favaro *et al*. 2006).

3.2.3 Western Blotting

Having established that mRNA for ANKHD1 could be detected in BMMCs, I went on to determine whether ANKHD1 protein could be detected in protein extracts from these cells, and cells from other patients. No full length ANKHD1 was detected in protein extracts from patient cells (Figure 3-1 B). In some cases actin was not detected in the protein extract, suggesting a problem with the method for sample preparation. However, even in the samples where actin was readily detected no 269kDa protein corresponding to full length ANKHD1 was seen. In some cases

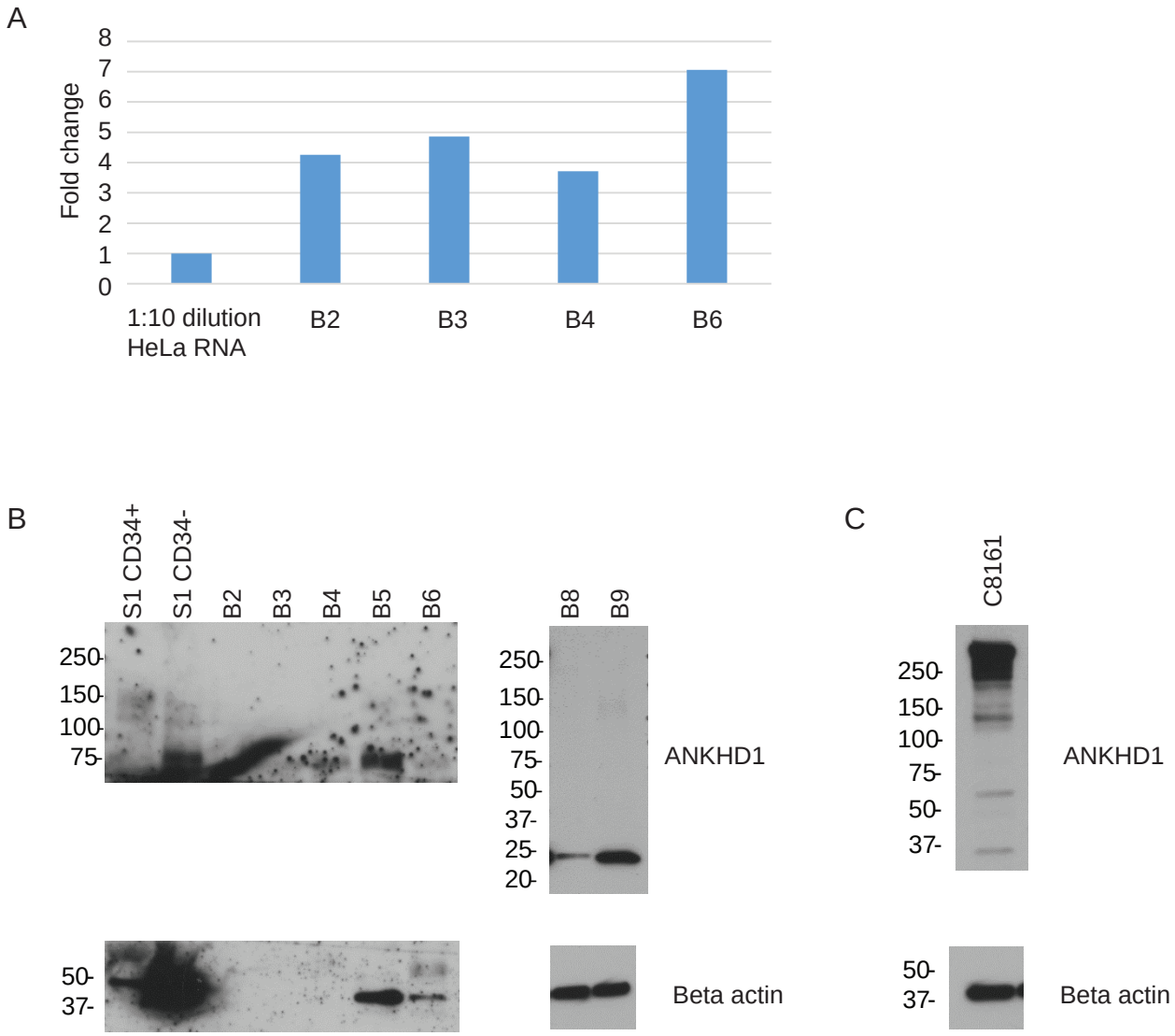


Figure 3-1. Detection of ANKHD1 in PBMCs and BMMCs. (A) ANKHD1 mRNA can be detected in BMMCs, but levels are lower than those in HeLa cells. Actin was used as a reference gene, and quantification performed using the delta delta ct method. (B) No protein of the molecular weight corresponding to full-length ANKHD1 (269kDa) can be detected in BMMCs and PBMCs using western blotting, although bands are seen at approximately 75, 100 and 150 kDa. See section 3.2.1 for details of the patients from whom these samples were obtained. C Full length ANKHD1 can be detected in primary cells from a patient with plasma cell leukaemia grown in culture using western blotting.

bands corresponding to proteins of lower molecular weight (approximately 150kDa, 100kDa, 75kDa and 25kDa) were seen. It is not clear if these correspond to shorter isoforms of ANKHD1 or if they are indicative of non-specific antibody binding. Some erythrocytes may have been retained in the BMMC separation procedure, and would contribute to the actin band.

It is possible that the failure to detect ANKHD1 in PBMC and BMMC samples is because of the lack of sensitivity of western blotting and the signal-to-noise ratio. BMMCs or PBMCs comprise a heterogeneous group of cells, and ANKHD1 could be expressed only in a sub-group of normal cells. Furthermore, ANKHD1 may be expressed only in certain malignancies but not in the corresponding normal cell type. The patients for whom bone marrow aspirate was examined at this stage of the study were clinically well and had their bone marrow biopsies taken for remission monitoring or prior to stem cell transplantation. The peripheral blood samples were from patients who had AML and circulating blast cells, but the percentage of blasts was relatively low. It is possible that in all these patients ANKHD1 was present in the malignant cells but not detected because the malignant cells were rare relative to other cells in the population.

To address this and examine whether ANKHD1 is expressed in malignant cells, primary cells growing in culture from a patient with plasma cell leukaemia were examined. Full length ANKHD1 was detected in these cells using western blotting (Figure 3-1 C.)

To further examine whether ANKHD1 was expressed only in particular sub-populations of normal or malignant blood cells flow cytometry and immunofluorescence microscopy methods were developed to examine ANKHD1 expression.

3.2.4 ANKHD1 expression in normal blood cells

Flow cytometry

ANKHD1 was is found in monocytes and lymphocytes, but absent from neutrophils, a result which was consistent across all five individuals (Figure 3-2).

Immunofluorescence

Immunofluorescent staining of blood films corroborated these results (Figure 3-3). Immunofluorescence showed qualitatively similar results across all five individuals

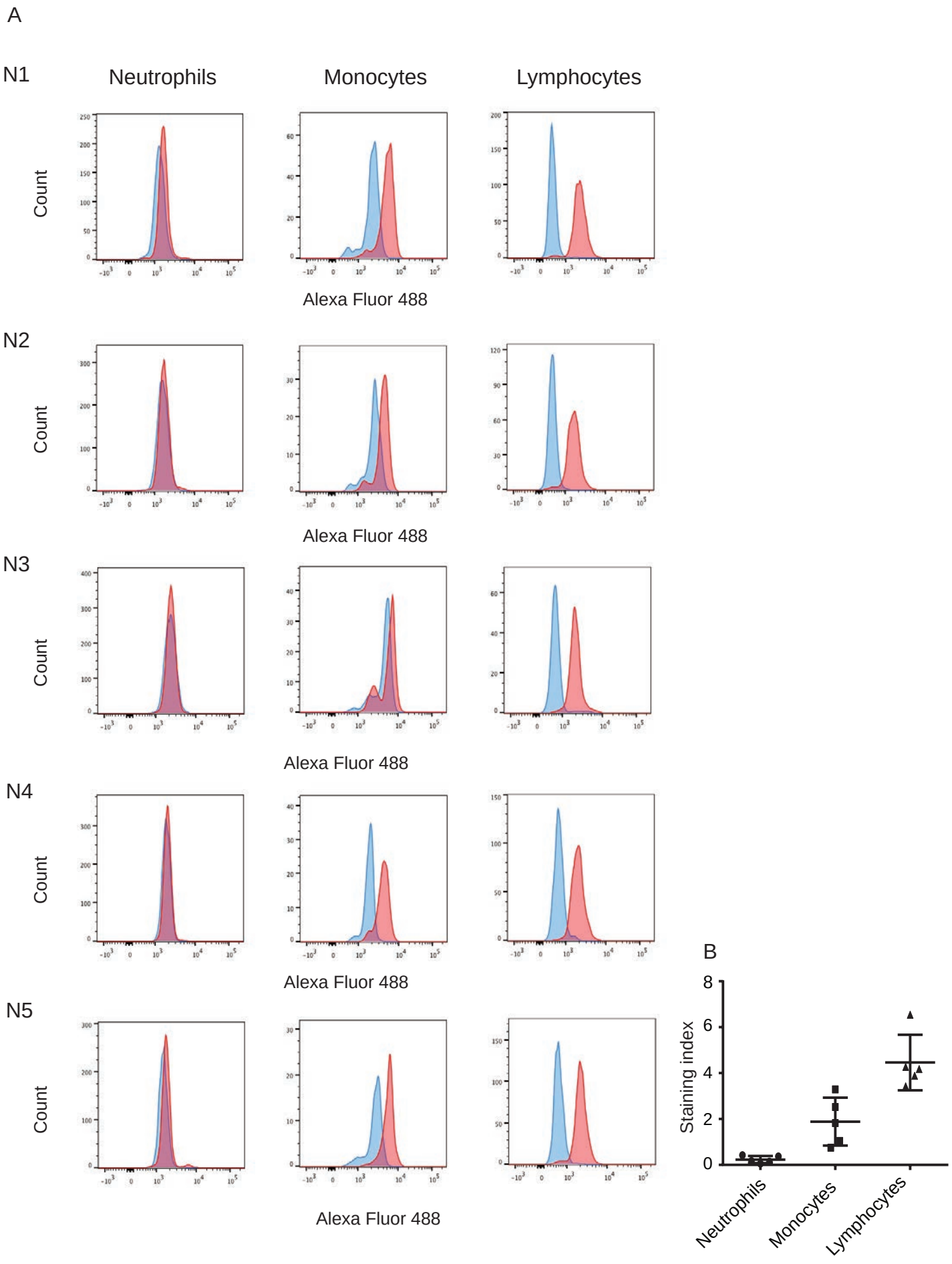


Figure 3-2. Flow cytometry shows ANKHD1 is absent from neutrophils but expressed in lymphocytes and monocytes. A Histograms show Alexa Fluor 488 fluorescence in the presence of anti-ANKHD1 primary antibody (red) or IgG isotype control primary antibody (blue). B Staining index for each cell type. Lines show mean and standard deviation

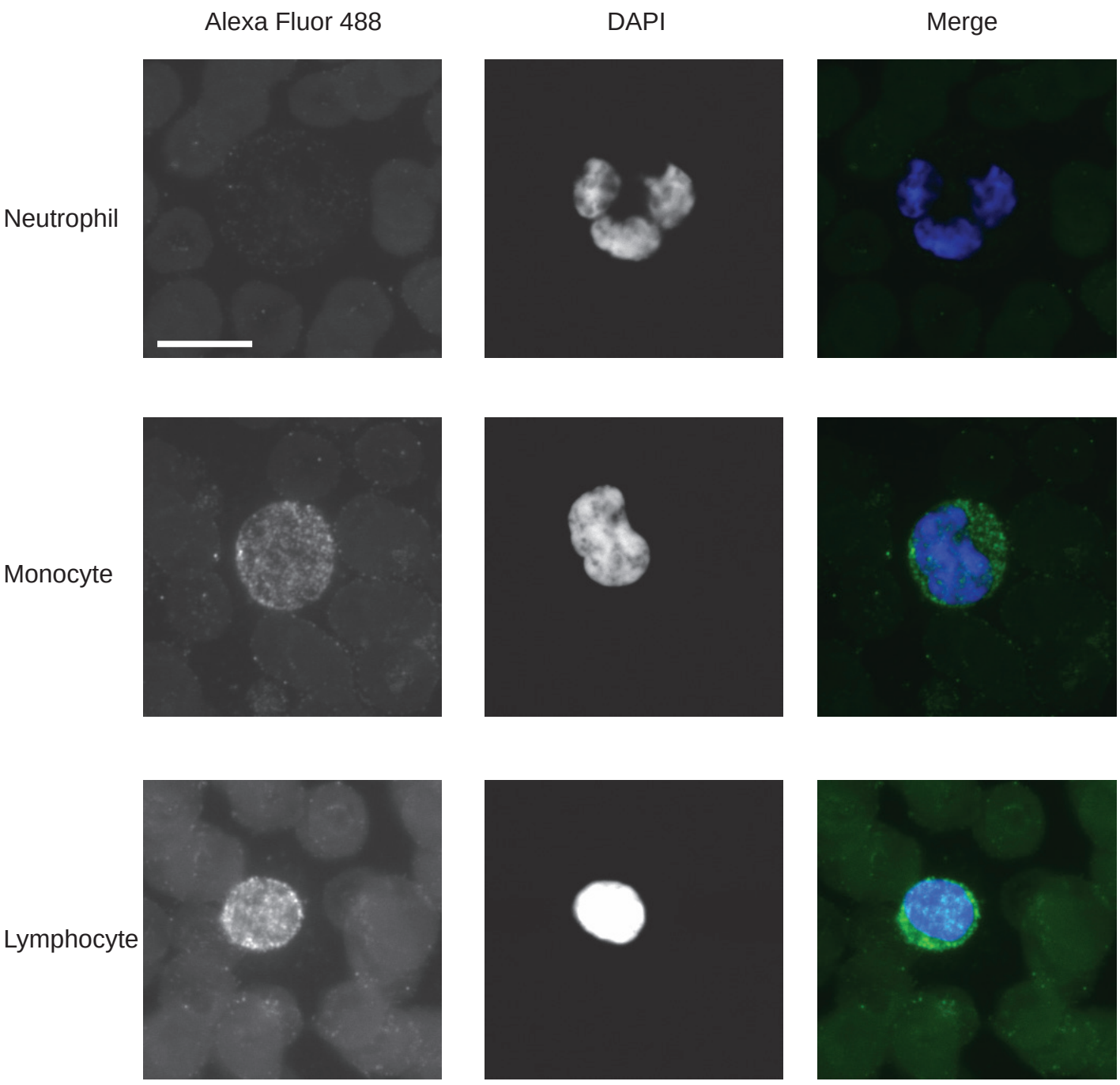


Figure 3-3. Immunofluorescence shows ANKHD1 is absent from neutrophils but found in monocytes and lymphocytes. No staining was seen with secondary antibody only (data not shown). Scale bar 10uM.

(data not shown). For the immunostaining, cell type was determined by examining nuclear morphology using DAPI staining. The characteristic nuclei of neutrophils enabled identification, but distinction between monocytes and lymphocytes, particularly activated lymphocytes, may have been less reliable.

3.2.5 ANKHD1 in haematopoietic stem cells

To consider whether ANKHD1 may play a role in blood cell development, I wished to determine the expression of ANKHD1 in haematopoietic stem and progenitor cells (HSCs). These cells are found within the population of cells in the blood and bone marrow expressing the cell-surface glycoprotein CD34, although CD34 is also found on epithelial cells.

Peripheral blood from individuals undergoing stem cell harvest for an autologous or allogeneic transplant was used to examine ANKHD1 expression in CD34+ cells. There was variation between individuals in the ANKKHD1 expression in CD34+ cells, with the cells of some individuals showing an increase in fluorescence when stained with anti-ANKHD1, and others showing only a small difference in fluorescence intensity compared to isotype control Figure 3-4). This may have been related to variations between experimental runs, as well as variations between individuals.

3.2.6 ANKHD1 in haematological malignancies

ANKHD1 expression was examined in the malignant clone of cells in haematological malignancies using flow cytometry. The samples examined represented a combination of samples from individuals who were recruited to the study and provided an additional sample specifically for the study, and samples obtained from the diagnostic haematology laboratory as de-identified samples. The diseases examined depended upon the samples which were being analysed by the diagnostic laboratory. Most samples were from individuals with acute leukaemia. However, the group also includes a sample from an individual with myelofibrosis and an individual with myelodysplasia, both of whom had high counts of circulating CD34+ cells.

In all samples from patients with acute leukaemia in whom blasts were identified, the median fluorescence intensity of the ANKHD1-stained blasts was

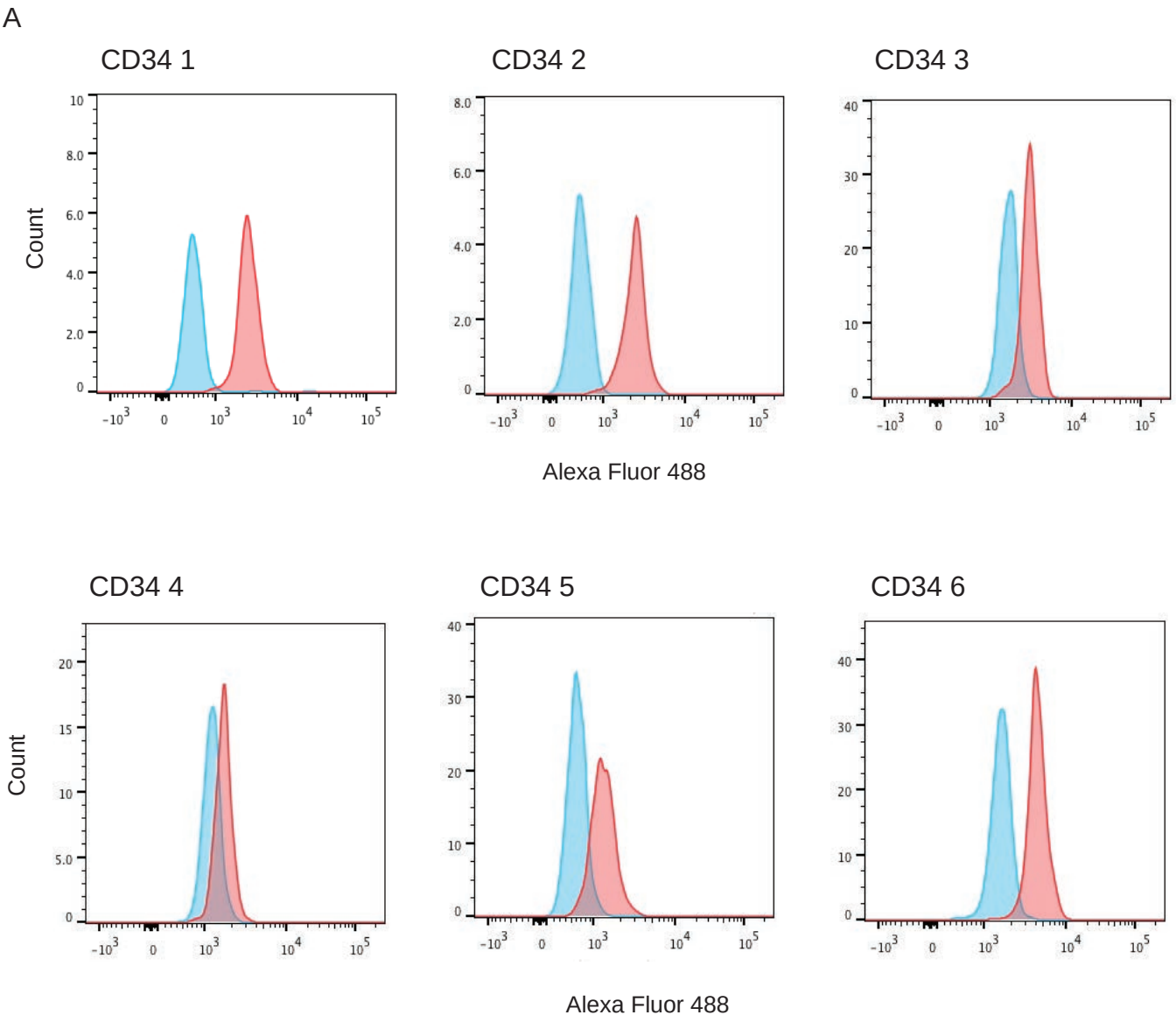


Figure 3-4. ANKHD1 is expressed in CD34+ cells in individuals undergoing harvests for stem cell transplants, but there is variation between individuals. A Histogams showing fluorescence with ANKHD1 primary antibody (red) compared to IgG isotype control antibody (blue). B Staining index for the six samples shown, mean and standard deviation.

higher than that of isotype-stained blasts (Figure 3-5). Immunofluorescence on a blood film from one patient showed blasts that expressed ANKHD1 (Figure 3-6).

For the two individuals with high counts of circulating CD34+ cells, ANKHD1 expression was examined in the CD34+ cells. ANKHD1 expression in the CD34+ cells in the individual with PMF was low, comparable to that in the cells from the individuals undergoing apheresis at the lower end of the range of ANKHD1 expression (Figure 3-7 A). For the individual with myelodysplasia and circulating CD34+ cells, the CD34+ population appeared to contain two sub-populations of cells, one of which expressed ANKHD1 and one of which was apparently ANKHD1 negative (Figure 3-7 B). However, in the IgG-isotype-stained cells from the same sample a similar bimodal pattern of fluorescence was seen and there was considerable overlap between the cell populations.

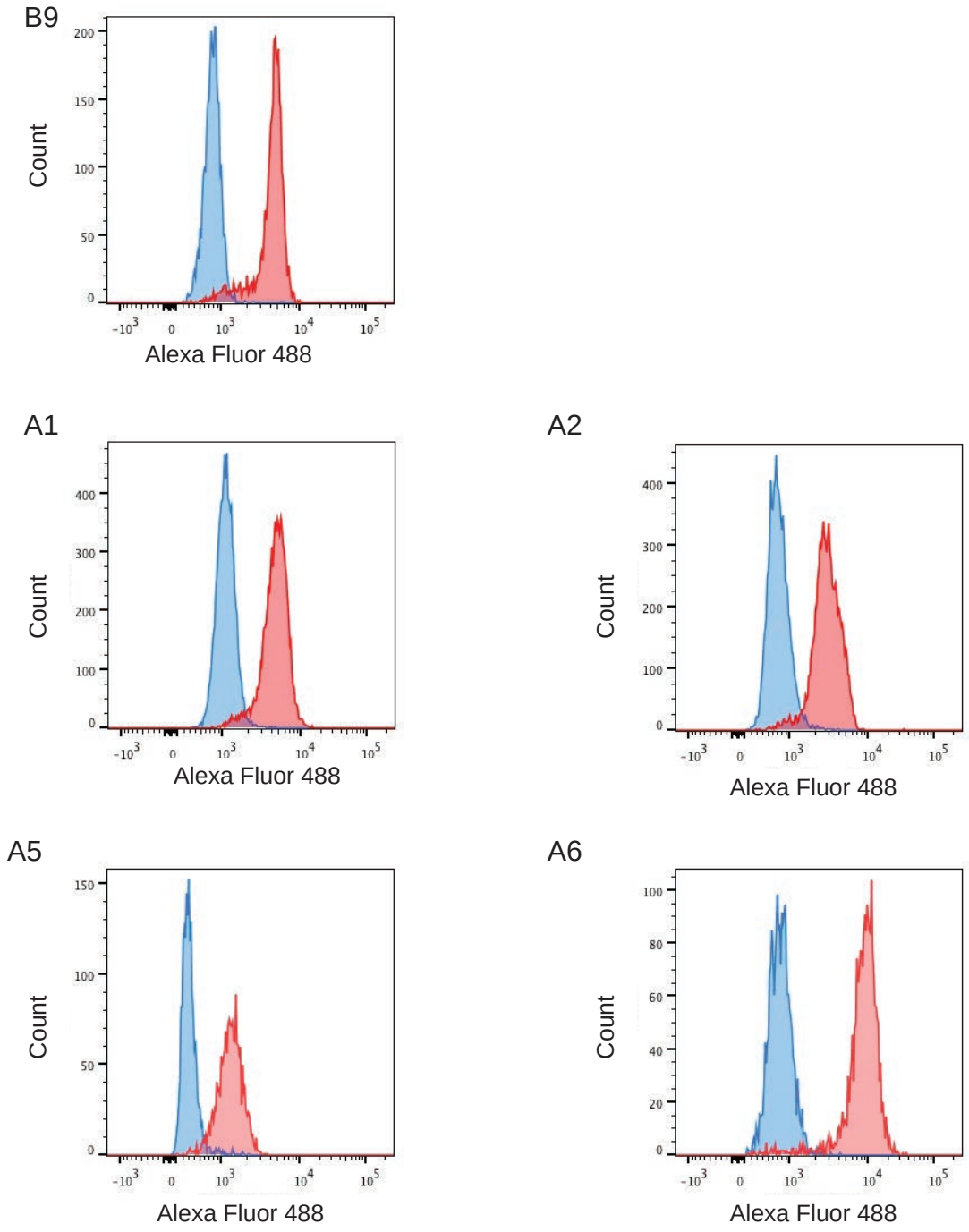
3.2.7 Summary of ANKHD1 expression in blood cells

The ANKHD1 staining indices of all the normal and malignant cell populations examined are summarised in Figure 3-8. It is notable that ANKHD1 expression in blasts from patients with acute leukaemia is higher than other cell types. Comparison of these populations (excluding the MF and MPN groups, which each included only one individual) showed a significant difference between them ($P = 0.0004$, one way ANOVA). The groups between which there were significant differences were acute leukaemia blasts and neutrophils ($P \leq 0.001$), acute leukaemia blasts and monocytes ($P \leq 0.01$) and acute leukaemia blasts and CD34+ HSCs ($P \leq 0.05$) (Tukey's multiple comparisons test).

3.3 Discussion

ANKHD1 mRNA expression is increased in acute leukaemia and myeloma, and in laboratory models contributes to the malignant behaviour of cancer cells. In this study I have established the expression of ANKHD1 protein in normal blood cells and investigated its expression in haematological malignancies.

A



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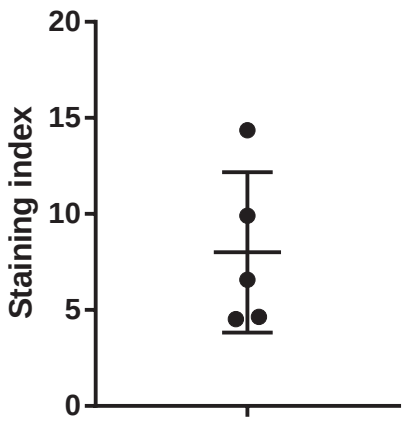


Figure 3-5 ANKHD1 protein is expressed in blast cells in patients with acute leukaemia. A Histograms showing the difference in fluorescence intensity in cells stained for ANKHD1 (red) or with IgG isotype control (blue). B Staining index for the five samples showing mean and standard deviation.

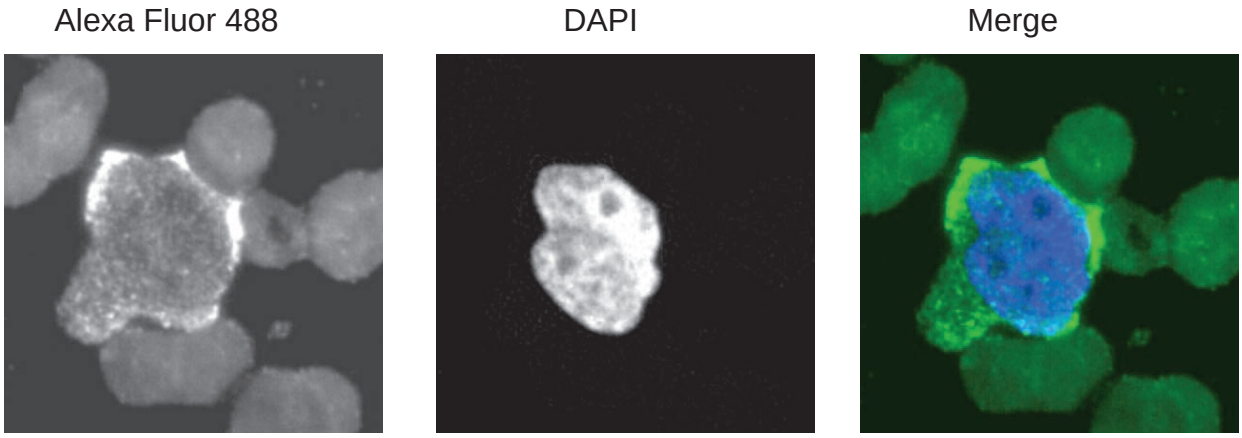
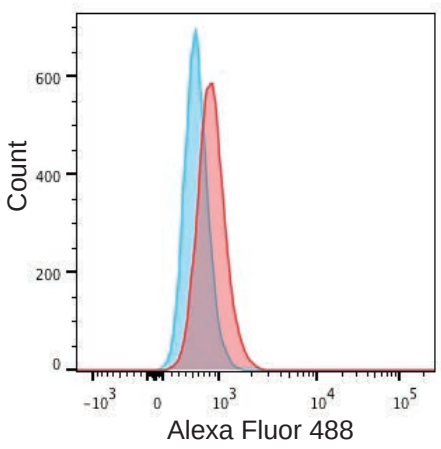


Figure 3-6. Immunofluorescence shows that ANKHD1 is expressed in blast cells in the peripheral blood of one patient with AML (patient B8).

A



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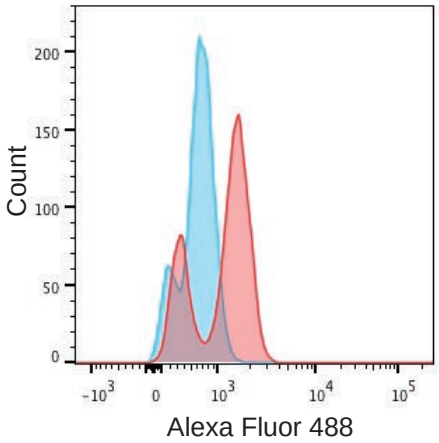


Figure 3-7. ANKHD1 expression in CD34+ cells from two patients with high CD34+ counts due to haematological malignancies. A Fluorescence of CD34+ cells from a patient with MF (Patient A8) stained with an anti-ANKHD1 antibody (red) is comparable to that of cells stained with polyclonal IgG isotype antibody (blue). The staining index is 0.76 and the fold change in median fluorescence intensity is 1.6, comparable to the CD34+ cells from patients undergoing apheresis at the lower end of the range of ANKHD1 expression. B Fluorescence of CD34+ cells from a patient with MDS (Patient A7) stained with anti-ANKHD1 antibody (red) appear to contain two sub-populations of cells, with different levels of ANKHD1 expression. Staining with IgG isotype (blue) shows a similar bimodal pattern of fluorescence, and there is considerable overlap between the fluorescence of the ANKHD1-stained and isotype-stained populations.

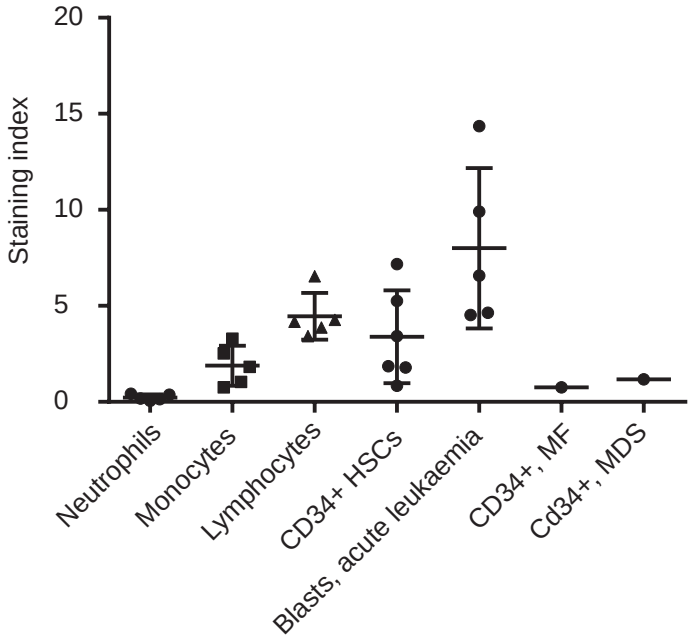


Figure 3-8. Summary of ANKHD1 staining indices for the different normal and malignant blood cell types examined using flow cytometry. Lines show mean and standard deviation.

3.3.1 Assessment of ANKHD1 expression with qPCR and western blotting, and modification of original experimental design

qPCR suggests that ANKHD1 is expressed in bone marrow at the mRNA level, albeit at about one tenth of the level seen in HeLa cells. No ANKHD protein was detected in BMMCs and PBMCs using western blotting, but it was highly expressed in primary cells derived from a patient with plasma cell leukaemia. It is possible that these differences were due to the amount of protein loaded. These results suggested that a technique that allowed assessment of individual cells in a heterogeneous population would be more informative, so a flow cytometry protocol was developed.

I had problems recruiting adequate numbers of patients. Specifically, it was difficult to obtain an additional sample of blood or marrow from patients at a time when the malignant clone of cells was likely to be large enough to be detected using western blotting. It was a requirement of the ethics permission that potential study participants had at least 24 hours to consider study participation. Most individuals undergoing bone-marrow biopsies as outpatients, who received study information in the post, were having their biopsies for remission monitoring or prior to transplant, so would be anticipated to have very low numbers of malignant cells. Many inpatients had their diagnosis made in a district hospital before being referred to Sheffield. For individuals undergoing a bone marrow biopsy in Sheffield, often there was insufficient time for participants to consider the study.

The development of flow cytometry as an assay for ANKHD1 expression partially overcame problems of patient recruitment as sufficient data could be gathered from small sample volumes (100µ1) using flow cytometry. This meant that additional bone marrow and blood draws were not necessary as samples which were surplus to requirements for clinical testing that would otherwise be discarded as waste could be used. Furthermore, if samples were de-identified by NHS laboratory staff it is considered ethically acceptable for such surplus ‘waste’ samples to be used for research without obtaining specific patient consent. I obtained approval from the Research Ethics Committee for a substantial amendment to my study that enabled the use of surplus de-identified samples from the NHS haematology lab, increasing the number of available patients.

3.3.2 Flow cytometry as an approach to examine ANKHD1 expression

I developed a method to examine ANKHD1 expression in normal blood cells and in haematological malignancies using flow cytometry. This technique was chosen as it enabled analysis of single cells, including rare populations, in part by utilising the expertise of the Sheffield Haematology-Oncology Diagnostic Service.

I encountered challenges developing a flow cytometry assay for ANKHD1 as no fluorescently-conjugated antibodies against ANKHD1 are commercially available. I used an approach employing a fluorescently labelled secondary antibody, and a polyclonal IgG as an isotype control. Although there are arguments against using an isotype control in flow cytometry, its use can be justified here. As samples were gathered prospectively and analysed in several batches, the isotype provides some control for variations in experimental runs, although the use of saved instrument settings and daily quality control of the cytometer in the diagnostic lab should have also minimised this. The protein concentration of both antibodies was known, so the same concentration could be used for test and control. The use of a secondary antibody reduces problems with fluorophore ratio that can occur when conjugated test and isotype antibodies are used. ANKHD1 is an intracellular antigen necessitating a permeabilisation step. The use of an isotype should have controlled for nonspecific retention of antibody within cells. When examining blood cells, antibody binding to Fc receptors is relevant. The isotype ought to control for this, although an Fc-blocking reagent could have been used as an alternative.

It has been difficult to choose an appropriate statistical analysis for the flow cytometry data. Other published work has shown superimposed histograms for isotype and test antibodies, or used the fold change in fluorescence intensity for comparison. However, I have been unable to find literature indicating what statistical test to use to determine whether the fold change is significant. Choice of statistical tests ought to take account of the non-parametric data produced by flow cytometry. The resolution index and staining index are two statistics used in flow cytometry to compare test and control antibodies, and they carry the advantage over consideration of fold changes in fluorescence intensity that the spread of the data is considered (Hulspas, O'Gorman *et al*. 2009). However, I have not been able to find information

on how to interpret these values to determine whether a difference is significant. In this work, the reduction in fluorescence intensity when ANKHD1 is knocked down in cultured cells, and the apparent lack of ANKHD1 in neutrophils acting as an internal negative control, must be used as an indicator of the validity of the approach.

3.3.3 ANKHD1 expression in normal peripheral blood cells

This work adds to our knowledge of ANKHD1 expression in blood cells. Previous studies of ANKHD1 in blood cells have been limited to mRNA. The only study to examine ANKHD1 protein failed to show ANKHD1 in PBMCs using western blotting, and demonstrated immunofluorescence only of PBMCs rather than specific cell types.

Based on my work, ANKHD1 appears to be expressed in lymphocytes and monocytes, but absent from neutrophils. This variation in expression suggests ANKHD1 function may relate to aspects of the behaviour of monocytes and lymphocytes which is not required in neutrophils. These results are consistent across samples from five patients, but a larger sample size would give a more reliable assessment of ANKHD1 expression.

ANKHD1 expression in erythrocytes was not examined using flow cytometry, with a red-cell-lysis step being included in the flow cytometry protocol. Some assessment of ANKHD1 in erythrocytes can be made from the immunofluorescence of blood films. The fluorescence of red cells stained with ANKHD1 did not seem qualitatively different to that in films with secondary antibody only. It did look as though some bright puncta of antibody staining were present at the periphery of the cells (Figure 3-3), but as there was not an IgG control for these slides it is not clear whether this is an artefact.

It would be interesting to perform a more detailed examination of ANKHD1 expression in sub-types of blood cells. Flow cytometry would be a suitable approach for this since antibodies against CD markers can be used to define cell types. In particular, examining variations in ANKHD1 expression amongst lymphocyte sub-types would be interesting. Both the flow cytometry data here, and immunohistochemical examination of ANKHD1 in tonsil performed whilst optimising antibody staining for melanoma tissues suggest that a small proportion of lymphocytes are ANKHD1 negative.

3.3.4 ANKHD1 in haematopoietic stem cells and haematological malignancies

Previous studies of ANKHD1 expression in haematological malignancies have used real-time PCR to examine mRNA expression. This work corroborates published data that showed expression of ANKHD1 mRNA in acute leukaemias by demonstrating that ANKHD1 protein is expressed in blasts in these patients. The limited clinical data available on the patients from whom these samples were obtained means comparison cannot be made with the corresponding normal myeloid or lymphoid lineage. This work also adds to our knowledge of ANKHD1 expression in myeloma, which had previously been examined using qPCR and in cell lines.

This work suggests that ANKHD1 is expressed in haematopoietic stem cells, although there is some apparent variation between individuals. Examination of samples from a much larger group is needed to establish this. Furthermore, some of the differences observed may be due to variations related to the experimental run, rather than variations between individuals. A caveat to my examination is that the cells I examined are from individuals who received G-CSF to mobilise stem cells. The stem cells mobilised by G-CSF differ slightly from bone-marrow resident stem cells, for example they are skewed towards the myeloid lineage. It should also be noted that G-CSF is a JAK/STAT pathway ligand, although I was not examining JAK/STAT activation in these cells. Examination of CD34+ stem cells from normal bone marrow would be informative here. Bone marrow considered to be near to normal, for example marrow obtained during orthopaedic surgery or normal bone marrow biopsies performed for disease staging in individuals with lymphoma might be easier to obtain.

The data on ANKHD1 expression in CD34+ cells in individuals with malignant diseases affecting the CD34+ compartment is interesting preliminary data, although a larger number of individuals must be examined to draw any conclusions. CD34+ cells from an MPN patient do not appear to have higher expression of ANKHD1 than normal CD34+ cells, suggesting it may not be involved in pathogenesis. The apparent presence of a population of CD34+ cells lacking ANHKD1 expression in an individual with myelodysplasia appeared notable, until the isotype control indicated that this was a feature of antibody binding, not specific

to the ANKHD1 antibody. ANKHD1 expression in myelodysplasia would nonetheless be an interesting avenue for future investigation. In some patients, myelodysplasia is associated with a deletion of the long arm of chromosome 5, the chromosomal location of ANKHD1. Furthermore, altered RNA metabolism appears to play a role in the pathogenesis of myelodysplasia, as mutations affecting components of the splicing machinery are common and altered ribosome biology is implicated in 5q syndrome. The KH domain in ANKHD1, a domain which binds single-stranded nucleic acids, suggests ANKHD1 may be involved in RNA metabolism.

3.4 Conclusions

This work is the first to examine ANKHD1 protein expression in sub-types of cells in peripheral blood, suggesting that it is expressed in lymphocytes and monocytes but absent from neutrophils. ANKHD1 appears to be expressed in haematopoietic stem cells, and in the malignant clone of cells in several haematological malignancies. Further work in well-defined cohorts of patients is needed to confirm these findings. It remains to be determined what cellular functions ANKHD1 performs in these cells, whether it affects intracellular signalling, and whether ANKHD1 expression carries any prognostic information for patients with haematological malignancies.

Chapter 4: ANKHD1 in melanoma

4.1 Introduction

Malignant melanoma is a malignant neoplasm of melanocytes, neural-crest-derived cells. Cutaneous primary tumours are the most common, although melanoma can also arise in other sites where melanocytes are present, for example ocular melanoma and mucosal melanomas. UV exposure is an important aetiological factor. Melanoma is the fifth commonest cancer in the UK, with an incidence of around 13,000 new cases per year. 10 year survival is about 90%, but this varies according to tumour stage. For patients with melanoma at an early stage, 5 year survival is comparable to matched populations. For those with melanoma that has metastasised beyond local lymph nodes, however, 5 year survival is 8% in men and 25% in women (CancerResearch-UK).

Primary melanoma is treated by surgical excision, and patients monitored for recurrence or metastasis. Patients with metastatic melanoma are treated with BRAF inhibitors and checkpoint inhibitors. At present, for patients who have had surgical excision of a primary melanoma there is no adjuvant treatment to reduce the risk of recurrence. However, BRAF inhibitors and checkpoint inhibitors are now being investigated in this setting. To guide investigation of adjuvant treatments, methods to identify patients at risk of recurrence are needed. Tumour thickness is the most important predictor of recurrence risk. It is reported in terms of the Breslow thickness, measured from the granular layer of the epidermis to the deepest point of tumour invasion. Histological features such as lymphovascular invasion, mitotic rate and the briskness of the tumour infiltrating lymphocyte response may add to this. However, better approaches to predict recurrence risk are needed.

Histopathology of melanoma

Melanoma can be classified into several histological sub-types, the major groups being superficial spreading melanoma, lentigo maligna melanoma, nodular melanoma and acral lentiginous melanoma. Superficial spreading melanoma is the commonest histological sub-type. It is found in young and middle-aged patients, on skin sites that are intermittently sun exposed. Lentigo maligna melanoma classically occurs in elderly patients, in skin sites which have been persistently exposed to

sunlight. Nodular melanoma occurs in middle age and is commonest on the trunk and limbs. Acral lentiginous melanomas are those on the palms, soles and around the nails. Rarer sub-types of melanoma include desmoplastic, naevoid and spizoid melanomas. The histopathological diagnosis of these melanoma types can be challenging.

An important histological feature in the assessment of melanoma is the Breslow thickness. This is related to the growth phase of the melanoma. In the radial, or horizontal, growth phase malignant cells are found only in the epidermis and superficial dermis. Melanoma cells at this stage are thought to lack the potential to metastasise. As the tumour progresses the properties of the cells change and they acquire the capacity to invade. In the vertical growth phase melanoma cells are found deeper in the dermis, where they proliferate and form an expanding tumour mass. Nodular melanoma consists entirely of tumour in the vertical growth phase, whereas the other common sub-types may consist of only radial growth phase, or have components of radial growth phase and vertical growth phase (McKee 2005, Underwood and Cross 2013, Elder 2015).

Signalling pathways in melanoma

Aberrant activation of intracellular signalling pathways plays a role in disease pathogenesis in melanoma. Targeting signalling via the Ras/Raf/MEK/ERK signalling pathway with BRAF inhibitors, and more recently combining a BRAF inhibitor and MEK inhibitor, has shown some success as a therapeutic strategy in patients with metastatic melanoma (Chapman, Hauschild *et al*. 2011, Long, Stroyakovskiy *et al*. 2014). However, development of resistance to these therapies prevents responses from being sustained. Further understanding of the mechanisms leading to activation and regulation of Ras/Raf/MEK/ERK signalling, and other intracellular signalling pathways activated in melanoma, should lead to improved treatment options for patients with melanoma. Strategies that use molecular approaches to classify disease and help to refine prediction of prognosis and treatment response may contribute to this.

Other signalling pathways activated in melanoma include JAK/STAT signalling and Hippo signalling. The presence of nuclear pSTAT3 is associated with increased risk of recurrence and death in patients with melanoma, and suppressing

STAT3 activation leads to apoptosis in melanoma cell lines (Messina, Yu *et al*. 2008, Bill, Fuchs *et al*. 2010). Nuclear localization of the transcriptional regulators YAP and TAZ, effectors of the Hippo pathway, has been described in some melanoma tissue samples. Cell and mouse models suggest YAP and TAZ promote tumour invasiveness in melanoma. The upstream signaling events that cause this nuclear localization in melanoma have not yet been identified. Interestingly, nuclear localization of YAP and TAZ is also observed in some benign naevi (Nallet-Staub, Marsaud *et al*. 2014).

Investigation of ANKHKD1 in melanoma

ANKHD1 has been identified as a positive regulator of JAK/STAT, Ras/Raf/MEK/ERK and Hippo signalling in *Drosophila* (Smith, Carroll *et al*. 2002, Müller, Kuttenkeuler *et al*. 2005, Sidor, Brain *et al*. 2013). ANKHD1 has also been shown to positively regulate JAK/STAT and Hippo signalling in human cancer cell lines (Müller 2012, Sansores-Garcia, Atkins *et al*. 2013). ANKHD1 promotes progression through the cell cycle, and suppresses expression and activity of the CDK inhibitor p21 (Dhyani, Machado-Neto *et al*. 2015).

As ANKHD1is a modulator of several intracellular signaling pathways known to be involved in melanoma, and promotes aspects of the malignant phenotype in other types of cancer cells, I hypothesized that ANKHD1 might play a role in melanoma. Little is currently known about ANKHD1 expression in melanoma. The human protein atlas shows immunohistochemistry for ANKHD1 for twelve samples of melanoma, three of which are metastatic, in a tissue microarray. Further information about histological sub-types is not available, however (Uhlen, Oksvold *et al*. 2010). To determine whether further investigation of ANKHD1 in melanoma would be informative, I wished to profile ANKHD1 expression in melanoma. I examined the expression of ANKHD1 and proteins in the signaling pathways with which it interacts in two melanoma cell lines. I then examined ANKHD1 expression in an *in vitro* model of melanoma invasion in engineered skin. Following these initial experiments in cell lines, ANKHD1 protein expression and localization in surgically excised melanoma tissue samples from 34 patients was investigated.

4.2 Results

4.2.1 ANKHD1 expression in melanoma cell lines

To investigate ANKHD1 expression in melanoma cells two cell lines, representing an invasive (C8161) and a less invasive (HBL) melanoma phenotype were examined.

Western blotting shows that ANKHD1 is expressed in both cell lines, and its expression can be knocked down using siRNA targeting ANKHD1 mRNA. The C8161 cell line shows a greater reduction in ANKHD1 protein levels following knockdown than the HBL line (Figure 4-1A). Cellular localisation of ANKHD1 in these two cell lines was examined using immunofluorescence. In both cell lines ANKHD1 was found in the cytoplasm and the nucleus, but in HBL cells it was found predominantly in the cytoplasm, whereas C8161 cells showed a more even distribution between nucleus and cytoplasm, and there is accentuation around the nucleus (Figure 4-1B).

4.2.2 Expression of signalling pathway proteins in melanoma cell lines

Proteins in signalling pathways modulated by ANKHD1 were also examined in these cell lines. Western blotting shows that both cell lines express JAK2, STAT1 and STAT3 (Figure 4-2A). However, no constitutive phosphorylation of these JAK/STAT pathway components could be detected with western blotting (data not shown).

YAP is seen in both cell lines, but in HBL cells it is predominantly located in the nucleus whereas in C8161 cells there is no prominent localisation of YAP to the cytoplasm (Figure 4-2B). It is difficult to make comparisons between the two cell lines as in these images the C8161 cells are more confluent than HBL cells. Contact with neighbouring cells is known to cause loss of nuclear localisation of YAP.

Western blotting shows that p21 can be detected in HBL cells, but appears to be absent from C8161 cells (Figure 4-3 A). Immunofluorescence to examine p21 in HBL cells shows it to be located in the nucleus and expression levels to vary between cells, as would be expected for a protein involved in cell-cycle regulation (Figure 4-3 B).

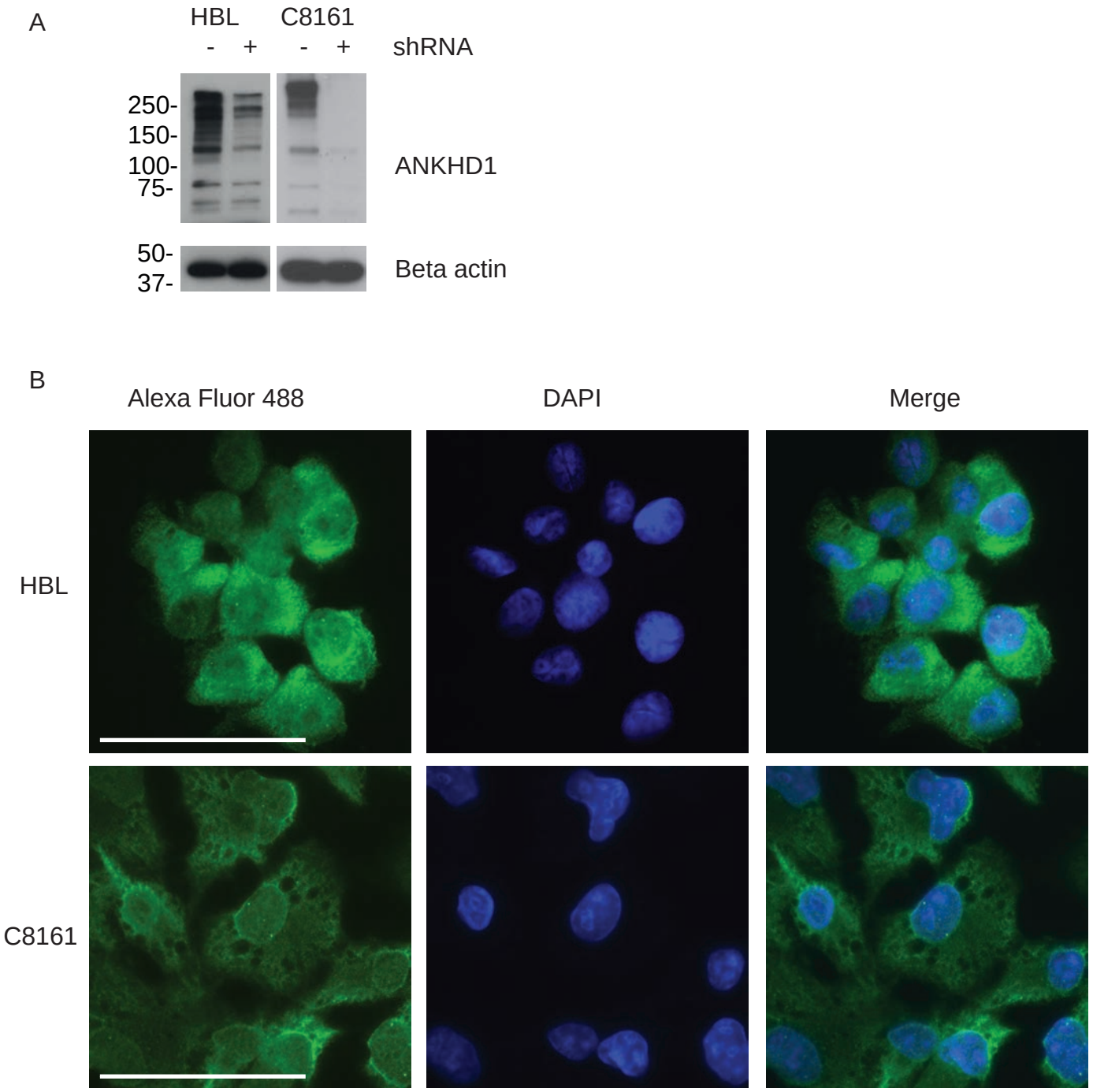
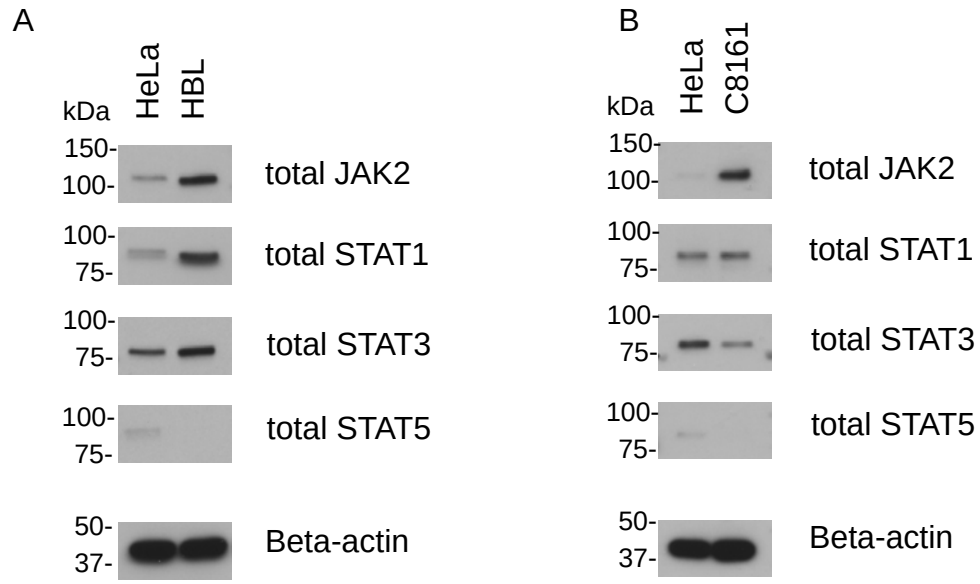


Figure 4-1. Examination of ANKHD1 expression in melanoma cell lines. (A) Western blotting shows that ANKHD1 is expressed in the melanoma cell lines HBL and C8161. ANKHD1 expression can be knocked down with shRNA targetting ANKHD1. Comparison is shown with cells treated with a non-target shRNA. A greater reduction in protein expression is seen in C8181 cells compared to HBL cells. (B) Immunofluorescence shows that in HBL cells ANKHD1 is found predominantly in the cytoplasm, but is also in the nucleus. In C8161 cells there is a more even distribution of ANKHD1 between the nucleus and cytoplasm, with some accentuation of the nuclear membrane. Green Alexa Fluor 488 anti-rabbit secondary antibody, anti ANKHD1 primary antibody. Blue DAPI. Scale bar 50µM.



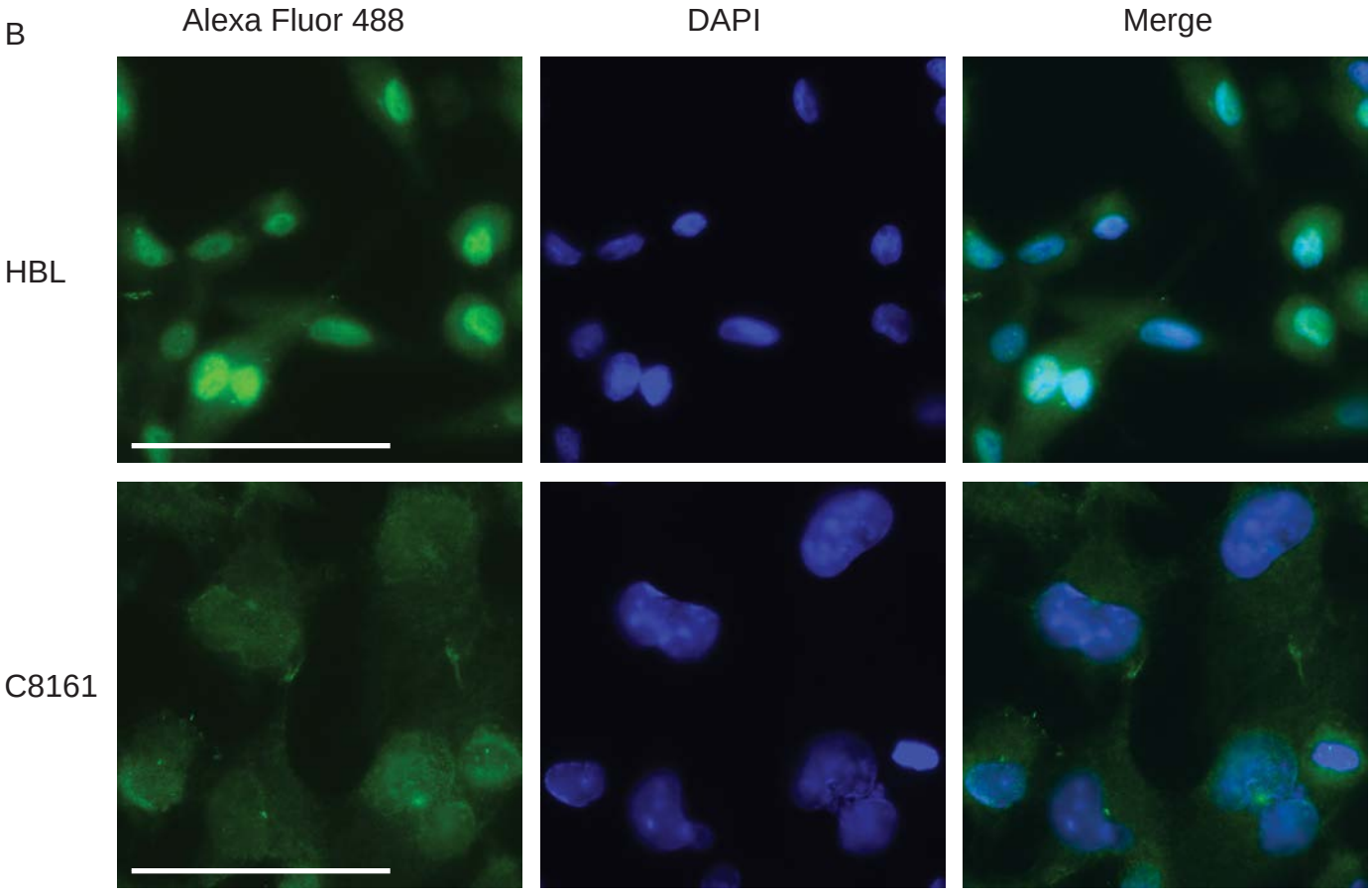


Figure 4-2. Expression of signalling pathway proteins in melanoma cell lines. Western blotting shows that the melanoma cell lines HBL and C8161 express JAK2 and STATs 1 and 3. Lysate from HeLa cells is loaded as a positive control. B Immunofluorescence shows that the Hippo pathway effector YAP is expressed in melanoma cell lines. Nuclear localisation of YAP is seen in HBL cells. There is no prominent nuclear localisation of YAP in C8161 cells, although in these images the C8161 cells are more confluent than the HBL cells. Green anti-rabbit Alexa Fluor 488 conjugated secondary antibody, anti-YAP primary antibody. Blue DAPI. Scale bar 50µm.

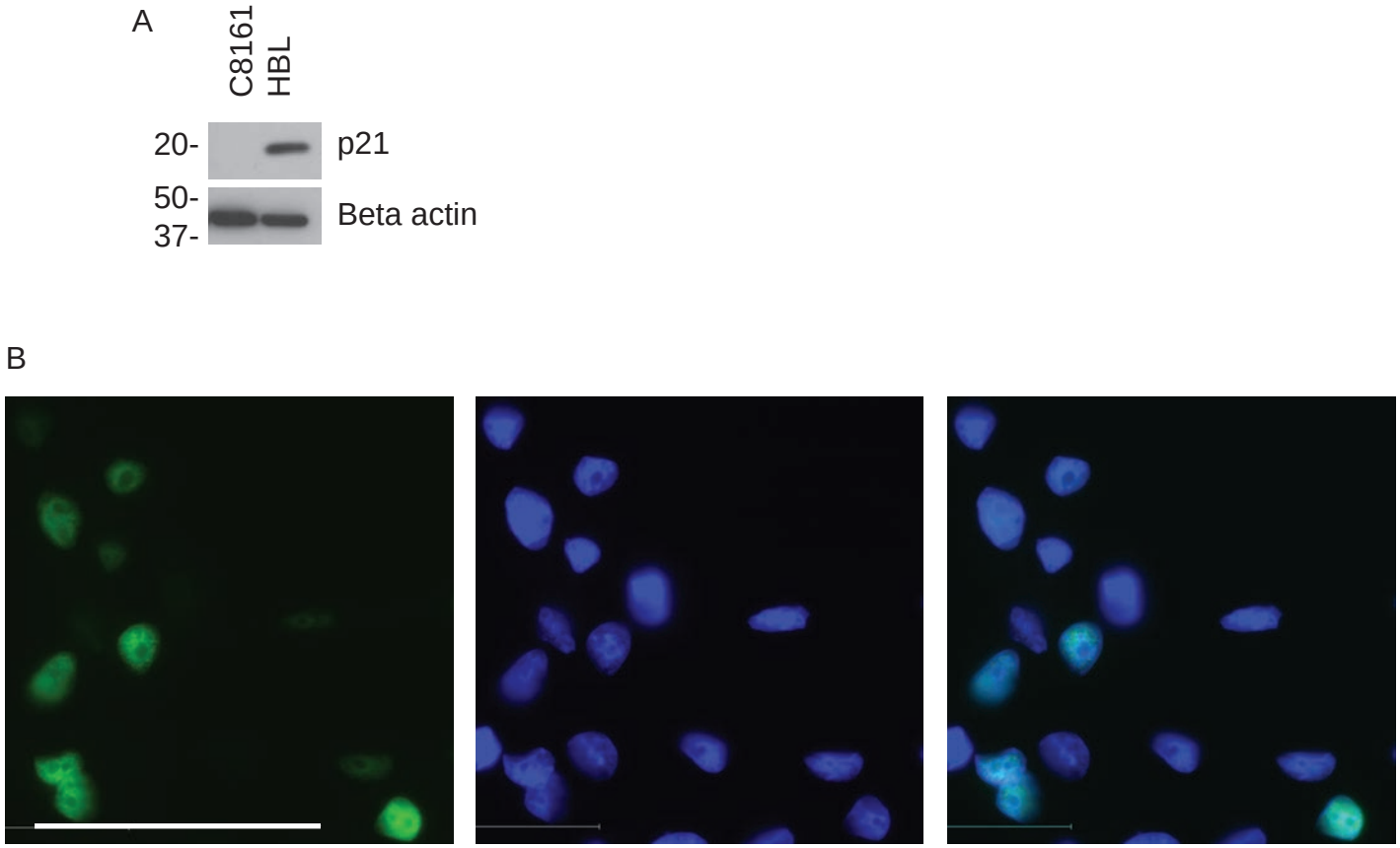


Figure 4-3 Examination of p21 protein expression in melanoma cell lines. (A) Western blotting shows that p21 can be detected in HBL cells but not C8161 cells. (B) Immunofluorescence shows p21 localised in the nucleus of HBL cells. Expression varies between cells, as would be expected of a cell-cycle protein. Green anti-rabbit Alexa Fluor 488 conjugated secondary antibody, anti p21 primary antibody. Blue Dapi. Scale bar 50µM.

4.2.3 ANKHD1 expression in invasive melanoma in engineered skin

There are limitations to the inferences about cell behaviour that can be made from examining cell lines growing in a monolayer in tissue culture vessels. To gain a better understanding of ANKHD1 expression and localisation in melanoma, samples of engineered skin containing invading cells from the melanoma cell line C8161 were examined (see section 2.1.1 for details of tissue engineered skin). Formalin-fixed paraffin-embedded samples were examined using immunohistochemistry. In this context, C8161 cells show ANKHD1 expression in the nucleus and cytoplasm, with stronger expression in the nucleus. Keratinocytes also show ANKHD1 expression, with stronger expression in the nucleus than the cytoplasm (Figure 4-4).

4.2.4 Patient Characteristics and histological features

Having established that ANKHD1 is expressed in two melanoma cell lines, I went on to examine ANKHD1 expression in surgically excised melanoma tissue samples from 34 patients. All patients had cutaneous melanoma. Patient characteristics are as follows:

Table 4-1. Patient characteristics of patients from whom melanoma samples were obtained

Age	Mean	60 years
	Range	24 to 86 years
	Standard deviation	16 years
Site	Arm	7
	Back	6
	Chest	2
	Face or head	7
	Leg	10
	Lymph node metastasis	1
	Lung metastasis	1
Histological sub-type	Desmoplastic	1
	Lentigo maligna	2
	Nevoid	3
	Nodular	3
	Nodular/spizoid	1
	Superficial spreading	20

	Metastatic	4
Growth phase (excluding metastatic)	Radial	16
	Vertical	14
Thickness (mm) (excluding metastatic)	Mean	1.4
	Standard deviation	2.3
	Range	0-11.5

4.2.4 Immunohistochemistry and tumour histological features

ANKHD1 expression and localisation was examined by visualising sites of anti-ANKHD1 antibody binding using diaminobenzidine (DAB), a brown stain. Melanin was rarely prominent in the sections, and where present could be distinguished from DAB by its granular distribution. Furthermore, each sample was compared to an adjacent section prepared with a polyclonal IgG rabbit antibody which also enabled consideration of nonspecific antibody binding and endogenous peroxidase activity. The cellular location and intensity of staining was assessed.

Slides were examined by Dr. Nick Tiffin, a consultant Dermatopathologist at the Royal Hallamshire Hospital. Histological features were assessed on a haematoxylin and eosin stained section. In addition to the histological features described above, an assessment was also made of other histological features which influence prognosis: mitotic rate, the presence of perineural and vascular invasion, the presence of microscopic satellites, whether regression had occurred, whether there was ulceration, and the tumour infiltrating lymphocyte response. The mitotic rate is the total number of mitotic figures in $1mm^2$ in the region of the tumour where they are most abundant. Higher mitotic rate is associated with worse prognosis. Vascular invasion, with tumour cells present within blood vessels, in the walls of vessels or around the exterior of vessels is associated with reduced survival. Perineural invasion is associated with increased risk of recurrence, although this may be partly attributable to its prevalence in desmoplastic melanoma, which carries a worse prognosis than some other histological variants. Microscopic satellites are nests of tumour cells separate from the main tumour mass and deep to the thickest region of the tumour. The presence of microscopic satellites is associated with increased risk of recurrence and reduced survival. Regression refers to the presence of areas within the tumour which have been replaced by fibrous tissue. It is

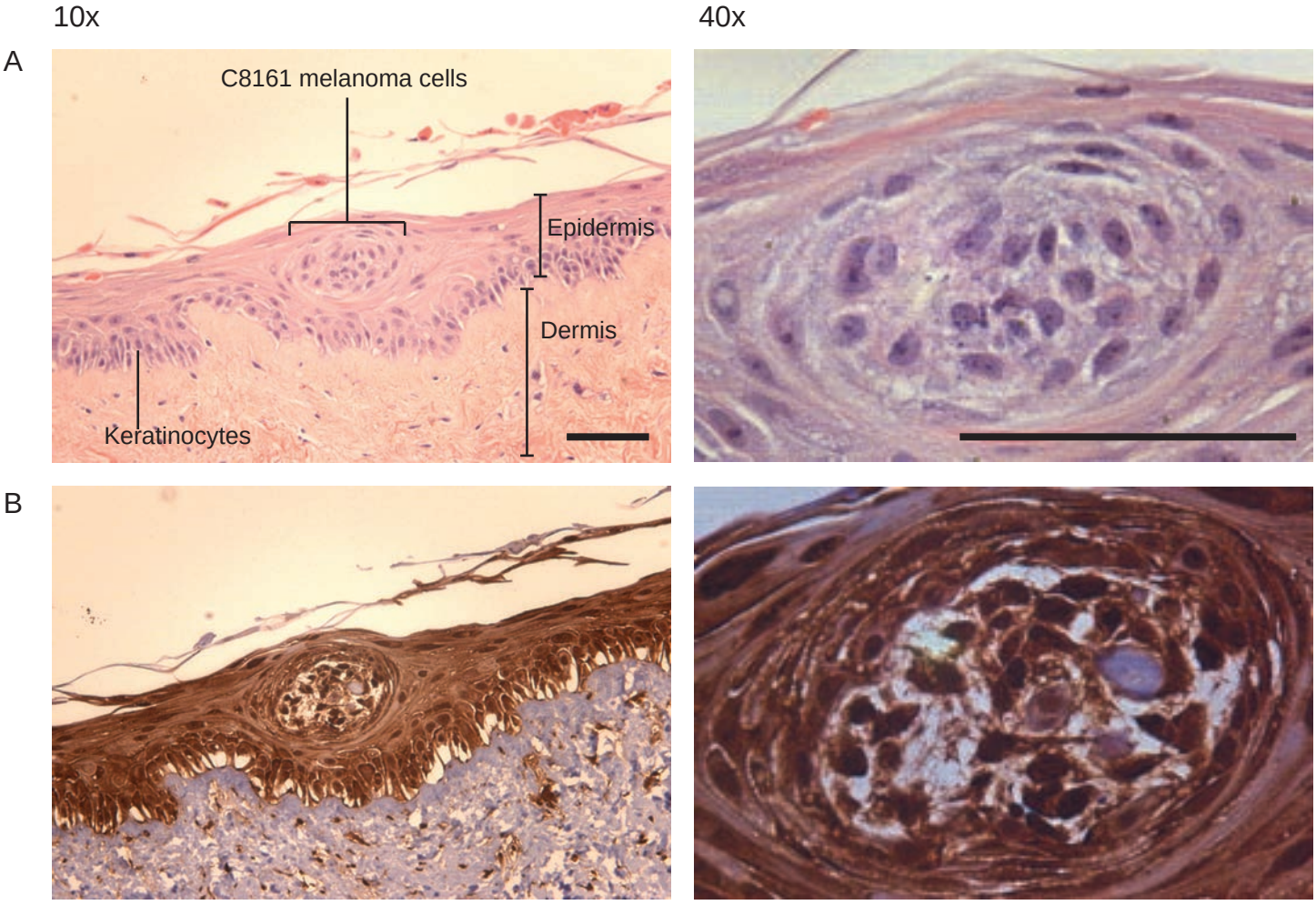


Figure 4-4. Cells of the melanoma cell line C8161 invading into tissue engineered skin show cytoplasmic and nuclear staining of ANKHD, with moderate to strong nuclear staining. Keratinocytes show a similar expression pattern. (A) H&E stained section. (B) Immunohistochemistry for ANKHD1 in adjacent section. Scale bar 100µm.

associated with worse prognosis, possibly because it is an indicator of previous tumour thickness. Ulceration, where the continuity of the epithelium over the tumour is lost, is associated with increased risk of metastasis. Tumour infiltrating lymphocytes (TIL), defined as lymphocytes which infiltrate the tumour and are in contact with the melanoma cells, carry prognostic significance. A brisk TIL response is associated with better survival than non-brisk or absent TIL (McKee 2005, Elder 2015).

4.2.5 ANKHD1 staining patterns

Qualitative assessment of staining patterns in histological sub-types

Superficial spreading melanoma is the commonest histological type at the population level and in the samples in this study. The melanoma cells in superficial spreading melanoma in radial growth phase often showed moderate or strong nuclear staining (Figure 4-5). Superficial spreading melanoma in vertical growth phase appeared to have a different staining pattern, with relatively low nuclear and cytoplasmic ANKHD1 (Figure 4-6). In superficial spreading melanoma where intra-epidermal cells were prominent these generally had stronger nuclear staining than the corresponding dermal tumour cells in the same patient. The three samples of nodular melanoma showed relatively low levels of nuclear and cytoplasmic ANKHD1 expression, similar to that in vertical growth phase superficial spreading melanoma (Figure 4-7). Four samples of metastatic melanoma were available although one of these samples consisted entirely of necrotic tumour and did not contain enough cells for analysis. The samples of metastatic melanoma show a similar pattern of ANKHD1 expression to that seen in nodular melanoma, with relatively low nuclear and cytoplasmic expression (Figure 4-8). Qualitatively the examination of ANKHD1 staining suggested that thinner melanomas and intra-epithelial melanoma cells showed more nuclear staining for ANKHD1 than thicker melanomas.

Quantitative assessment of staining patterns in histological sub-types

To make a quantitative assessment of ANKHD1 expression a scoring system was devised. Nuclear staining and cytoplasmic staining were assessed separately, and each was categorised as strong, moderate, weak or absent. Examples of these categories of staining are shown in Figure 4-9. In each slide the category of nuclear

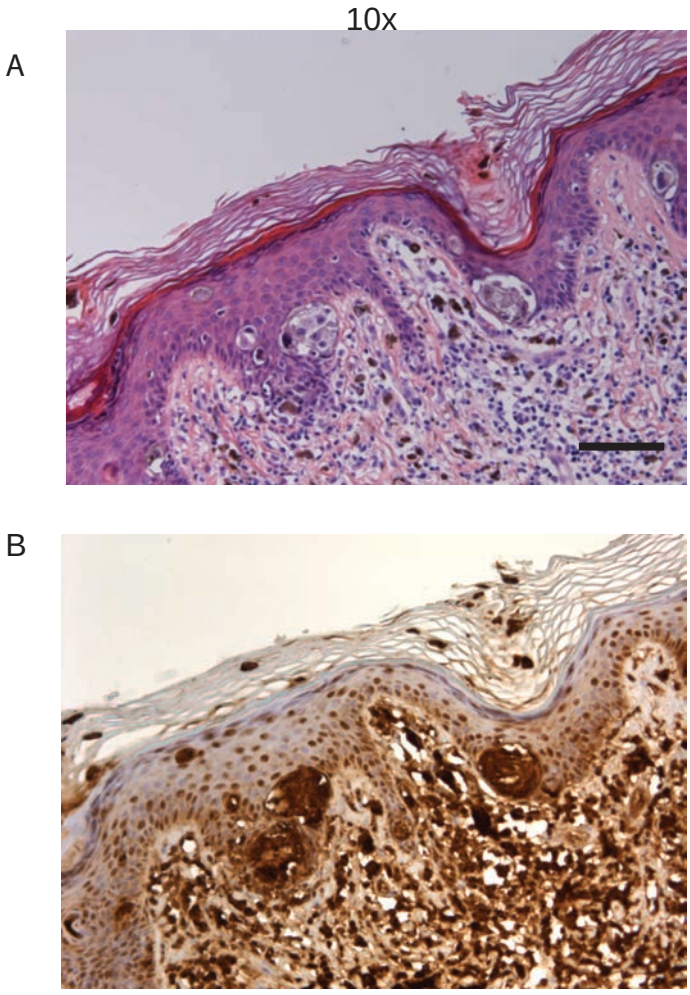
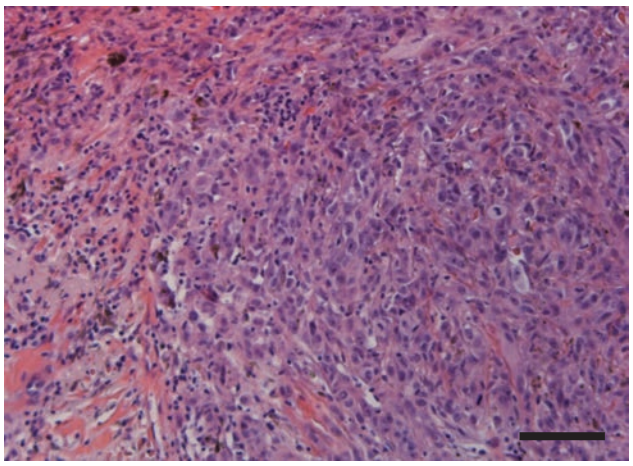


Figure 4-5. Superficial Spreading Melanoma in radial growth phase showing some cells with strong nuclear staining. Melanoma thickness in this specimen was 0.6mm. A H&E stained section. B Immunohistochemistry for ANKHD1 in adjacent section. Scale bar 100µM.

10x

A



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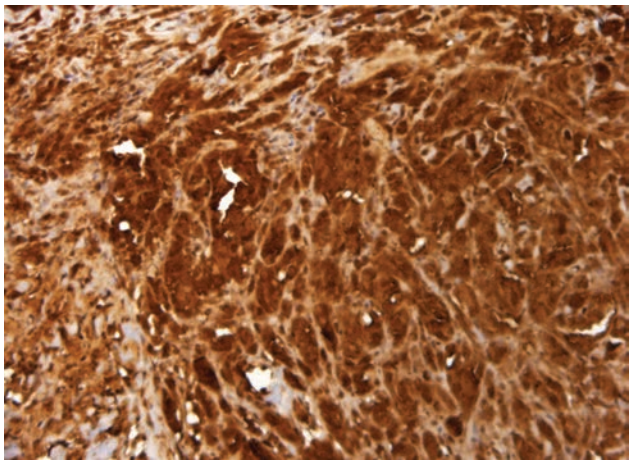


Figure 4-6. Superficial Spreading melanoma in vertical growth phase, showing mostly weak nuclear staining for ANKHD1 and moderate and weak cytoplasmic staining. Melanoma thickness in this specimen was 1.1mm. A. H&E stained section B. Immunohistochemistry for ANKHD1 in adjacent section. Scale bar 100µm.

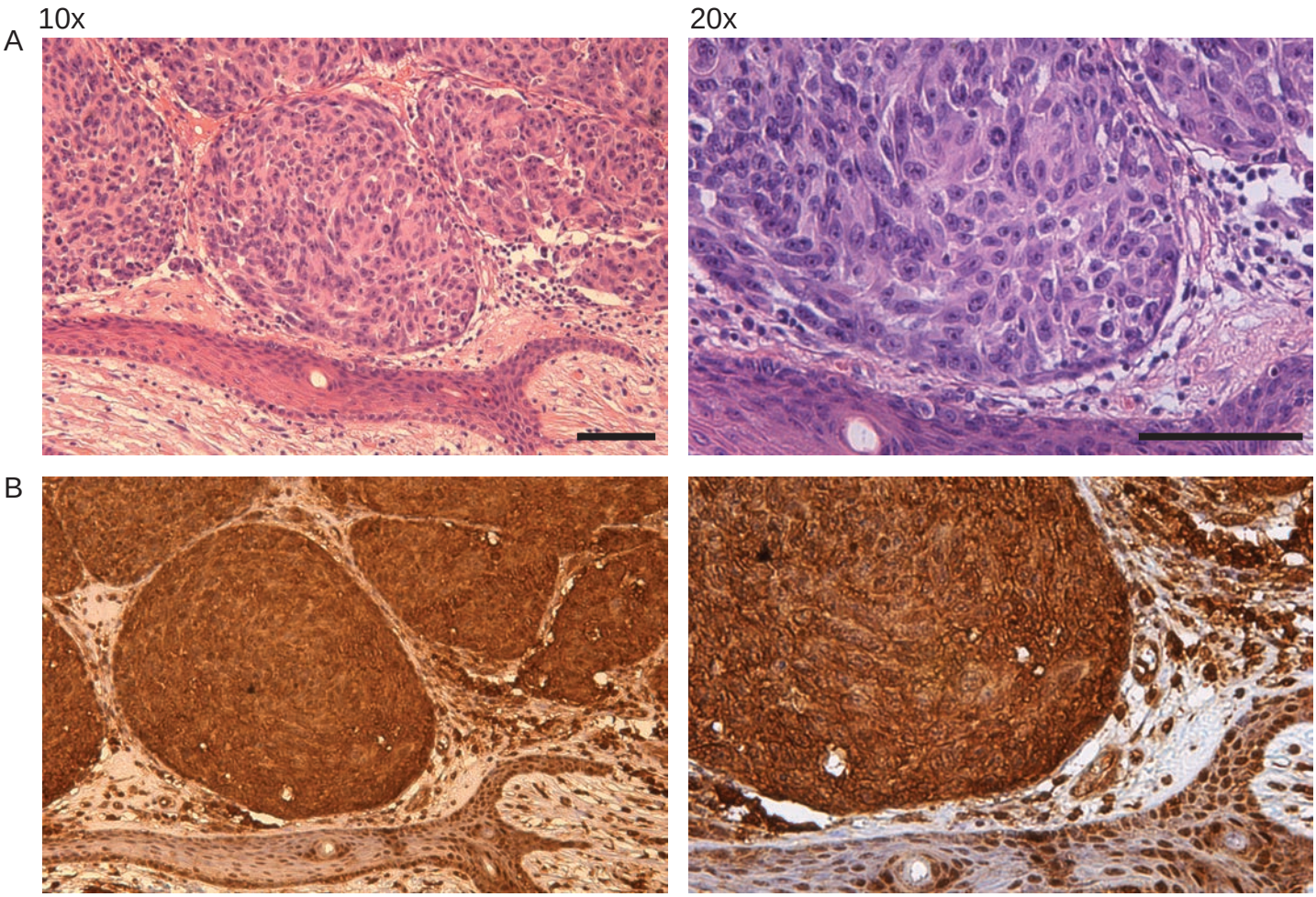


Figure 4-7. Nodular melanoma shows uniform weak cytoplasmic staining for ANKHD1 and uniform weak nuclear staining, note the visibility of the nuclear counterstain in many cells. This specimen was a thick melanoma, 7mm thick. a H&E stained section, b immunohistochemistry for ANKHD1 in adjacent section. Scale bar 100µm.

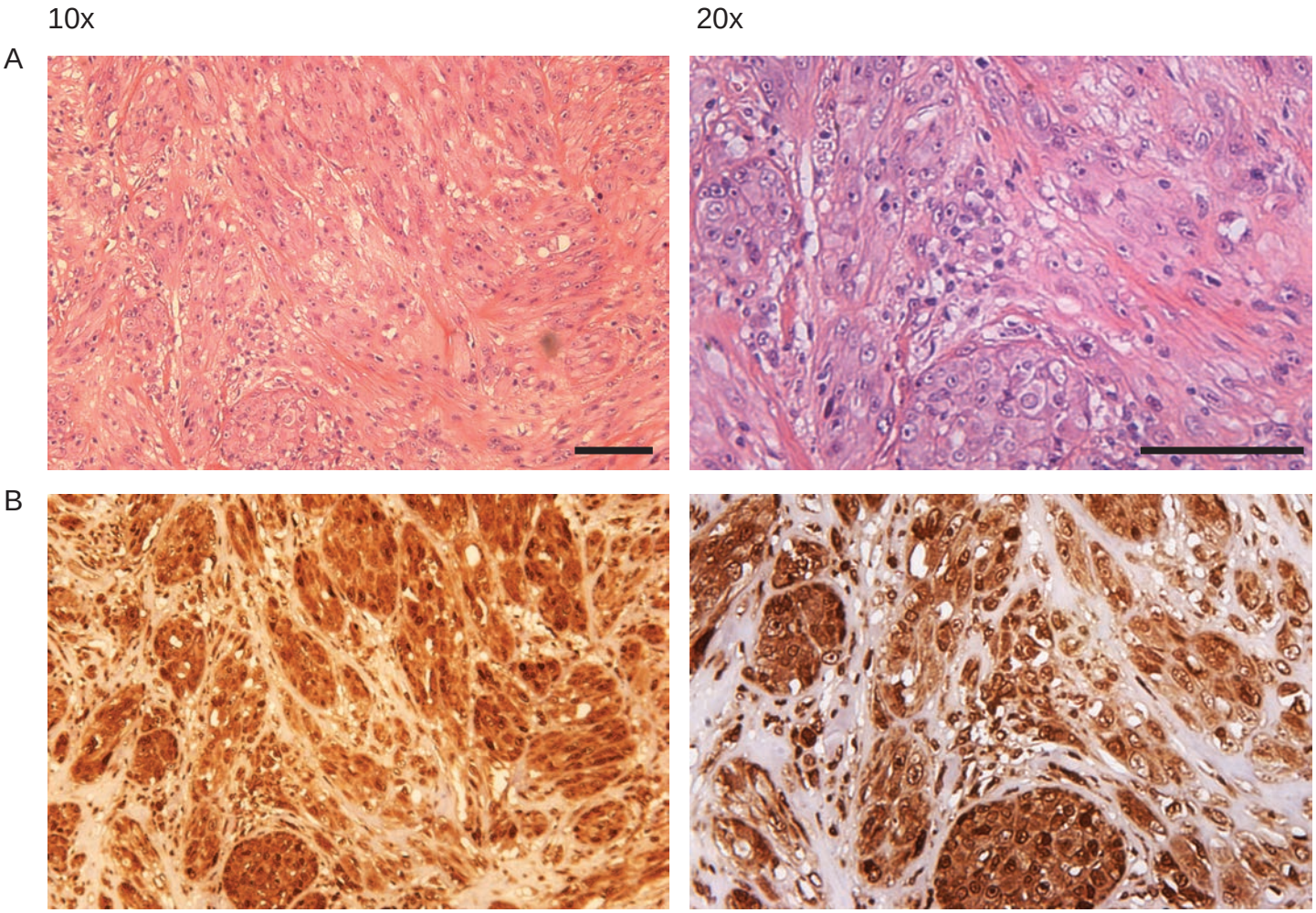


Figure 4-8. Metastatic melanoma shows uniform cytoplasmic staining for ANKHD1 and variable nuclear staining. A H&E stained section, B immunohistochemistry for ANKHD1 in adjacent section. Scale bar 100µm.

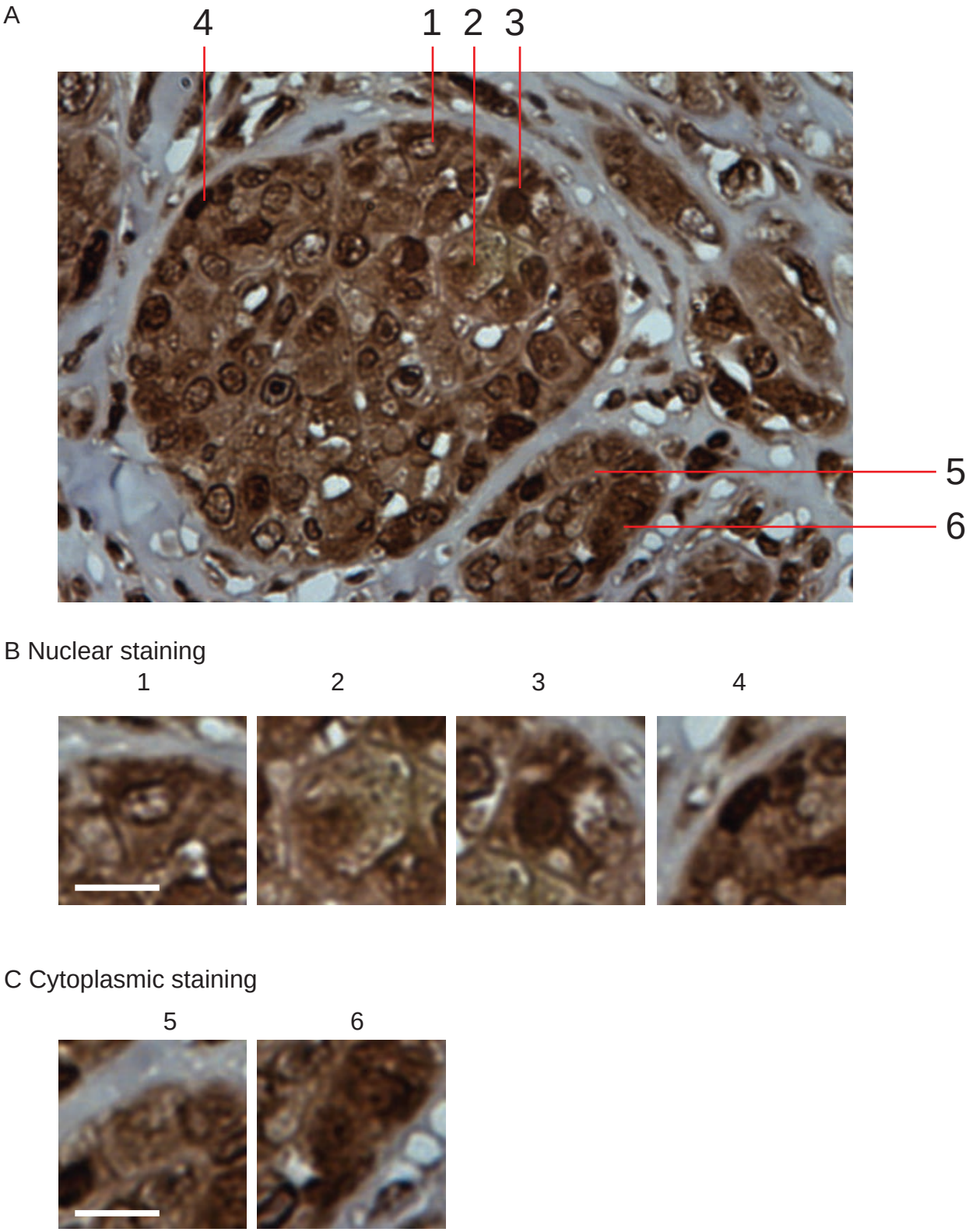


Figure 4-9. Four categories of staining intensity were assigned to the nucleus and cytoplasm, most of which are shown in this 40x magnification image from an individual with metastatic melanoma. Scale bar 10µM. A Whole image. B Expanded images of cells with examples of nuclear staining categories. B Expanded images of cells with examples of cytoplasmic staining categories.
1. Nuclear staining absent. 2. Nuclear staining weak - details of nuclear architecture clearly visible 3. Nuclear staining moderate - nuclear arthitecture can be distinguished, intermediate between weak and strong.4. Nuclear staining strong - no nuclear architecture discernable.
5. Cytoplasmic staining weak. 6. Cytoplasmic staining moderate. Absent cytoplasmic staining and strong cytoplasmic staining not seen in this image.

and cytoplasmic staining was assessed in 100 melanoma cells. For samples in which intra-epidermal tumour cells were prominent, these were assessed separately to cells in the dermal component of the tumour.

To give a visual overview of ANKHD1 expression patterns across all samples in the study the percentage of cells with each category of cytoplasmic and nuclear staining in each patient is displayed in Figure 4-10 and Figure 4-11 (the three samples of metastatic melanoma have not yet been quantified, and are not included in this analysis). Almost all tumour cells across the histological sub-types examined showed cytoplasmic ANKHD1 expression, and most showed some nuclear staining for ANKHD.

Statistical comparisons

The quantitative data was used to test three hypotheses suggested by the qualitative data: 1) Radial growth phase superficial spreading melanoma shows stronger nuclear staining than vertical growth phase superficial spreading melanoma or nodular melanoma. 2) Thinner melanomas show stronger nuclear staining than thicker melanomas. 3) Intra-epidermal tumour cells show stronger nuclear staining than the corresponding dermal tumour cells.

Statistical analysis did not support these hypotheses. The mean percentage of cells with strong or moderate nuclear staining in nodular melanoma was 6%, in radial growth phase superficial spreading melanoma it was 36% and in vertical growth phase superficial spreading melanoma it was 35%. There was no significant difference between these values when examined with a one-way ANOVA ($P = 0.36$) although this may be related to the small number of samples in the nodular melanoma group. To examine the relationship between melanoma thickness and nuclear ANKHD1 staining intensity the percentage of cells with moderate or strong nuclear staining was plotted against melanoma thickness (Figure 4-12). This did not show a clear relationship. The correlation coefficient for the relationship was -0.33, but this was not statistically significant ($P = 0.08$) (Pearson's correlation coefficient, two-tailed significance test). The percentages of intra-epithelial cells with moderate or strong nuclear staining were compared to the percentage of dermal cells with moderate or strong nuclear staining using a paired *t* test. The difference between these groups was not significant ($P = 0.27$).

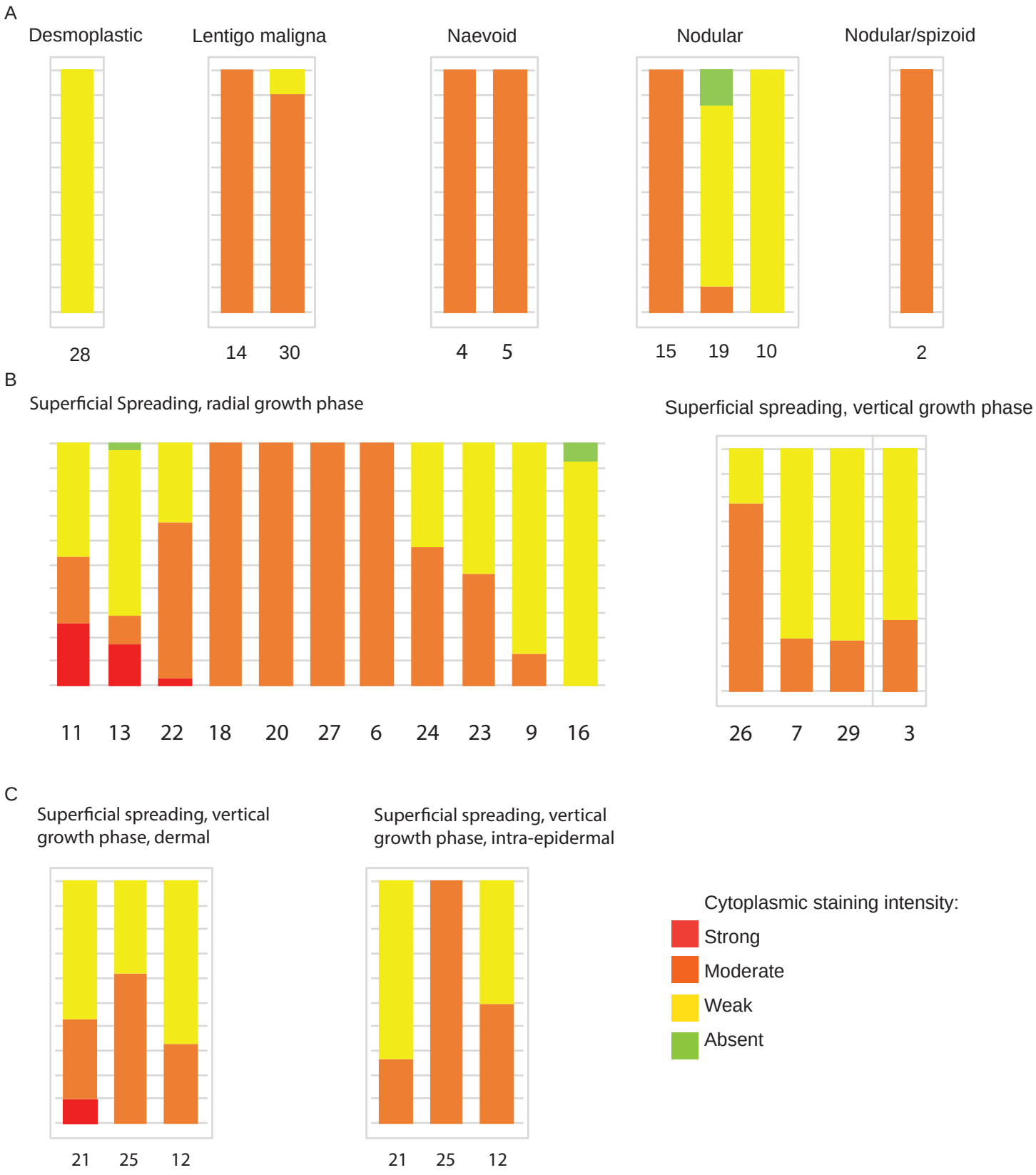


Figure 4-10. Pattern of cytoplasmic staining in malignant melanoma. 100 tumour cells were examined in each sample. Each bar represents one patient, identified by the numbers below the bars. The bar represents 100 cells and is coloured to show the proportion of cells with each grade of cytoplasmic staining intensity. Within each group samples are arranged in order of staining intensity, with the samples with the highest proportion of cells staining strongly on the left. (A) Staining in histological sub-types which are rare in this cohort. (B) Staining in superficial spreading melanoma, with tumours in radial growth phase and vertical growth phase separated. (C) Staining in vertical growth phase superficial spreading melanoma in which intra-epidermal tumour cells are present. Cells in the dermal component of the tumour are compared to the intra-epithelial tumour cells.

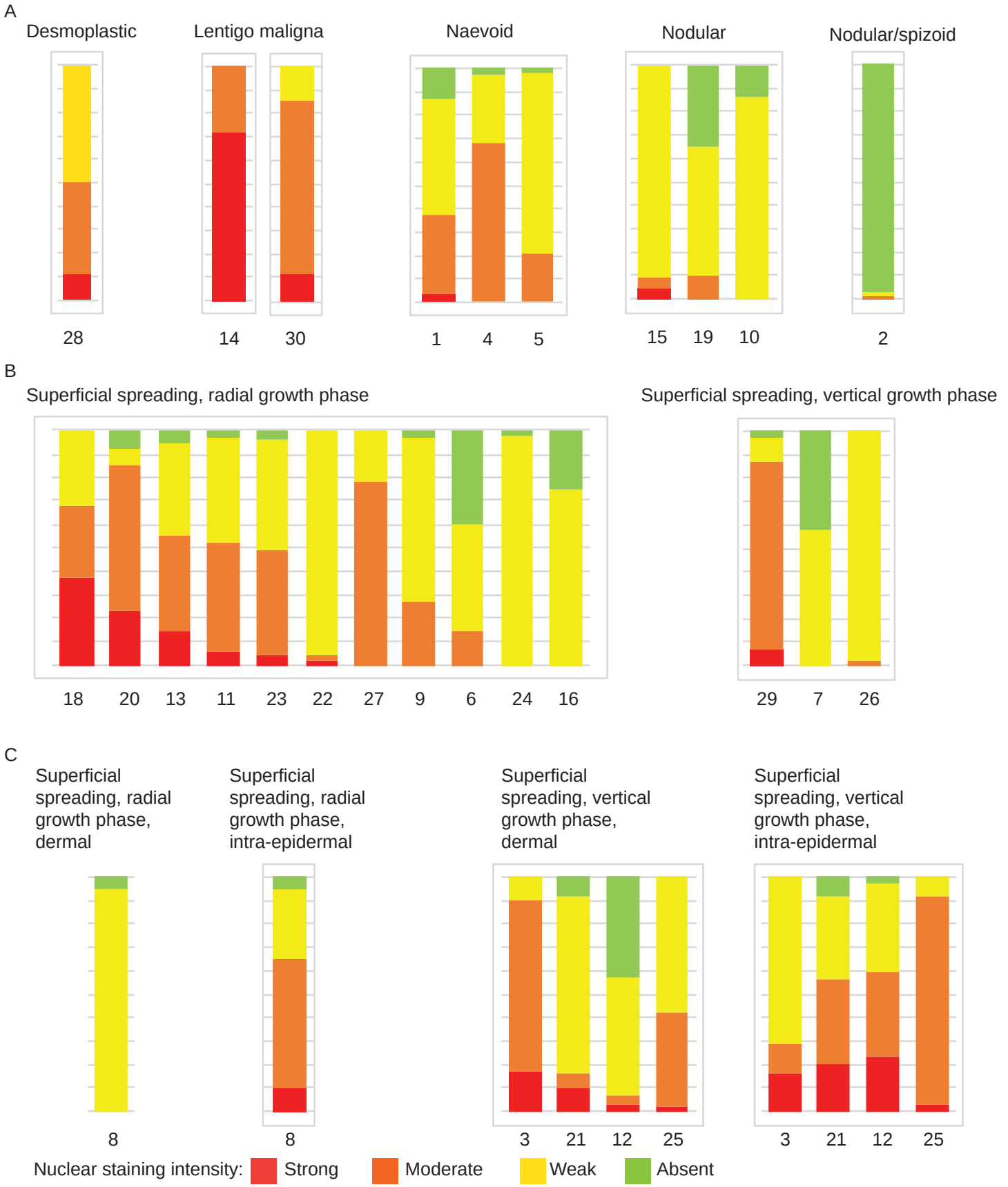


Figure 4-11. Pattern of nuclear staining in malignant melanoma. 100 tumour cells were examined in each sample, bars show the proportion of cells with each category of staining intensity. Numbers identify individual patients. (A) Staining in histological sub-types which are rare in this cohort. (B) Staining in superficial spreading melanoma, with tumours in radial growth phase and vertical growth phase separated. (C) Staining in superficial spreading melanoma in which intra-epidermal tumour cells are present. Cells in the dermal component of the tumour are compared to the intra-epidermal tumour cells, with radial growth phase and vertical growth phase tumours separated.

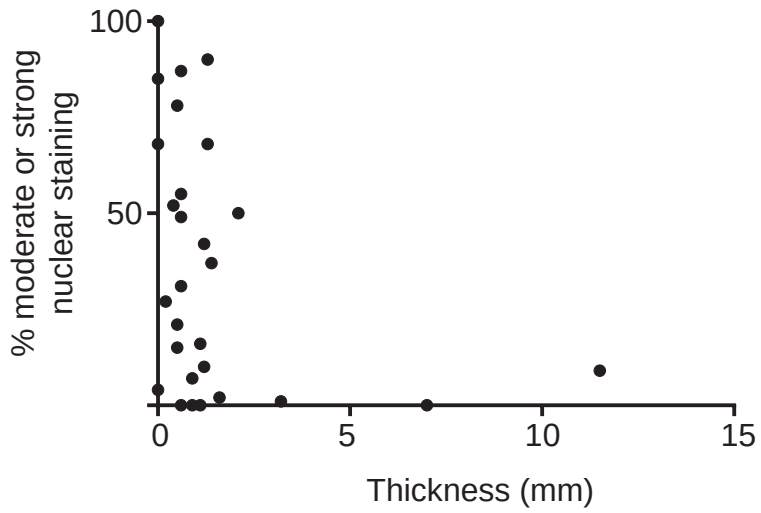


Figure 4-12. Scatter plot to examine for a correlation between melanoma thickness and percentage of cells with moderate or strong nuclear staining for ANKHD1. *r* = -0.33 (95% confidence interval -0.62 to 0.03, P = 0.08).

4.3 Discussion

In laboratory models, ANKHD1 promotes activation of several intracellular signalling pathways thought to contribute to disease pathogenesis in melanoma. In this study I have profiled the expression of ANKHD1 in tissue samples from patients with a range of histological sub-types of malignant melanoma, to determine whether further investigation of the role of ANKHD1 in melanoma is likely to be informative.

4.3.1 Expression and cellular localisation of ANKHD1 in melanoma

ANKHD1 is expressed in melanoma. It is found in the nucleus and cytoplasm. There are variations in ANKHD1 expression within and between individuals, and histological sub-types. Initial examination suggested that there were qualitative differences in ANKHD1 distribution according to histological sub-type, with stronger nuclear staining for ANKHD1 in thinner melanomas, and in intra-epidermal tumour cells. However, a semi-quantitative assessment of staining intensity did not confirm this. The number of samples examined in this study was relatively small so it is possible that these apparent trends might have been statistically significant had a larger sample size been examined. However, as there is no clear pattern in this variation of ANKHD1 expression that would suggest it provides an insight into disease pathogenesis or would be diagnostically useful, examination of further samples would probably not be worthwhile.

Caveats to the quantitative analysis of ANKHD1 expression are associated with the use of a scoring system to categorise staining intensity. In particular, a continuous range of levels of ANKHD1 expression have been arbitrarily divided into categories, and this study has not included a consideration of intra and inter-observer variability (Cross 1998).

A notable feature of the data presented here is the observation of nuclear localised ANKHD1. Early studies of ANKHD1 described it as a cytoplasmic protein but more recently ANKHD1 has also been observed to be localised to the nucleus, a feature that relates to its function in modulation of the Hippo pathway (Sansores-Garcia, Atkins *et al*. 2013, Sidor, Brain *et al*. 2013, Machado-Neto, Lazarini *et al*. 2014) and as a repressor of transcription of the cell-cycle regulator p21/WAF/CIP (Dhyani, Machado-Neto *et al*. 2015). The observation of nuclear localised ANKHD1

here contrasts to the previous description of ANKHD1 in melanoma in the Human Protein Atlas, where it is seen only in the cytoplasm.

4.3.2 Ongoing and possible future work

The samples from individuals with metastatic melanoma remain to be quantitatively analysed. An investigation of ANKHD1 expression in benign naevi is also underway. Samples from 20 benign naevi have been stained for ANKHD1. An initial examination of these slides indicates that ANKHD1 is expressed in melanocytes in benign naevi, and that there is less heterogeneity in expression in naevi compared to melanoma. The samples are awaiting quantitative analysis.

This study used only one anti-ANKHD1 antibody. Polyclonal rabbit IgG was used as a control for nonspecific antibody binding, but it does not control for antibody cross-reactivity. Repeating the immunohistochemistry with an anti-ANKHD1 antibody directed against a different epitope of the protein would be an important experiment to increase our confidence that the results truly represent ANKHD1 distribution. Erroneous data produced by antibody cross-reactivity is a prevalent problem in research, and in cancer biology in particular (Baker 2015).

It would be interesting to determine whether there is any relationship between ANKHD1 expression and JAK/STAT and Hippo pathway activation in melanoma. Studies of STAT3 activation and YAP/TAZ nuclear localisation have shown that this occurs only in some cells in some melanoma samples – it is possible that these are samples with high levels of ANKHD1 expression and ANKHD1 nuclear localisation, respectively. Immunohistochemistry could be carried out on adjacent tissue sections to determine this. A greater insight could be gained from performing two-colour immunohistochemistry, to try to determine whether there is a relationship between ANKHD1 expression and pSTAT3 levels or YAP/TAZ localisation at the level of individual cells.

The data from this study does not indicate that further investigation of ANKHD1 in melanoma is likely to be clinically useful. However, it would still be of interest to examine whether ANKHD1 might contribute to the malignant phenotype of melanoma cells. Investigation of the effects of ANKHD1 knockdown and overexpression upon proliferation, migration and apoptosis in melanoma cell lines might be of interest.

4.4 Conclusions

This work has shown that ANKHD1 is expressed in melanoma. However, the wide variation in ANKHD1 expression patterns even within a single histological sub-type of melanoma suggests that further investigation of ANKHD1 in melanoma is unlikely to give a clinically useful insight into melanoma biology.

Chapter 5: Methotrexate is an inhibitor of JAK/STAT signalling

5.1 Introduction

Prior to the start of my PhD, the Zeidler group identified methotrexate and aminopterin as suppressors of JAK/STAT signalling in a *Drosophila* cell-based screen of drugs, natural products and bioactive compounds (Figure 5-1).

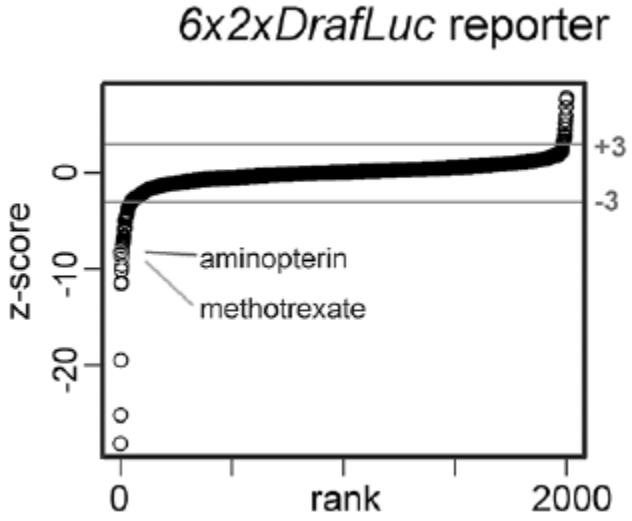


Figure 5-1. Methotrexate and aminopterin cause significant suppression of JAK/STAT pathway activation, assessed using a reporter of STAT responsive transcription. z score for firefly luminescence/Renilla luminescence = median/ median absolute deviation on a plate-by plate basis. z score values are considered significant when >3 or <3. Data generated by Katie Fisher and Steve Brown (Thomas, Fisher *et al*. 2015).

Low-dose methotrexate is used in the treatment of inflammatory conditions such as rheumatoid arthritis, but its mechanism-of-action in this setting is incompletely understood. Given that the JAK/STAT signalling pathway is involved in the inflammatory response (O'Shea and Plenge 2012) and that an inhibitor of JAK3 has shown benefit in patients with rheumatoid arthritis (Fleischmann, Kremer *et al*. 2012), this result suggested that inhibition of JAK/STAT signalling might contribute to the action of methotrexate in inflammatory conditions. Further investigation of this effect might also lead to the use of methotrexate to treat other conditions associated with increased activation of JAK/STAT signalling.

The screen result provided a starting point for a more detailed investigation of the effect of methotrexate on JAK/STAT signalling. The JAK/STAT signalling pathway in *Drosophila* is simpler than that in humans, with the major components

being three ligands (Unpaired, Unpaired 2 and Unpaired 3), one receptor (Domeless) one JAK (Hopscotch) and one STAT (STAT92E) (Zeidler and Bausek 2013). This relative simplicity and lack of redundancy makes the *Drosophila* JAK/STAT pathway a good model in which to perform screens. However, further investigation is needed to determine effects in the more complex JAK/STAT system of humans.

I investigated the effects of methotrexate on different mechanisms of JAK/STAT pathway activation in *Drosophila* cells, and extended this to investigate the effect of methotrexate on JAK/STAT signalling *in vivo* in a fly strain with an activating mutation in Hop. I then determined the effect of methotrexate upon JAK/STAT activation in human cell lines. To establish the relevance of the effect of methotrexate on JAK/STAT signalling in disease I investigated the effects on pathway activation caused by the JAK2V617F mutation found in patients with MPNs.

5.2 Results

5.2.1 Methotrexate reduces JAK/STAT activation caused by ligand-binding and activation caused by constitutive kinase activation in *Drosophila* cultured cells

In the screen which identified methotrexate and aminopterin as suppressors of JAK/STAT pathway activation, activation of the pathway was produced by overexpression of Unpaired (Upd). To test whether the effects of methotrexate and aminopterin were specific to pathway activation caused by ligand activation, the effect on JAK/STAT activity in cells where the pathway is activated by Upd secretion was compared to its effect in cells where the pathway is activated by overexpression of a constitutively active mutant of Hopscotch (Hop). $Hop^{Tum\text{-}l}$ contains a glycine to glutamic acid substitution at residue 341 in the JH4 domain, which causes JAK/STAT activation (Luo, Hanratty *et al*. 1995). When JAK/STAT pathway activation was assessed using a STAT responsive transcriptional luciferase assay, methotrexate and aminopterin both caused a dose-dependent reduction in JAK/STAT activation when the pathway was activated by expression of $Hop^{Tum\text{-}l}$ as well as when it was activated by Upd (Figure 5-2).

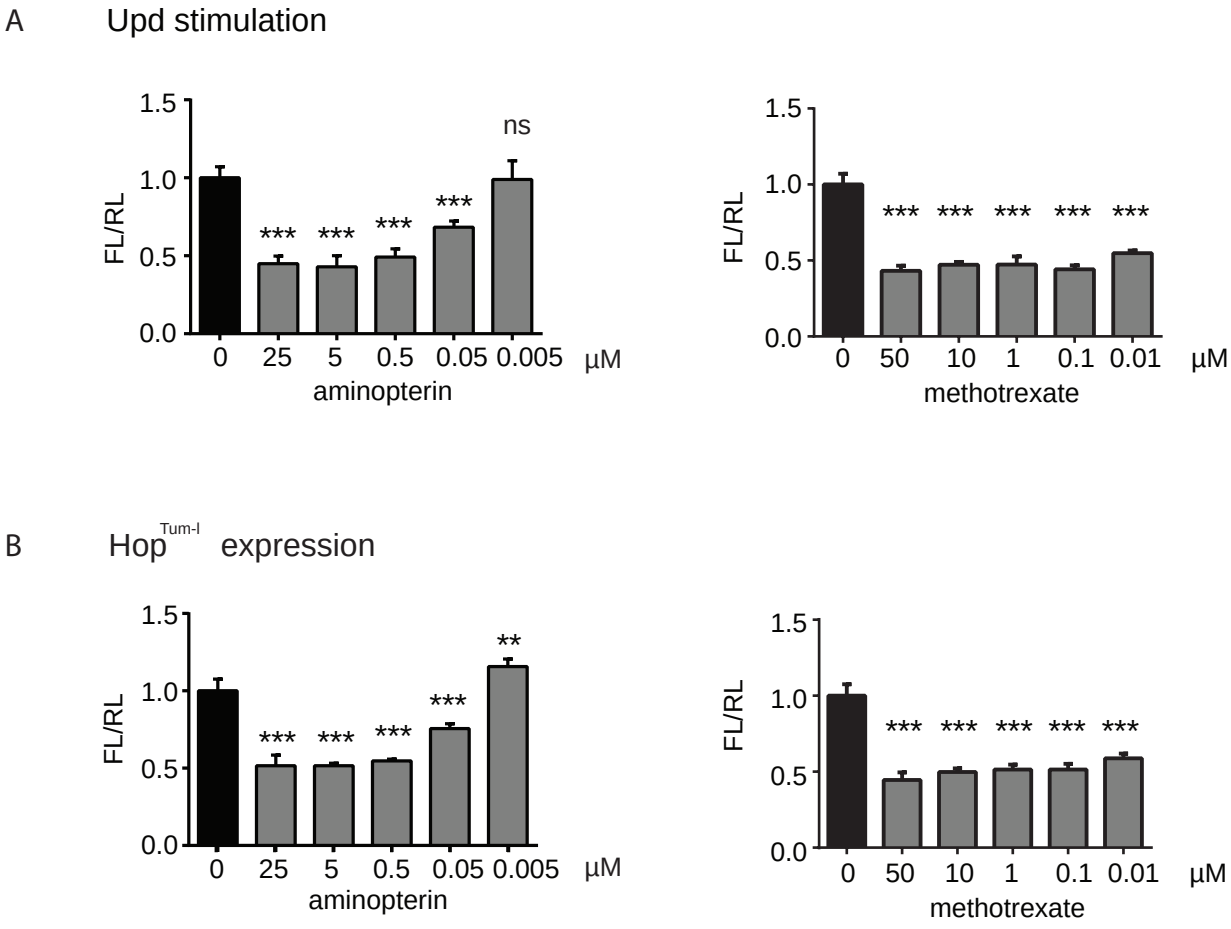


Figure 5-2. Methotrexate and aminopterin suppress STAT responsive transcription in *Drosophila* Kc_{167} cells when the JAK/STAT pathway activation is caused by Upd secretion (A) or expression of a constitutively active Hop (B). FL/RL = firefly luciferase luminesence/ *Renilla* luciferase luminescence. Values are normalised to those in cells treated with DMSO alone. Bars show mean and standard deviation of four replicates. ns $P > 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

5.2.2 Methotrexate does not affect JAK/STAT dependant tumour formation *in vivo* in Drosophila

The original screen which identified methotrexate as a JAK/STAT inhibitor was performed in a *Drosophila* cell line. I wished to establish whether methotrexate affects JAK/STAT signalling *in vivo* in flies. Flies possessing a mutation in *hop* which causes a glutamic acid to lysine substitution at position 695, in the JH2 domain, develop melanotic tumours due haematopoietic abnormalities (Luo, Rose *et al*. 1997). JAK/STAT activation causes proliferation of plasmatocytes, a phagocytic haemocyte present in embryos, and their premature differentiation into lamellocytes, cells that participate in encapsulation reactions. These lamellocytes form melanised tumours, and alterations in the number and size of these tumours have been used to identify modulators of JAK/STAT signalling in flies (Shi, Calhoun *et al*. 2006). I hypothesised that methotrexate would reduce tumour formation in hop^{T42} flies.

I first established what range of methotrexate concentrations could be tolerated by wild-type larvae without substantially reducing the number of adult flies that hatch (Figure 5-3A). Based on this data, concentrations of 0.1µM, 0.5µM and 0.75µM were selected for further investigation.

Methotrexate did not have any significant effect on tumour formation in flies heterozygous for hop^{T42} (Figure 5-3B).

It is possible that no effect was observed because of the pharmacokinetics of methotrexate in flies, if concentrations of methotrexate in the tissues of the developing flies were substantially lower than concentrations in the food. However, flies at the 0.5µM and 0.75µM methotrexate concentrations showed wing abnormalities that have previously been described as a consequence of methotrexate treatment (Affleck, Neumann *et al*. 2006), suggesting that methotrexate was being absorbed in sufficient quantities to affect development (Figure 5-3C).

5.2.3 Examination of expression and phosphorylation of JAK/STAT pathway components in human cell lines

To examine the effect of methotrexate on JAK/STAT signalling in human cells I used the Hodgkin lymphoma cell lines HDLM-2 and L-540 which have previously been found to show constitutive phosphorylation of certain JAK/STAT pathway components (Cochet, Frelin *et al*. 2006), and used to examine the effects of drugs on

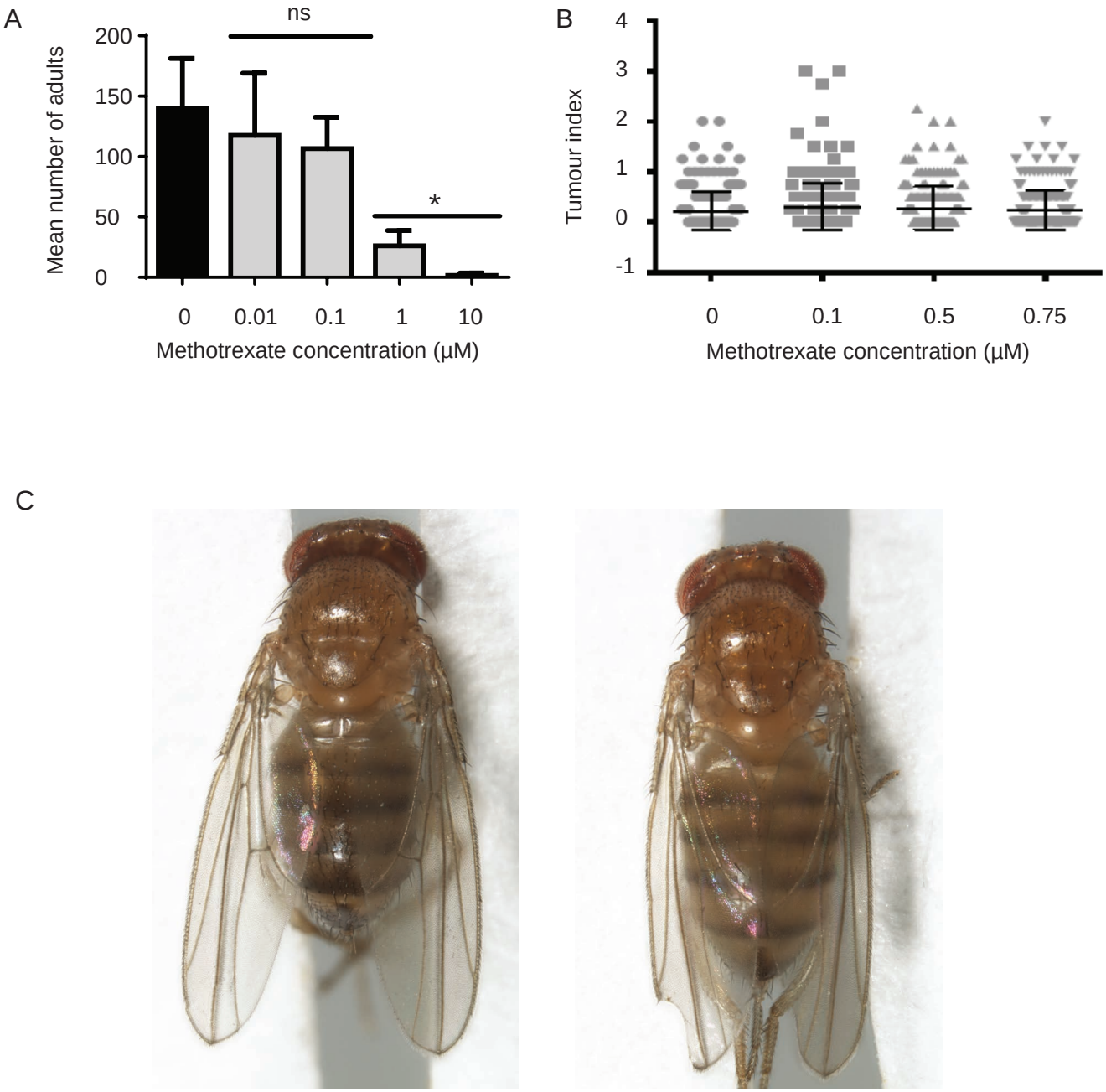


Figure 5-3. Effect of methotrexate in vivo in Drosophila. (A) High doses of methotrexate significantly reduce survival of wild-type Drosophila from egg laying to adulthood. Bars show mean and standard deviation of two replicates. One-way ANOVA with Dunnetts multiple comparisons test. ns $P > 0.05$, * $P \leq 0.05$. (B) Methotrexate does not affect tumour index in *hop*^{T42} mutants. Bars show mean and standard deviation (median in each group is 0). One-way ANOVA does not show a significant difference between groups. (C) A proportion of flies raised on methotrexate have wing notching, as shown on the image on the right of a fly raised on 0.5µM methotrexate. This is not present in flies raised on food containing drug vehicle alone, shown on the left.

JAK/STAT signalling (Kim, Oh *et al*. 2010). Prior to using these cell lines to examine the effects of methotrexate and aminopterin on JAK/STAT signalling I determined the expression and phosphorylation of JAK and STAT family members using western blotting. These were compared to JAK and STAT phosphorylation in HeLa cells with and without JAK/STAT pathway stimulation with oncostatin M (Figure 5-4A).

Constitutive phosphorylation of JAK3 has previously been observed in L-540 cells, but no pJAK3 was detected in these cells in my hands (Kim, Oh *et al*. 2010). pJAK3 was detected after cells were stimulated with IL-2, confirming a lack of constitutive JAK3 phosphorylation rather than a problem with the antibody used in western blotting (Figure 5-4B).

5.2.4 Methotrexate and aminopterin reduce JAK phosphorylation

Methotrexate and aminopterin reduce levels of pJAK1 and pJAK2 in HDLM-2 cells at higher drug concentrations (Figure 5-5A and B). This effect is more marked for pJAK1 than pJAK2.

The effect of methotrexate on JAK3 phosphorylation was investigated in L-540 cells in which JAK3 phosphorylation was stimulated by IL-2 treatment. However, methotrexate treatment affected total JAK3 in these cells, appearing to produce protein degradation, making the blots difficult to interpret (Figure 5-5C).

5.2.5 Methotrexate does not affect the activity of JAK2 in *in vitro* kinase assays, and has little effect on other kinases

The reduction in JAK phosphorylation produced by methotrexate treatment suggested methotrexate may be acting as a direct inhibition of JAK activity. This was examined in a cell free assay of kinase activity (Hastie, McLauchlan *et al*. 2006). A kinome screen examining the effect of methotrexate upon 50 kinases was performed by the International Centre for Kinase profiling at the University of Dundee (Table 5-1). In this assay methotrexate had no effect upon JAK2 activity - the mean activity in the presence of methotrexate was 102% of usual activity, with a standard deviation of 10%. Methotrexate had little effect on most of the kinases examined, where the mean percentage kinase activity was close to 100%, and the standard deviations overlapped with 100%. A few kinases showed a modest increase

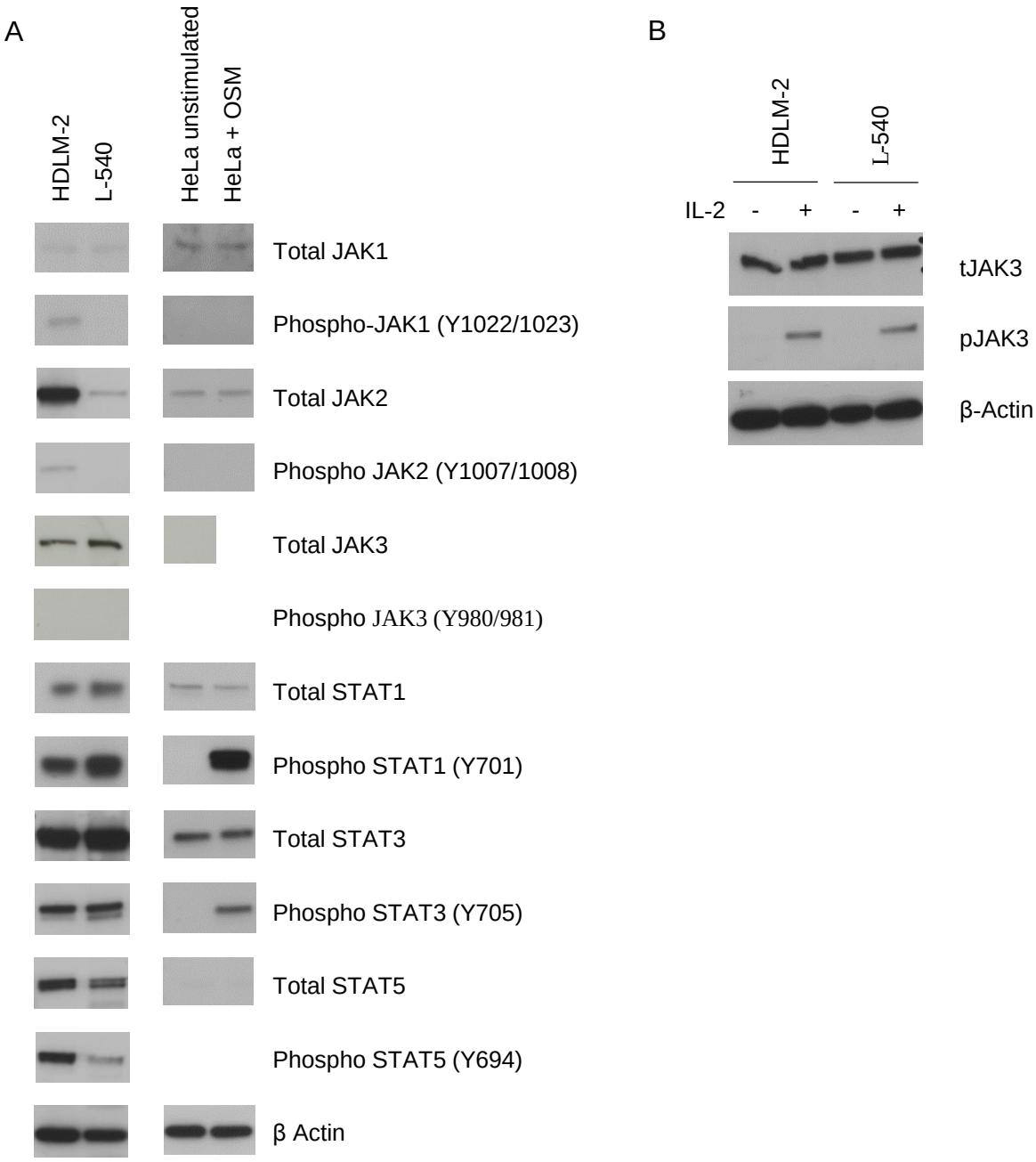


Figure 5-4. Hodgkin lymphoma cell lines HDLM-2 and L-540 show constitutive phosphorylation of certain JAK and STAT family members. (A) Basal expression and phosphorylation of JAKs and STATs in the the Hodgkin lymphoma cell lines is compred to that seen in unstimulated HeLa cells and in HeLa cells in which the JAK/STAT pathway has been activated by stimulation wih Oncostatin M at 10ng/ml for 30 minutes. HDLM-2 cells show constitutive phosphorylation of JAK1 and JAK2. Both lines express JAK3 but no phospho-JAK3 was detected. Both Hodgkin lymphoma cell lines show constitutive phosphorylation of STATs 1, 3 and 5. (B) Stimulation with IL-2 leads to JAK3 phosphorylation in both cell lines

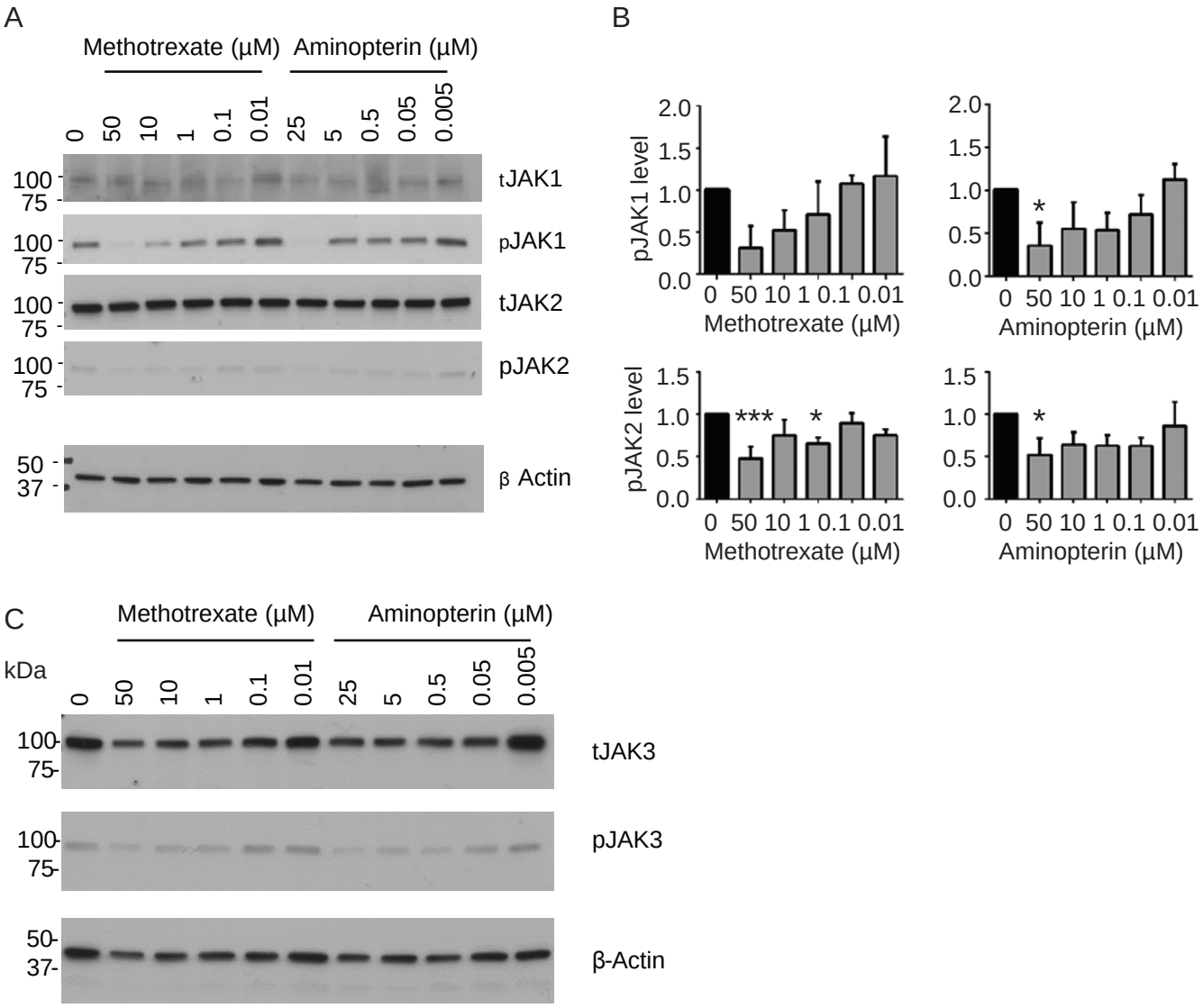


Figure 5-5. Methotrexate and aminopterin reduce constitutive JAK phosphorylation. (A) Western blotting shows a reduction in pJAK1 and pJAK2 at high drug concentrations, but little effect on total JAK levels. (B) Quantification of western blot band intensities. Bars show mean and standard deviation of three independent experimental replicates. Values were adjusted for loading using actin and normalised to the values for cells treated with vehicle alone. One-way ANOVA with Dunnett's multiple comparisons test. *** $P \leq 0.001$, * $P \leq 0.05$. (C) Methotrexate and aminopterin reduce pJAK3 in L-540 cells stimulated with IL-2. tJAK3 is also reduced, however, making this result difficult to interpret.

Table 5-1. Methotrexate does not affect JAK2 activity in an *in vitro* kinase assay, and has modest effects on other kinases. Columns show mean and standard deviation (SD) of the percentage activity remaining after exposure to 10μM methotrexate, for duplicate experiments. Kinases have been arranged according to the degree to which they are affected by methotrexate. Only those kinases for which mean - SD or mean + SD did not cross 100 were considered as having increased or decreased kinase activity respectively. JAK 2 is highlighted.

A. No Change

Kinase	Mean	SD
TBK1	86	15
LKB1	94	10
SGK1	95	11
MKK1	95	12
DYRK1A	96	13
NEK6	98	10
RSK1	98	6
SYK	99	18
PKA	99	8
p38a MAPK	100	9
MST2	101	2
TAK1	101	4
IGF-1R	102	12
JAK2	102	10
PLK1	103	5
VEG-FR	103	15
PDK1	103	6
GSK3b	103	17
PKBa	104	21
HER4	104	5
HIPK2	105	6
PKCa	107	17
MARK3	107	14
IRAK4	107	7
MLK3	107	34
PRK2	110	11
CHK2	115	30

B. Increased activity

Kinase	Mean	SD
BTK	126	3
EPH-A2	124	19
CK1_	118	7
SmMLCK	117	14
ROCK 2	114	2
JNK1	114	1
CAMKKb	112	1
Src	111	7
MSK1	111	2
RIPK2	111	1
TrkA	108	3
S6K1	105	0
PAK4	105	1
PKD1	103	1

C. Decreased activity

Kinase	Mean	SD
EF2K	48	2
Lck	68	3
CAMK1	83	5
AMPK (hum)	85	6
PIM1	88	0
Aurora B	91	2
TTK	93	5
SRPK1	96	2
CK2	98	1

or decrease in activity. Most notable amongst these is Lck (Lymphocyte Specific Protein Tyrosine Kinase), for which kinase activity was reduced to 68% with a standard deviation of 3%. Lck is a Src family kinase involved in T cell receptor activation. This relationship is consistent with the anti-inflammatory properties of methotrexate. Based on a literature search and search in the EMBL-EBI IntAct Molecular Interaction Datbase (Orchard, Ammari *et al*. 2014) no interaction between Lck and methotrexate has previously been described. Of significance for the interpretation of my results, Lck is known to phosphorylate and activate STAT3 and STAT5.

5.2.6 Methotrexate and aminopterin have selective effects on STAT phosphorylation in HDLM-2 cells

Given that methotrexate and aminopterin substantially reduce STAT-responsive transcription in *Drosophila* cells and reduce JAK phosphorylation in human cell lines, I next examined whether these drugs affect STAT phosphorylation in HDLM-2 cells. Both drugs produced a dose-responsive reduction in STAT1 phosphorylation and STAT5 phosphorylation, but had little effect on STAT3 phosphorylation. Levels of total STATs appeared unaffected by the drug treatments (Figure 5-6 A and B).

5.2.7 The effects of methotrexate on STAT phosphorylation are not the consequence of a global reduction in protein phosphorylation

It is possible that the reduction of STAT1 and STAT5 phosphorylation could be an indication that methotrexate has non-specific effects on intracellular protein phosphorylation, rather than evidence of a selective effect on JAK/STAT signalling. To address this I examined the effect of methotrexate on three serine/threonine or tyrosine phosphorylated proteins in other intracellular signalling pathways - ERK 1/2, Akt and c Jun). Phosphorylation of these proteins was not affected by methotrexate, even at the highest concentrations tested (Figure 5-6C).

5.2.8 STAT phosphorylation caused by the JAK2 V617F mutation is reduced by methotrexate

In *Drosophila* cell culture experiments methotrexate reduced JAK/STAT signalling when the pathway was activated by $hop^{Tum\text{-}l}$, a JAK mutation that causes constitutive

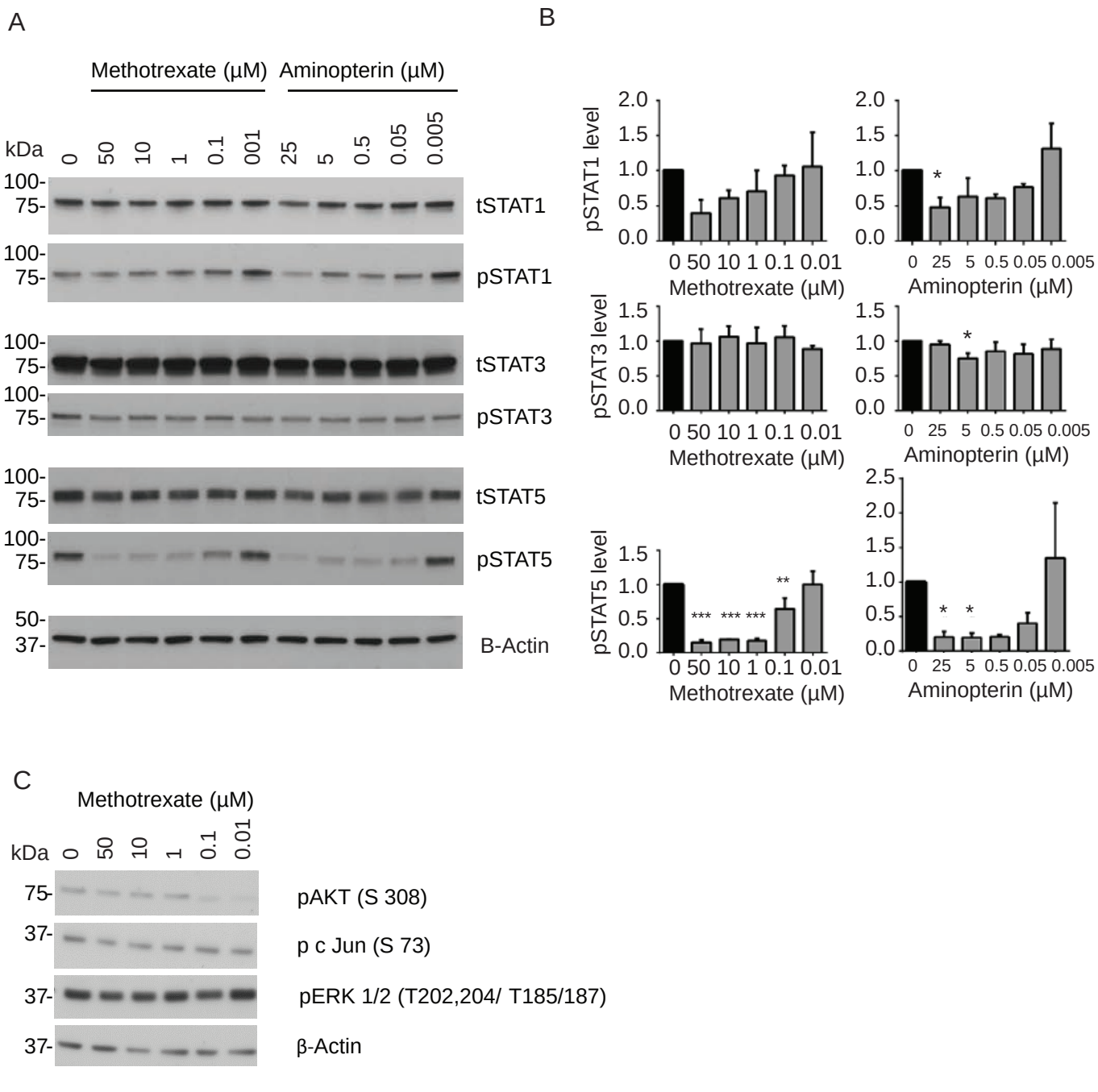


Figure 5-6. Methotrexate and aminopterin reduce STAT1 and STAT5 phosphorylation, with little effect on total STATs, STAT3 phosphorylation or other intracellular phosphorylated proteins. (A) Western blots showing the effect of methotrexate and aminopterin treatment on total and phosphorylated STATs. (B) Quantification of western blot band intensities. Bars show mean and standard deviation of three independent experimental replicates. Values were adjusted for loading using actin and normalised to the values for cells treated with vehicle alone. One-way ANOVA with Dunnett's multiple comparisons test. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$. (C) Western blots showing the effect of methotrexate on phosphorylation of Akt, c jun and ERK 1/2.

kinase activation. To investigate whether methotrexate produces the same effect in human malignancies caused by JAK mutations which confer constitutive kinase activation I examined the effect of methotrexate on phosphorylation of JAK/STAT pathway components in the HEL cell line. This cell line is homozygous for the JAK2 V617F mutation (Baxter, Scott *et al*. 2005), and shows constitutive STAT phosphorylation that is dependent on JAK2 activity (Anand, Stedham *et al*. 2011).

Methotrexate treatment led to a reduction in STAT3 and STAT5 phosphorylation in HEL cells. There was also a mild reduction in the levels of the un-phosphorylated form of these STATs (Figure 5-7A). The changes in pSTAT levels were statistically significant, whereas the changes in total STAT levels were not (Figure 5-7B).

The effect of methotrexate on STAT5 phosphorylation in HEL cells becomes apparent after 24 hours of methotrexate treatment (Figure 5-7C).

5.2.7 The effects of methotrexate on STAT phosphorylation appear after 24 hours of methotrexate treatment

The time taken for methotrexate to affect STAT phosphorylation may give information about its likely mechanism of action. In HDLM-2 cells it takes 24 hours for a reduction in STAT phosphorylation to be observed (Figure 5-7A).

5.2.8 The effect of methotrexate on STAT phosphorylation is not reversed by folinic acid

Methotrexate exerts its effects as a chemotherapy drug through impairment of folate metabolism, via competitive inhibition of dihydrofolate reductase (DHFR). This inhibition reduces intracellular levels of intermediate compounds required for nucleotide synthesis, resulting in impaired DNA replication and repair that ultimately causes cell death (Li and Kaminskas 1984). Folinic acid is given following methotrexate in chemotherapy regimens, in order to bypass the block in folate metabolism caused by inhibition of DHFR (Visentin, Zhao *et al*. 2012). Patients taking low dose methotrexate for rheumatoid arthritis are also usually given folate supplementation to reduce adverse effects of folate antagonism, and this does not have a marked effect on the efficacy of methotrexate in these patients (Whittle and Hughes 2004).

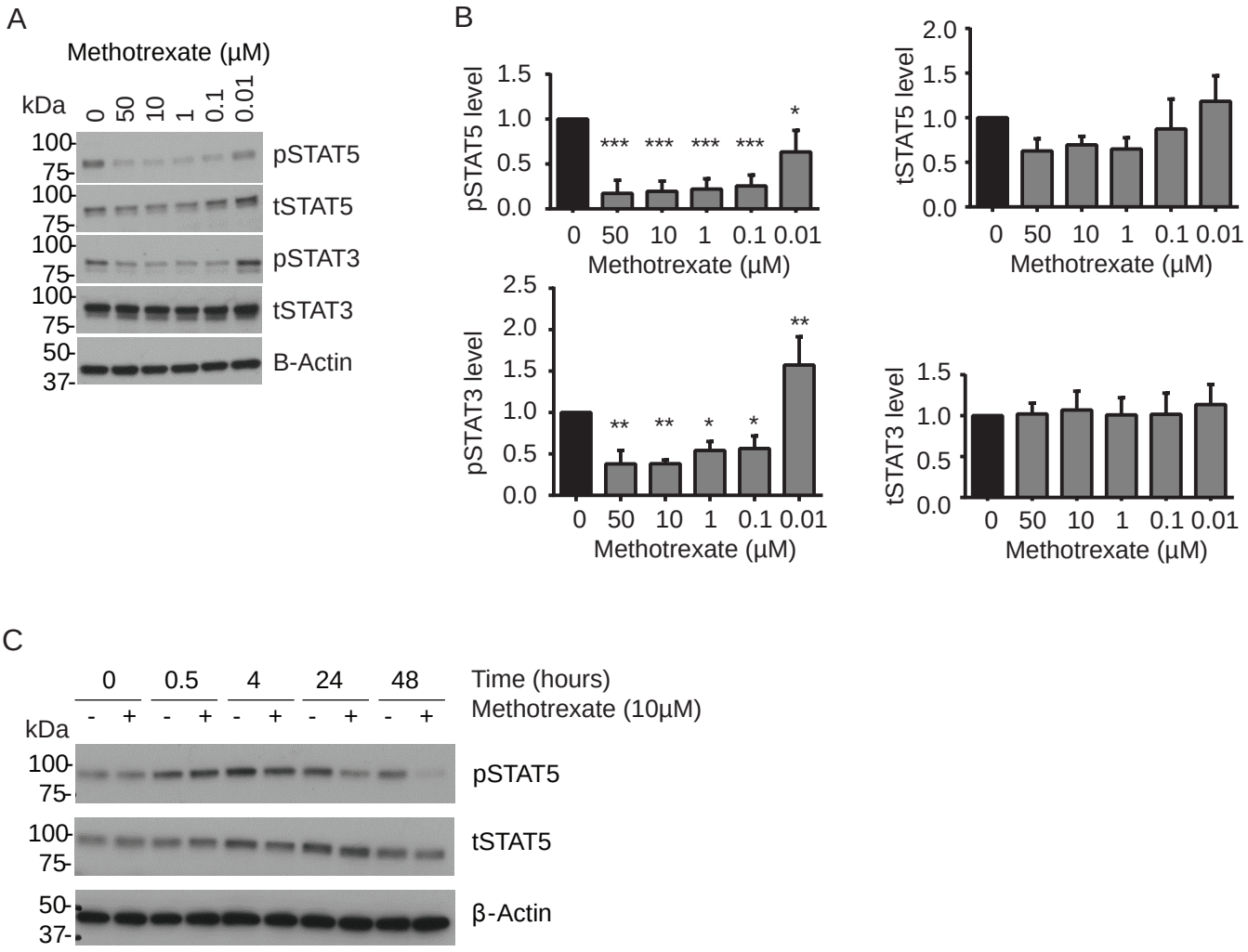


Figure 5-7. Methotrexate reduces STAT3 and STAT5 phosphorylation in HEL cells, and this effect becomes apparrent after 24 hours. (A) Western blots showing the effect of methotrexate on total and phosphorylated STATs in HEL cells. (B) Quantification of western blot band intensities. Bars show mean and standard deviation of three independent experimental replicates. Values were adjusted for loading using actin and normalised to the values for cells treated with vehicle alone. One-way ANOVA with Dunnett's multiple comparisons test. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$. (C) The reduction in pSTAT5 becomes apparent after 24 hours of methotrexate treatment, and is more marked at 48 hours. Total STAT5 levels are similar in treated and control cells.

To establish whether the effects of methotrexate on JAK/STAT signalling might be linked to inhibition of DHFR I examined the effect of folinic acid on STAT phosphorylation in methotrexate treated HEL cells. The ability of methotrexate to suppress STAT5 phosphorylation persisted in the presence of folinic acid (Figure 5-8A). However, the magnitude of the suppression was reduced. These results suggest that the inhibition of JAK2/STAT5 in these cells is at least partly independent of perturbation of folate metabolism. It is also consistent with the continuing anti-inflammatory and immunosuppressive effects of methotrexate in patients with rheumatoid arthritis receiving folate supplementation.

5.2.9 Methotrexate-treated cells can activate JAK/STAT signalling in response to EPO

JAK/STAT pathway activity is essential for multiple developmental and physiological processes including haematopoiesis and immunity. As a result, thrombocytopaenia, anaemia and susceptibility to infection have been significant side effects of JAK inhibitors used in clinical practice (Fleischmann, Kremer *et al*. 2012, Harrison, Kiladjian *et al*. 2012). If methotrexate were to be used clinically to treat patients with MPNs it would be desirable for the suppression of JAK/STAT signalling to occur in such a way that pathway inhibition can be overcome by physiological stimuli. To test whether this was the case for methotrexate-induced pathway suppression we stimulated methotrexate-treated HEL cells with recombinant EPO. The normal range for serum EPO is large, so to recapitulate EPO levels produced by the physiological stimulus of hypoxia we used an EPO concentration calculated to correspond to that measured in the serum of individuals with secondary erythrocytosis (Spivak 2002). Strikingly, STAT5 phosphorylation in methotrexate-treated cells was increased following EPO stimulation, and was comparable to that observed in control cells (Figure 5-8B).

5.2.10 Suppression of STAT5 phosphorylation by methotrexate is comparable to that produced by ruxolitinib

I wished to determine whether the effects of methotrexate on STAT phosphorylation in HEL cells were comparable to the effects of ruxolitinib, which is used clinically to treat patients with MF. As expected, both ruxolitinib and methotrexate treatment

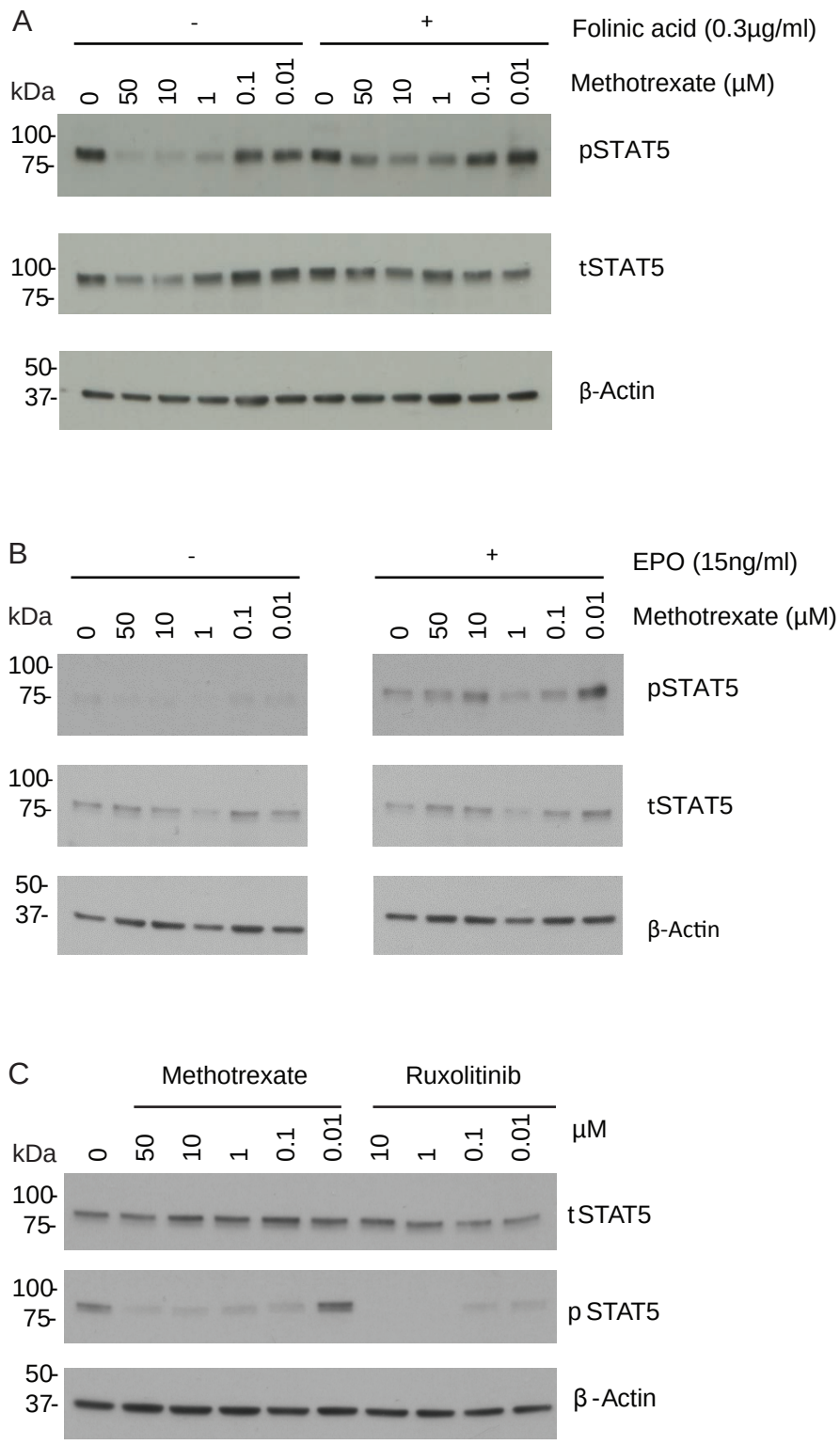


Figure 5-8. (A) Western blots showing the effect of exogenous folinic acid on the suppression of STAT5 phosphorylation in HEL cells treated with methotrexate. (B) Western blots showing the effect of EPO stimulation of the JAK/STAT pathway in HEL cells treated with methotrexate. (C) Comparison of the effects of ruxolitinib and methotrexate on STAT5 phosphorylation in HEL cells.

resulted in a clear suppression of STAT phosphorylation (Figure 5-8C). Taking into account peak serum concentrations measured in patients taking the drugs orally, 0.4 - 0.8μM for methotrexate and 1μM for ruxolitinib (Shilling, Nedza *et al*. 2010, Hobl, Mader *et al*. 2012), ruxolitinib produced a more profound suppression of STAT phosphorylation than methotrexate. However the effects of both drugs on STAT5 phosphorylation were comparable at pharmacologically realistic concentrations.

5.3 Discussion

This study has established that methotrexate suppresses activation of the JAK/STAT signalling pathway, including JAK/STAT activation caused by JAK2V617F. These results have implications for understanding the biology of methotrexate when used as an anti-inflammatory drug, and suggest that it may provide a novel treatment option for patients with malignancies associated with JAK/STAT pathway activation.

5.3.1 JAK/STAT suppression as a mechanism of action for methotrexate as an anti-inflammatory drug

Methotrexate has been used for many years to treat inflammatory disorders including rheumatoid arthritis, but its mechanism of action in these conditions is not fully understood (Wessels, *et al* 2008). The JAK/STAT signalling pathway is involved in inflammation and immunity (O'Shea and Plenge 2012) and activation of JAK/STAT signalling is seen in many inflammatory diseases. JAK/STAT activation is known to contribute to disease pathogenesis in rheumatoid arthritis (Walker and Smith 2005), and a specific inhibitor of JAK3 has shown efficacy in clinical trials in patients with rheumatoid arthritis (Fleischmann, *et al* 2012). It is also notable that leflunomide, used in the treatment of inflammatory conditions including rheumatoid arthritis, has been observed to inhibit JAK/STAT signalling (Siemasko, Chong *et al*. 1998).

Our data suggests that suppression of JAK/STAT activation may contribute to the action of methotrexate in inflammatory conditions. Methotrexate produces a clear suppression of STAT phosphorylation at drug concentrations equivalent to the peak plasma concentrations measured in patients taking oral methotrexate to treat rheumatoid arthritis (0.4uM) (Hobl, Mader *et al*. 2012). Caution is needed when extrapolating results from *in vitro* drug treatments to speculate on what might occur

in patients, however. The plasma concentrations referred to are peak plasma concentrations. Methotrexate has a relatively short plasma half-life (4 hours) due to uptake into cells and excretion by the liver and kidneys. It is possible that adding methotrexate to media for 48 hours exposes cells to higher concentrations of methotrexate for longer periods than would occur in patients. Conversely, as methotrexate is rapidly and actively absorbed into cells by folate carriers, whose expression is upregulated on cancer cells, it may be that all the methotrexate is absorbed into cells within a short time frame and the methotrexate concentration in the media for most of the experiment is negligible. To better recapitulate physiological conditions methotrexate-containing media could be replaced with fresh media after a few hours.

5.3.2 What is the mechanism through which methotrexate suppresses JAK/STAT signalling?

This work has not established the mechanism through which methotrexate reduces the activation of the JAK/STAT pathway. Nonetheless, some inferences can be drawn to generate hypotheses. The data in *Drosophila* cells comparing STAT-responsive transcriptional activation due to Upd and $Hop^{Tum\text{-}l}$ suggests that methotrexate acts downstream of ligand secretion. Examination in HDLM-2 cells suggests that it selectively affects levels of phosphorylated JAK/STAT pathway intermediates without having a global effect on all intracellular phosphorylated proteins. The persistence of the suppression of STAT phosphorylation in the presence of folinic acid suggests that the effect of methotrexate is at least partially independent of its known effects upon folate metabolism.

One possible mechanism through which methotrexate might reduce JAK/STAT pathway activation is by acting as a direct inhibitor of JAK kinase activity. The dose dependent reduction in JAK1 phosphorylation produced by methotrexate in HDLM-2 cells supports this hypothesis (although the effect on JAK1 phosphorylation is not statistically significant). Modelling of the possible molecular interactions between methotrexate and the kinase pocket of JH1 of JAK2 (John Sayers and Martin Zeidler, unpublished data) suggests methotrexate could dock in the kinase pocket under physiological conditions, in a manner comparable to ruxolitinib and ATP (Figure 5-9).

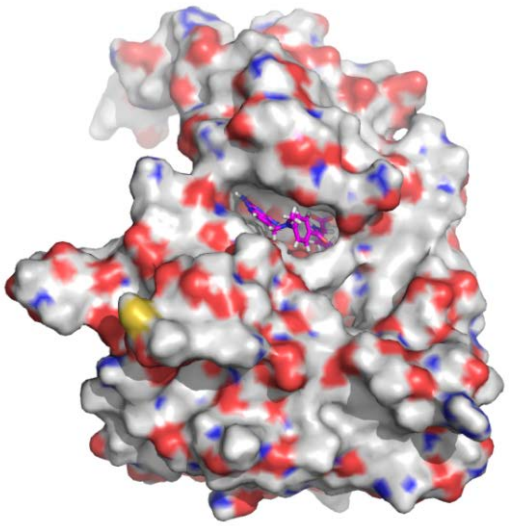


Figure 5-9. Modelling of methotrexate occupying the ATP-binding pocket of JH1 of JAK2. John Sayers and Martin Zeidler, unpublished data.

However, some evidence is not consistent with this hypothesis. Firstly, the effects of methotrexate on JAK2 and JAK3 were less clear than the effect on JAK1, and in general the effect on JAK phosphorylation is less marked than the effect on STAT phosphorylation. Secondly, 10uM methotrexate did not affect JAK2 activity in *in vitro* kinase assays. It is possible that a metabolised form of methotrexate, such as methotrexate polyglutamates, is the biologically active drug, accounting for the lack of effect in kinase assays. Consistent with this, methotrexate treatment takes 24 hours to produce a change in STAT phosphorylation when examined with western blotting.

One intriguing possible mechanism through which methotrexate may suppress JAK/STAT signalling is as an indirect result of its effects on adenosine metabolism. Methotrexate causes adenosine accumulation indirectly via inhibition of the enzyme 5-aminoimidizole 4-carboxamideribonucleotide (AICAR) (Tian and Cronstein 2007). Recently adenosine analogues have been shown to act as JAK inhibitors (Liu 2014).

When considering possible mechanisms for the effect of methotrexate on JAK/STAT signalling, the role of folates merits further discussion. Administration of folinic acid, which bypasses the block in folate metabolism caused by inhibition of DHFR, reduced but did not reverse the suppression of STAT phosphorylation observed here. The concentration of folinic acid was equivalent to that which is found in patients taking folinic acid to treat methotrexate toxicity (Whittle and

Hughes 2004). This suggests that the effects of methotrexate may be independent of effects on folate metabolism. This is supported by RNAi screening for genes that modulate JAK/STAT pathway activation in *Drosophila*, where there is no interaction between multiple enzymes in the folate biosynthetic pathway and STAT transcriptional activity (Fisher, Wright *et al*. 2012). The partial restoration of STAT phosphorylation in the presence of folinic acid could be due to reduced cellular uptake of methotrexate, since the compounds compete for uptake via the same transporter (Dixon 1991, Visentin, Zhao *et al*. 2012). Conversely, there is evidence in the existing literature to support the hypothesis that the methotrexate effects are related to DHFR inhibition since pyrimethamine, an anti-parasitic drug which inhibits DHFR, has been shown to reduce STAT phosphorylation (Takakura, Nelson *et al*. 2011).

Further work will be required to determine the mechanism through which methotrexate inhibits JAK/STAT activation. This does not negate its potential clinical usefulness as a treatment for JAK/STAT associated disorders, however, especially as it is a drug whose safety profile is already well understood from its use in inflammatory conditions.

5.3.2 Methotrexate as a potential treatment for patients with MPNs

The demonstration that methotrexate reduces JAK/STAT pathway activation caused by the JAK2V617F mutation suggests that methotrexate may be a suitable treatment for patients with myeloproliferative neoplasms. Although the cytoreductive drugs busulphan and hydroxyurea are used to treat patients with MPNs, a literature search has not identified any reports of the use of methotrexate in these conditions. Our data shows that suppression of STAT5 phosphorylation occurs at methotrexate concentrations equivalent to those measured in the plasma of patients, both those receiving methotrexate for chemotherapy and those taking methotrexate for rheumatoid arthritis (Hobl, Mader *et al*. 2012, Radtke, Zolk *et al*. 2013).

These effects are comparable to the effects of ruxolitinib, although ruxolitinib caused greater suppression of STAT phosphorylation at comparable concentrations *in vitro*. Methotrexate-treated cells retained the capacity to activate the JAK/STAT pathway in response to ligand stimulation, which has implications for its possible use to treat patients with MPNs. Methotrexate may ‘dampen’ the pathological basal

over-activation of the JAK/STAT pathway sufficiently to control the disease, without preventing physiological activation when needed for haematopoiesis and the response to infection. Levels of STAT5 phosphorylation in CD34+ cells from patients with MPNs are only about 1.5 fold greater than in CD34+ cells from normal healthy individuals (Anand, Stedham *et al*. 2011), suggesting that a relatively mild suppression of pathway activation may be sufficient to control the disease. This is important in the context of the effects of ruxolitinib, which produces a more profound inhibition of STAT phosphorylation, but for which thrombocytopaenia and to a lesser extent anaemia and susceptibility to infection are significant side effects (Harrison, Verstovsek *et al*. 2012).

There is a need for new treatments for patients with myeloproliferative neoplasms. Ruxolitinib is recommended by the British Committee for Standards in Haematology (BCSH) for patients with myelofibrosis and splenomegaly or constitutional symptoms (Reilly, McMullin *et al*. 2014). However, the high cost of ruxolitinib means that the National Institute for Clinical Excellence (NICE) has placed limitations on its use (NICE 2013). There is also evidence from a phase 3 trial that ruxolitinib benefits patients with PV (Vannucchi, Kiladjian *et al*. 2015), although it is not currently recommended for patients with PV in the UK. Hydroxyurea is widely used, but a proportion of patients do not respond to hydroxyurea or experience intolerable side-effects (Hernandez-Boluda 2010, Alvarez-Larrán, Pereira *et al*. 2012). The ability of methotrexate to inhibit JAK/STAT pathway activation might may make it a suitable alternative treatment for patients with myeloproliferative neoplasms.

5.3.3 Methotrexate as a potential treatment for patients with other haematological malignancies associated with activation of JAK/STAT signalling

The suppression of STAT phosphorylation observed was not specific for JAK/STAT pathway activation caused by JAK2V617F. This suggests that methotrexate may benefit patients with other haematological malignancies in which JAK/STAT activation plays a role in pathogenesis, such as those arising from fusion genes of JAK2 with PCM1, ETV6 and BCR (Bain and Ahmad 2014), T-cell large granular lymphocytic leukaemia (Koskela, Eldfors *et al*. 2012), chronic lymphoproliferative

disorders of natural killer cells (Jerez, Clemente *et al*. 2012) Waldenstrom's Macroglobulinaemia (Hodge 2014) chronic myeloid leukaemia (Warsch 2013) and chronic lymphocytic leukaemia (Rozovski, Wu *et al*. 2014)). Low-dose methotrexate is already used for the treatment of large granular lymphocytic leukaemia, which is associated with activating mutations in STAT3, where its effectiveness may result at least partly from its capacity to suppress JAK/STAT pathway activation (Lamy and Loughran 2011). In a recent phase 2 study of immunosuppressive drugs in LGL leukaemia the presence of the commonest activating STAT3 mutation, Y640F (a tyrosine to phenylalanine substitution), was strongly predictive of a clinical response to methotrexate (Loughran 2014).

5.4 Conclusions

This work has established that methotrexate suppresses JAK/STAT signalling in human cell lines *in-vitro.* The effect occurs following treatment at drug concentrations equivalent to those measured in patients taking high dose methotrexate as a chemotherapy drug, and the lower oral doses used to treat rheumatoid arthritis. This suggests that inhibition of JAK/STAT signalling may contribute to the mechanism through which methotrexate suppresses inflammation. It also suggests that methotrexate treatment might be beneficial in haematological malignancies associated with JAK/STAT pathway activation. Further work is needed to establish to what extent these effects observed after *in vitro* treatment of cell lines are applicable to pathway activation in disease.

Chapter 6: Effects of methotrexate on patient cells

6.1 Introduction

In order to determine whether the effects of methotrexate on JAK/STAT signalling observed in *Drosophila* and human cell lines were relevant for diseases involving increased JAK/STAT activation, I wished to examine the effect of methotrexate upon primary human cells. Primary cells involved in disease pathogenesis from patients with two categories of disease involving JAK/STAT activation were examined; fibroblast-like synoviocytes from patients with rheumatoid arthritis and haematopoietic stem and progenitor cells from patients with myelofibrosis.

Rheumatoid arthritis fibroblast-like synoviocytes

In patients with rheumatoid arthritis, the synovium of affected joints contains a characteristic cell type, the rheumatoid arthritis fibroblast-like synoviocyte (RA-FLS) which is not present in healthy joints, and is thought to be central to disease pathogenesis. These cells produce matrix-degrading enzymes and cytokines which contribute to the recruitment and activation of inflammatory cells (Huber, Distler *et al*. 2006). Synovial fibroblasts can be isolated from synovial tissue of patients with arthritis during arthroscopic surgery and maintained in culture. In contrast to synovial fibroblasts from patients with osteoarthritis, a type of arthritis in which immunity plays little role in the pathogenesis, RA-FLS show increased expression and phosphorylation of STAT1 when assessed using western blotting (Kasperkovitz, Verbeet *et al*. 2004).

Haematopoietic stem and progenitor cells

Myeloproliferative neoplasms arise from haematopoietic stem cells (HSC), and patients with MPNs have increased numbers of circulating cells with characteristics of HSCs, compared to normal individuals (Jamieson, Gotlib *et al*. 2006). The cell-surface glycoprotein CD34 is expressed on early haematopoietic stem and progenitor cells and used as a marker to identify HSCs, since CD34+ cell populations are enriched for cells which morphologically resemble blasts and have colony-forming abilities (Krause, Fackler *et al*. 1996). CD34 positive cells can be isolated from

patients with MPNs and show increased levels of STAT5 phosphorylation compared to CD34 positive cells obtained from healthy individuals (Anand, Stedham *et al*. 2011).

Patients with myelofibrosis who are undergoing a stem cell transplant provide an opportunity to obtain HSCs for research. The best outcomes for these patients are achieved when they undergo an allogeneic transplant, receiving stem cells from a donor. However, they also have their own cells harvested by apheresis and stored as a 'back-up' so that an autologous transplant can be performed if there are problems with the allogeneic transplant. For individuals in whom the allogeneic transplant has been successful, this apheresis material is surplus to requirements for clinical care, and provides a source from which the cells involved in disease pathogenesis can be isolated.

6.2 Results

6.2.1 RA-FLS show constitutive phosphorylation of STAT1

Patient characteristics of the three individuals from whom RA-FLS were obtained are not known. RA-FLS from the first patient grown in media containing 5% FBS show constitutive phosphorylation of STAT1, and some phosphorylation of STAT3. Stimulation with OSM leads to a massive increase in STAT1 phosphorylation and increased STAT3 phosphorylation, and pJAK1 becomes detectable (Figure 6-1).

6.2.2 The suppression of STAT1 phosphorylation by methotrexate varies between patients

The effect of methotrexate on constitutive STAT1 phosphorylation was examined in two further patients. In one patient methotrexate appeared to suppress STAT1 phosphorylation, whereas in the second STAT1 phosphorylation appeared to be unaffected (Figure 6-2).

6.2.3 MF Patient characteristics

Five patients were recruited, four of whom had samples analysed. Patient characteristics are summarised in Table 6-1.

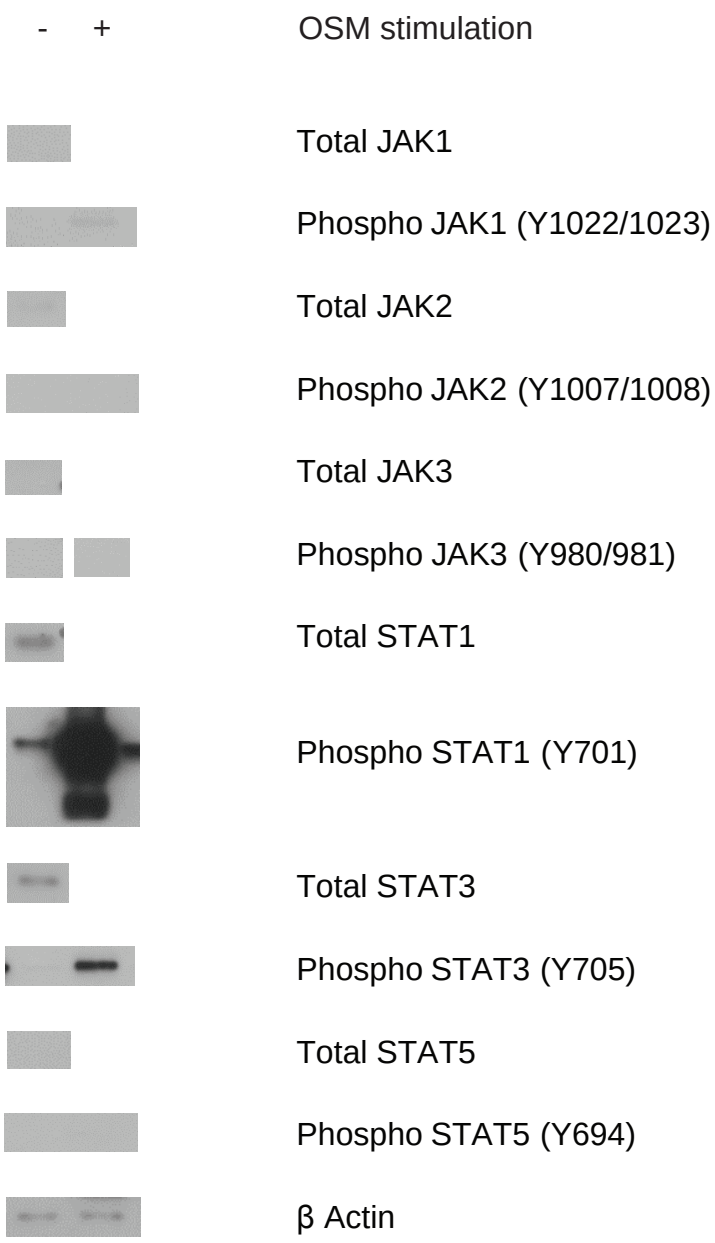


Figure 6-1. Profile of JAK and STAT expression, basal phosphorylation and phosphorylation following stimulation with OSM in RA-FLS (rheumatoid arthritis fibroblast-like synoviocytes) from one patient. JAK1 is not detected, but some pJAK1 is seen following OSM stimulation suggesting that it is present. JAK2 is seen but there is no basal or OSM-stimulated phosphorylation. JAK3 is not detected. STAT1 is present and shows constitutive phosphorylation. OSM stimulation leads to a massive increase in STAT1 phosphorylation. STAT3 is present and shows constitutive phosphorylation, and an increase in phosphorylation following OSM stimulation. No STAT5 is detected in these cells.

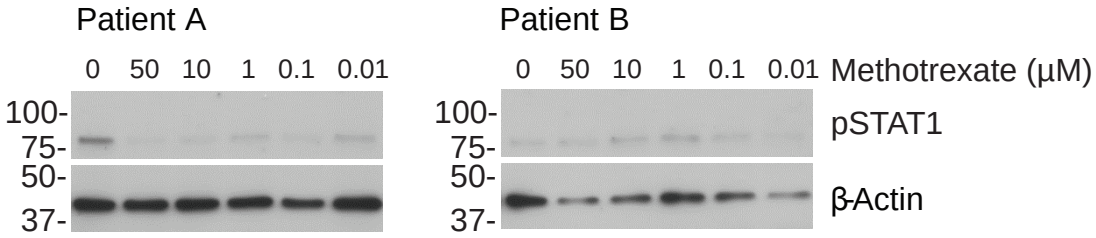


Figure 6-2. Methotrexate treatment reduces constitutive STAT1 phosphorylation in patient-derived RA-FLS (rheumatoid arthritis fibroblast-like synoviocytes), although this effect varies between patients. In cells from patient A, pSTAT1 levels are reduced at all methotrexate concentrations examined, with a more marked effect at higher concentrations. In cells from patient B uneven loading makes the blot difficult to assess, but methotrexte appears to have little effect on levels of pSTAT1.

Table 6-1. Patient characteristics of patients from whom apheresis material was used for isolation of HSCs (details deleted from library copy to protect patient anonymity).

Patient	**Age**	**Gender**	**Diagnosis**	***JAK2* mutation status**	***CALR* insertions/ deletions**	**Storage time (months)**	**CD34 count pre-harvest (cells/µl)**
Patient 1		2F, 2M	3 PMF, 1 post-PV MF	2 negative, 1 V617F, 1 unknown	1 negative, 3 unknown		Unknown
Patient 2							491
Patient 3							41
Patient 4							237
Mean	55					13	256
Standard deviation	4.8 years					7	225

6.2.4 Isolation of CD34 positive cells

Magnetic labelling was successfully used to isolate CD34 positive cells[1] from cryopreserved apheresis material from patients with MPNs. From 0.5ml aliquots of apheresis material the number of cells initially isolated in the CD34 positive fraction ranged from 0.3 x 10^6 to 1.2 x 10^6 (mean 0.8 x 10^6 standard deviation 0.47 x 10^6) with the number of cells isolated being roughly proportional to the patient's CD34+ count prior to harvest. The isolated cells were large round cells which grew in suspension, as expected for these cells (Figure 6-3).

Flow cytometry was used to determine the proportion of CD45+ cells which were CD34+, following culture for 48 – 72 hours. In the first patient analysed, the proportion of CD34+ cells in the mixed apheresis material prior to magnetic sorting was 12%. Following sorting, this percentage rose to 74% in the CD34 positive fraction, a 6 fold enrichment. In the CD34 negative fraction 2% of cells were CD34+ (Figure 6-4).

Comparison of the forward-scatter and side-scatter properties of the pre-sort, CD34 positive and CD34 negative fractions showed that the CD34 positive fraction

[1] Note on terminology: 'CD34+' will be used to refer to cells which are identified with CD34-PerCP using flow cytometry. 'CD34 positive' will be used to refer to the cell *population* obtained following magnetic sorting which is enriched for CD34+ cells but also contains variable numbers of CD34- cells.

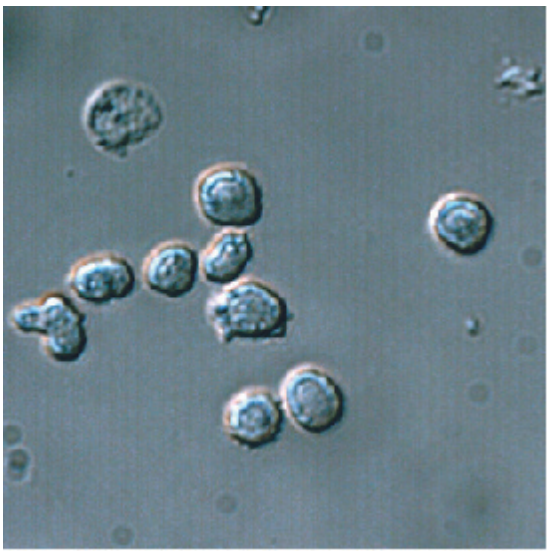
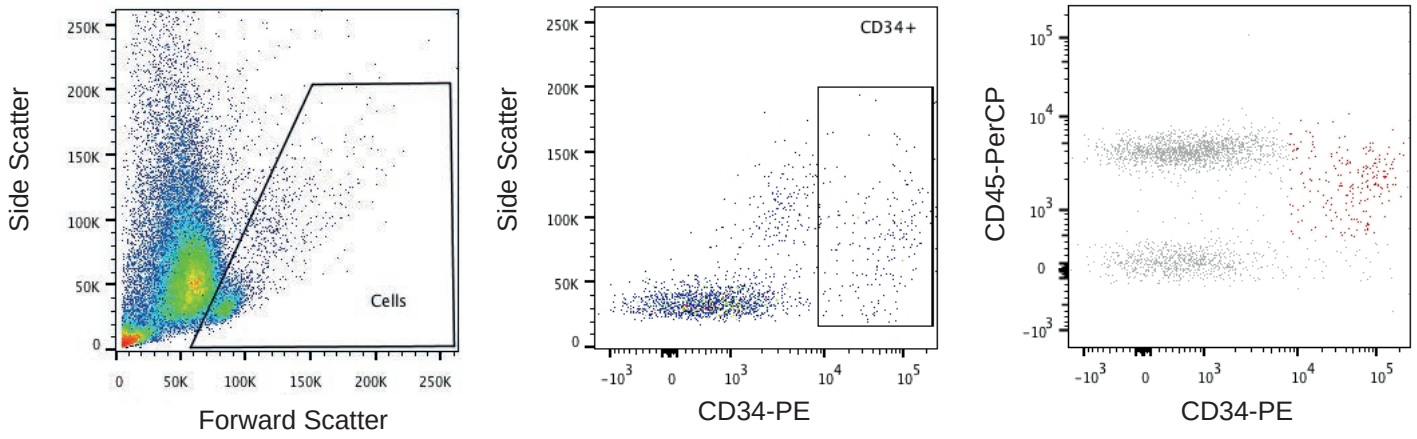
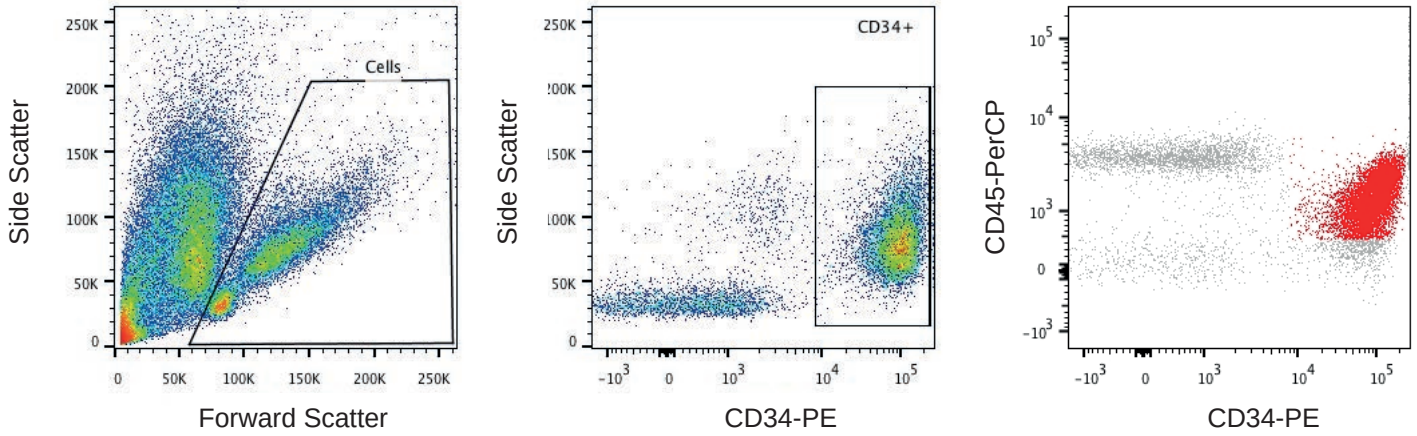


Figure 6-3. The cells in the CD34 positive fraction following magnetic sorting are round cells that grow singly in suspension (note that the brightness and contrast of the image have been altered to display better in the printed page).

A. Mixed



B. CD34 positive



C. CD34 depleted

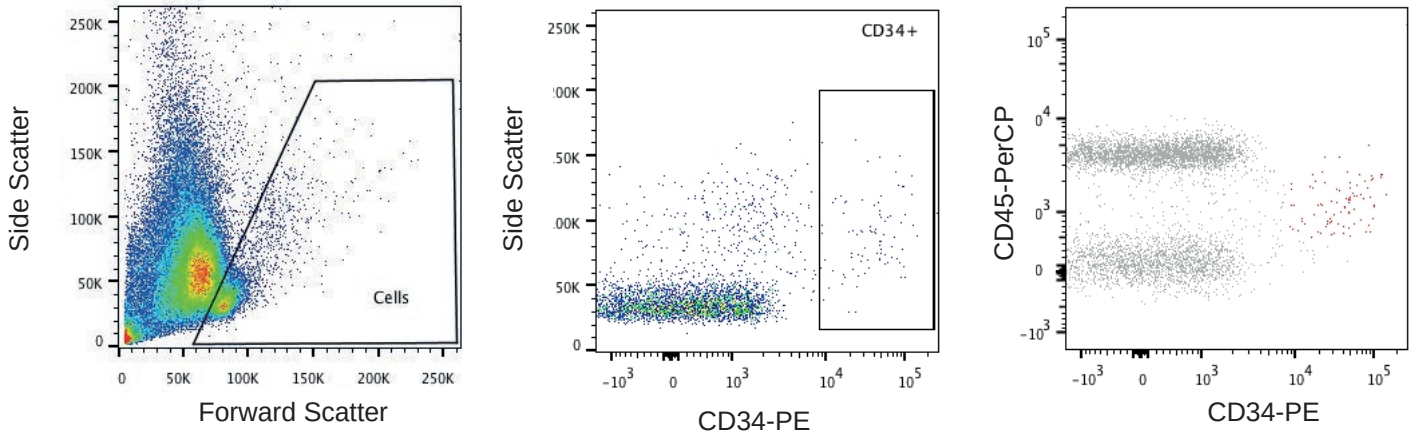


Figure 6-4. Magnetic sorting produces a cell population enriched for CD34+ cells. See materials and methods for gating strategy. Note the events to the left of the 'cells' gate are debris and dead cells. (A) In the mixed population of cells prior to magnetic sorting 12% of CD45+ cells are CD34+. (B) Following magnetic sorting, in the cell fraction that should be enriched for CD34+ cells this proportion rises to 74%. (C) In the cell fraction that should be depleted of CD34+ cells the proportion falls to 2%. Back gating shows that the CD34+ population (red) is CD45 dim, when compared to the overall population of single cells (grey). The CD34+ cells have distinctive forward scatter and side scatter characteristics, being larger and more granular than the other cells obtained from apheresis material.

contained a large and distinctive population of large, relatively granular cells which were CD45 dim, as expected for HSCs (Figure 6-4).

In the samples from three subsequent patients these enrichments were sufficient to produce an 89 – 99% pure population (Figure 6-5).

6.2.4 Viability of CD34 positive cells

Flow cytometry with Annexin V-FITC and propidium iodide was used to assess viability in the CD34 positive cell fraction at the time of the phenotyping analysis described above, i.e. after 48 – 72h in culture following isolation. The cultures contained a large proportion of dead cells (Figure 6-6). The dead cells had a characteristic pattern of forward scatter and side scatter and could be excluded from viability analyses in subsequent patients. As there was such extensive cell death in this initial culture phase it was not meaningful to look at percentages of live cells, however, a substantial proportion of the isolated cells from each patient were live (Figure 6-7). High numbers of dead cells were expected at this stage, when it is considered that cells must survive not only the freeze-thaw cycle, but also several hours of processing to isolate CD34+ cells immediately after thawing.

6.2.5 Maintenance of phenotype in culture

The proportion of cells in the CD34 positive fraction which were CD34+ fell during prolonged culture, with CD34+ cells no longer detected amongst the CD45+ population after 14 days in culture (Figure 6-8). This change is not unexpected as culture conditions are very different to the bone marrow microenvironment, but suggests that the degree to which the cells in culture indicate what happens in patients is limited.

6.2.6 Flow cytometry as a method to detect changes in STAT5 phosphorylation

It was anticipated that the number of cells available to examine drug effects of methotrexate in primary samples would be low. A flow cytometry protocol to determine pSTAT5 levels was developed, as this would allow examination of cells in numbers potentially too small to detect with western blotting.

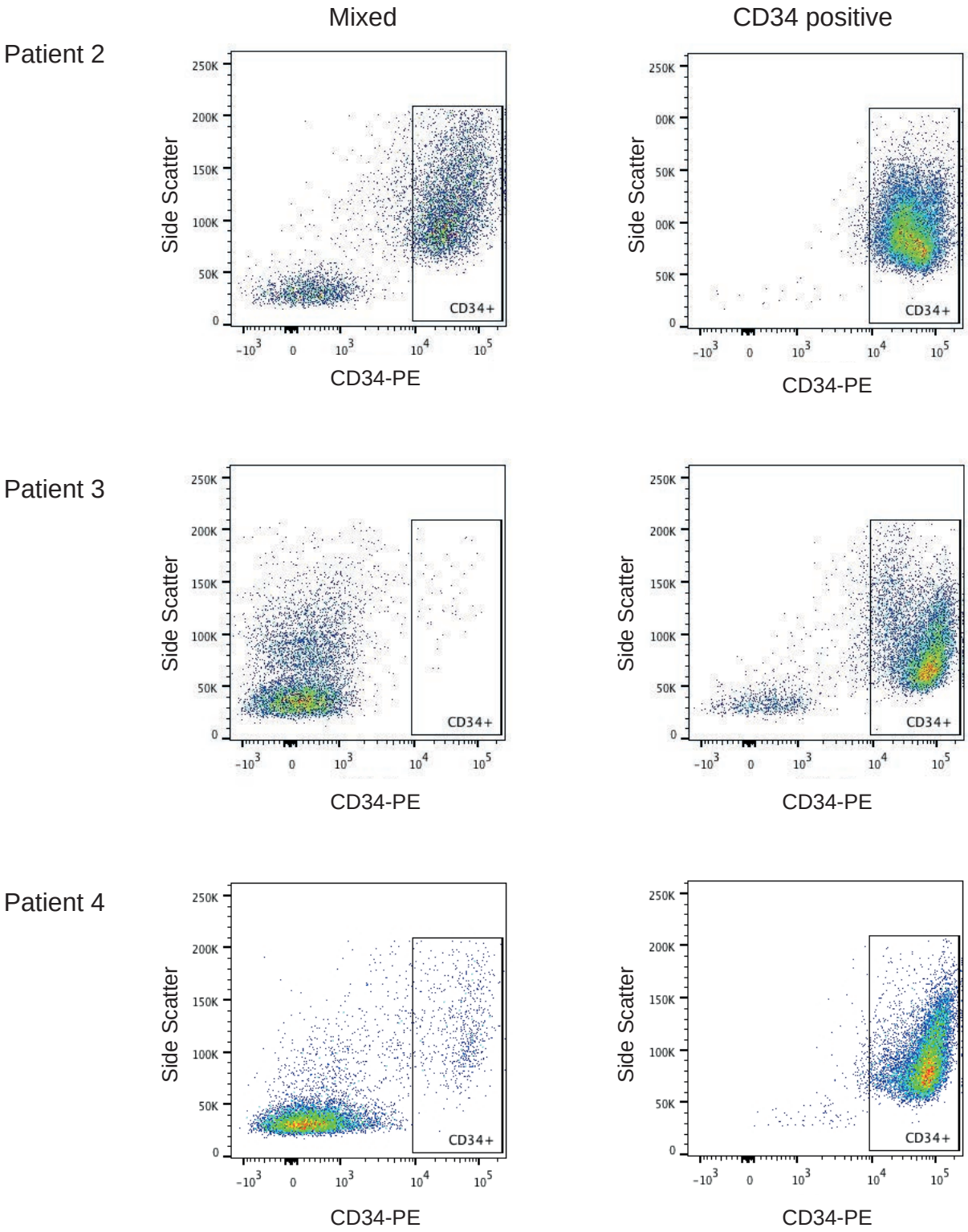


Figure 6-5. In three further patients, magnetic sorting produces a cell population enriched for CD34+ cells. Plots shown are for CD45+ cells (see materials and methods for gating strategy). For patient 2 the proportion of CD34+ cells rises from 72% to 99% (1.4 fold enrichment), for patient three the proportion rises from 0.65% to 89% (136 fold enrichment) and for patient four the proportion rises from 7.6% to 98.4% (13 fold enrichment).

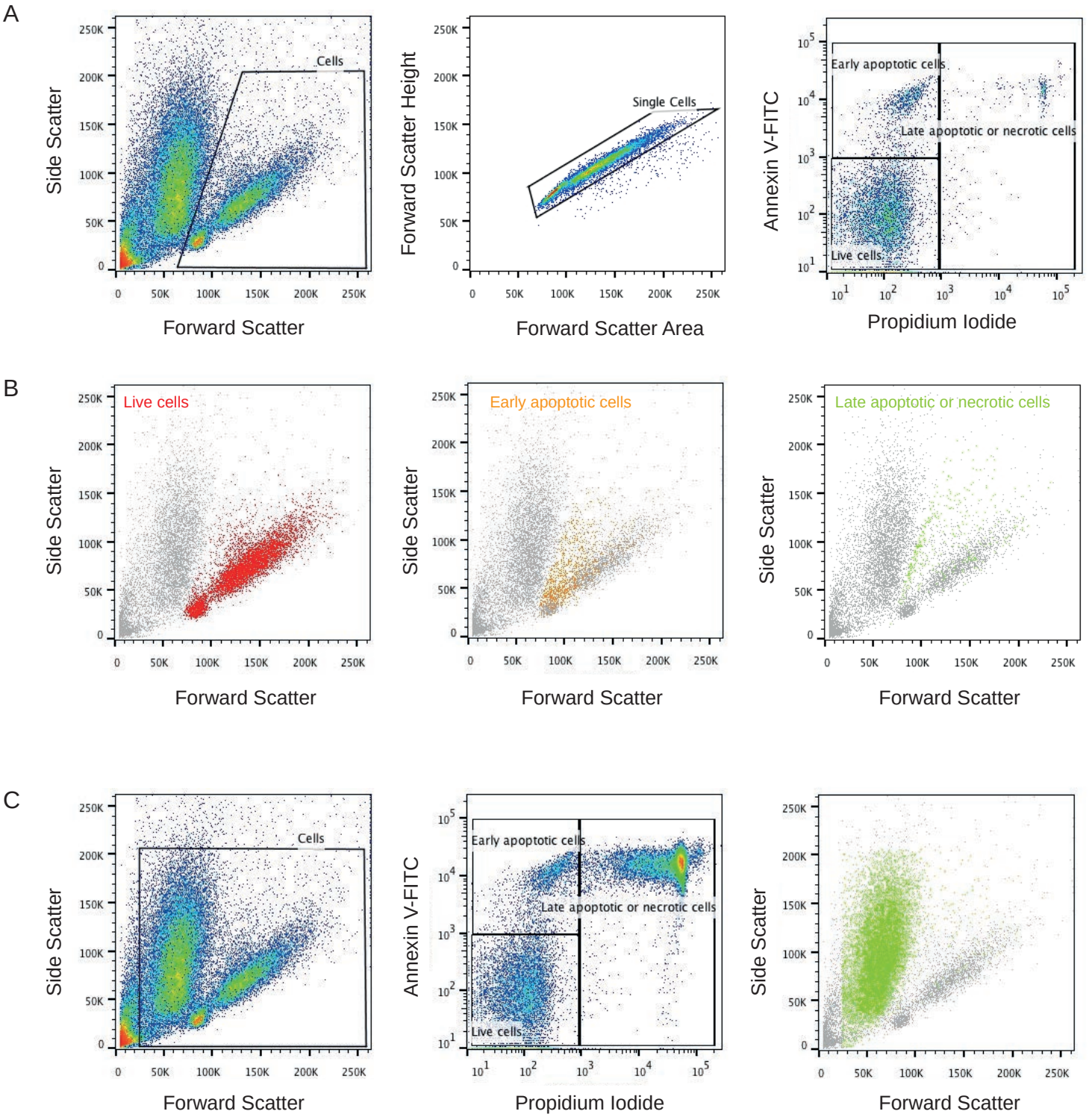
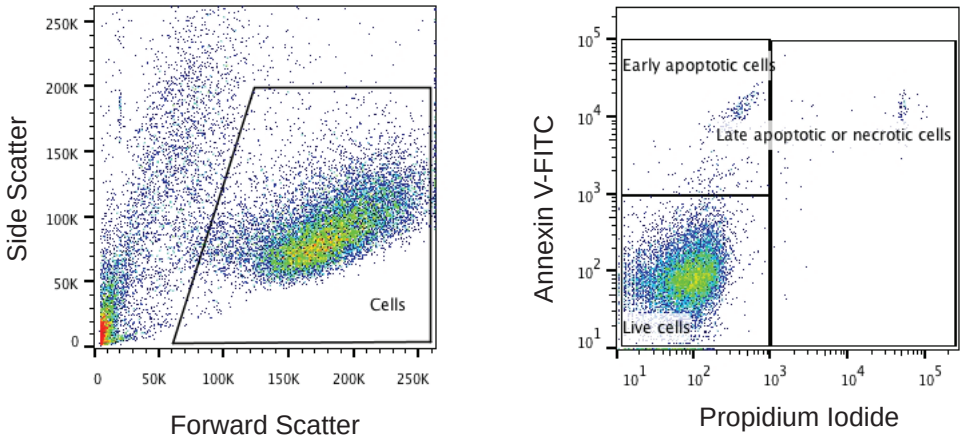
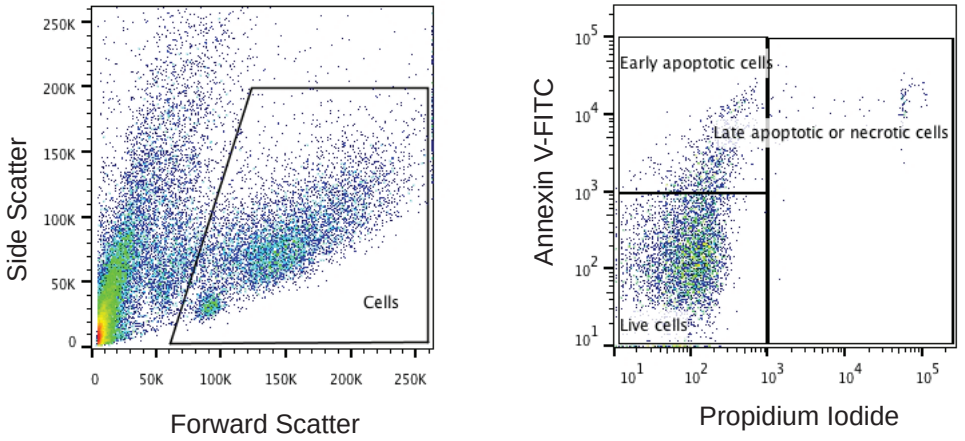


Figure 6-6. Live cells are found in the CD34 positive fraction following magnetic sorting. Figures are from the data from patient 1. (A) gating strategy to identify cells. Live cells do not take up propidium iodide and do not bind Anexin V. Cells that have entered apoptosis bind Annexin V, and those which are dead also take up propidium iodide. (B) Projection of the cell populations identified based on Annexin V and propidium iodide indicates that these populations of cells can also be identified by distinctive pattrns of forward scatter and side scatter. Grey shows all events represented on the forward scatter/side scatter plot, red live cells, orange early apoptotic cells and green late apoptotic and dead cells. (C) Re-positioning the gates confirms that the large number of events with low forward scatter (excluded by the gating strategy in A) represent dead cells.

Patient 2



Patient 3



Patient 4

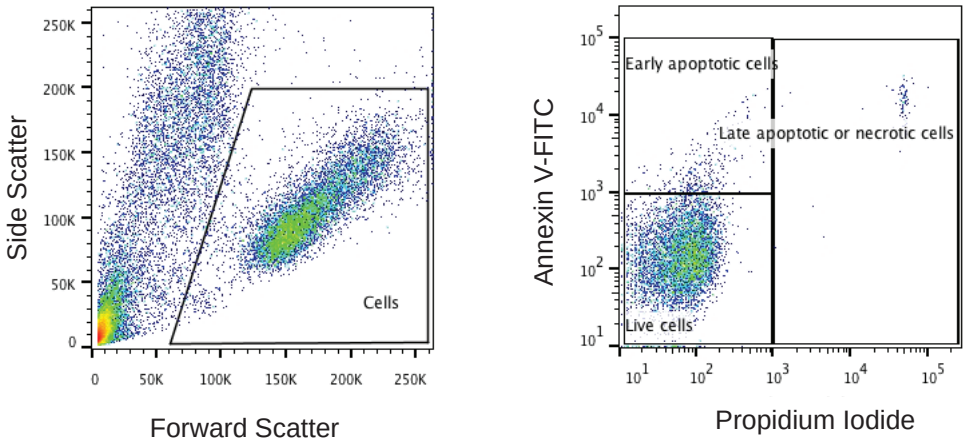


Figure 6-7. Live cells are obtained following magnetic isolation of CD34 positive cells from three subsequent patients.

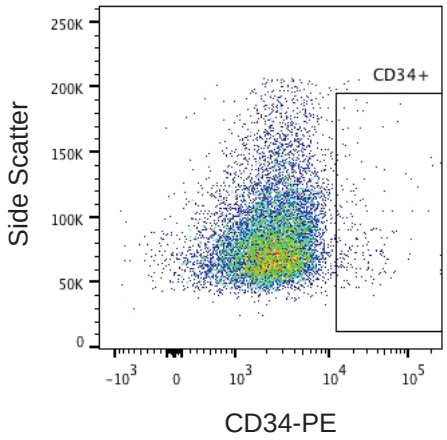


Figure 6-8. After fourteen days in culture, the CD45+ cell population originally derived from the CD34 positive fraction following magnetic separation contains very few CD34+ cells.

In HEL cells, flow cytometry could detect a dose-responsive reduction in pSTAT5 following treatment with ruxolitinib. At low drug concentrations the two techniques were comparable, whereas at higher drug concentrations the magnitude of the reductions measured with flow cytometry was smaller than that seen with western blotting done in cells from the same population (Figure 6-9).

Following treatment with methotrexate, a dose responsive reduction in pSTAT5 in HEL cells was seen with flow cytometry. The reduction was smaller than that observed when western blotting was used as the detection method (Figure 6-10). The forward-scatter and side-scatter properties of methotrexate-treated cells differed from those in DMSO-treated or ruxolitinib-treated cells. The pattern of pSTAT5 staining was also different, with a biphasic staining pattern indicating two populations of cells, with high and low levels of staining for pSTAT5. This may be indicative of a true difference in STAT5 phosphorylation, or may be a consequence of uneven staining or of the gating strategy used. In particular, it is possible that inclusion of dead cells might give this pattern.

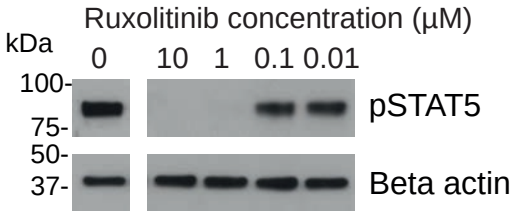
For the CD34 positive cells from patients with MF, flow cytometry was chosen to examine the effects of methotrexate in the cells available 48 hours after isolation. If cells were available in subsequent passages, they were used for western blotting.

6.2.7 Effect of methotrexate on STAT5 phosphorylation in HSCs

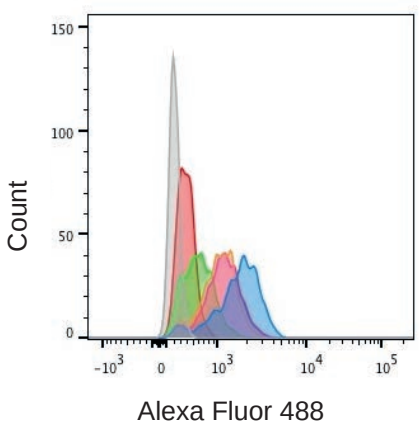
In the first patient analysed there was a substantial difference in fluorescence in cells stained with anti-pSTAT5 compared to isotype (a fourfold change in median fluorescence intensity). However, there was no decrease in pSTAT5 in cells treated with methotrexate, or the control cells treated with ruxolitinib. Median fluorescence intensity was in fact slightly higher in the ruxolitinib-treated and methotrexate-treated cells compared to DMSO-treated cells. However, this was probably due to a change in nonspecific antibody binding, since fluorescence in these cells stained with isotype antibody was also increased. The apparent increase in pSTAT5 is lost when the fold-change in median fluorescence intensity is calculated for each drug treatment (Figure 6-11).

In the three subsequent patients analysed the fluorescence intensity in the cells stained with anti-pSTAT5 was very low, comparable to that in the isotype-

A



B



	Ruxolitinib concentration (µM)	Western blotting % inhibition pSTAT5	Flow cytometry % inhibition pSTAT5
	0, Isotype		
	0		
	0.01	37	44
	0.1	45	38
	1	98	74
	10	100	87

C

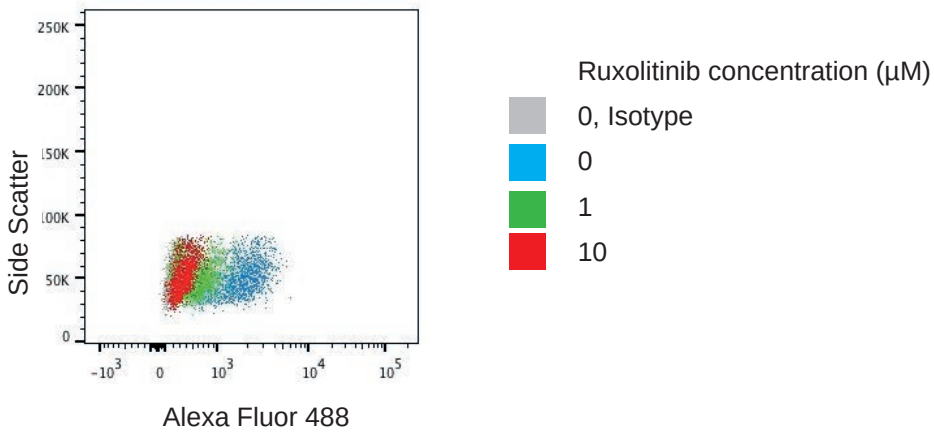
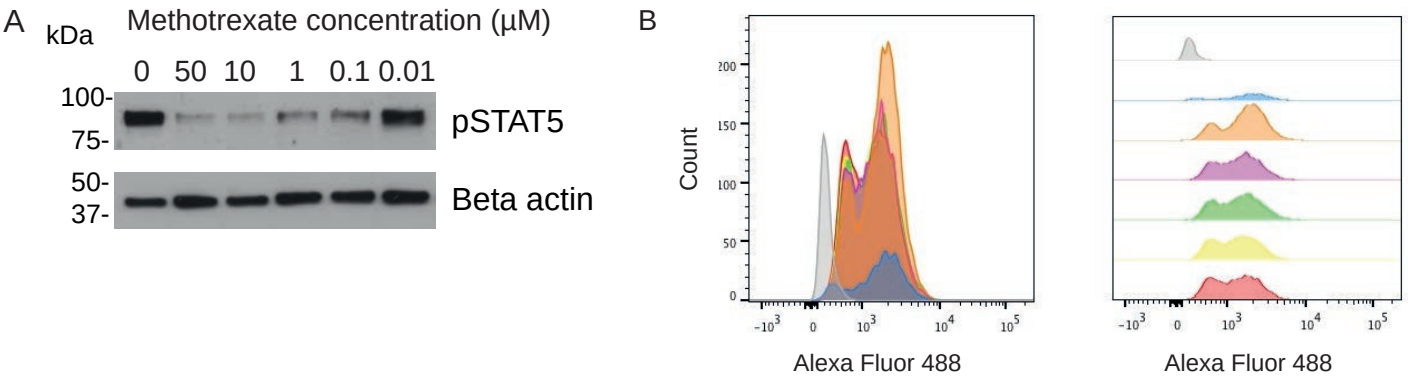


Figure 6-9. Flow cytometry can be used to detect dose-responsive changes in STAT5 phosphorylation in HEL cells treated with ruxolitinib.
A. Western blot showing reduction in pSTAT5 in populations of HEL cells treated with the indicated concentrations of ruxolitinib, compared to cells treated with DMSO alone. Note that the control and drug treated lanes are imaged from the same experimental replicate and blot, the intervening lanes which have been removed for clarity show methotrexate treated cells from the same experiment (see figure 7-10).
B. Histograms showing the dose-responsive reduction in pSTAT5 which can be detected with flow cytometry. Histograms have been smoothened to facilitate comparisons between doses. The percentage reductions in pSTAT5 measured using flow cytometry are comparable to those measured using western blotting in figure A at low drug concentrations, but at higher drug concentrations the inhibition seen with western blotting is greater than that seen with flow cytometry.
C. The dose-responsive reduction in STAT5 phosphorylation which can be detected with flow cytometry is apparrent when dot-plots of cells treated with a range of drug concentrations are superimposed.



	Methotrexate concentration (µM)	Western blotting % inhibition pSTAT5	Flow cytometry % inhibition pSTAT5	Flow cytometry MFI
	0, Isotype			234
	0			1917
	0.01	38	20	1846
	0.1	75	40	1506
	1	83	40	1509
	10	91	46	1407
	50	91	47	1381

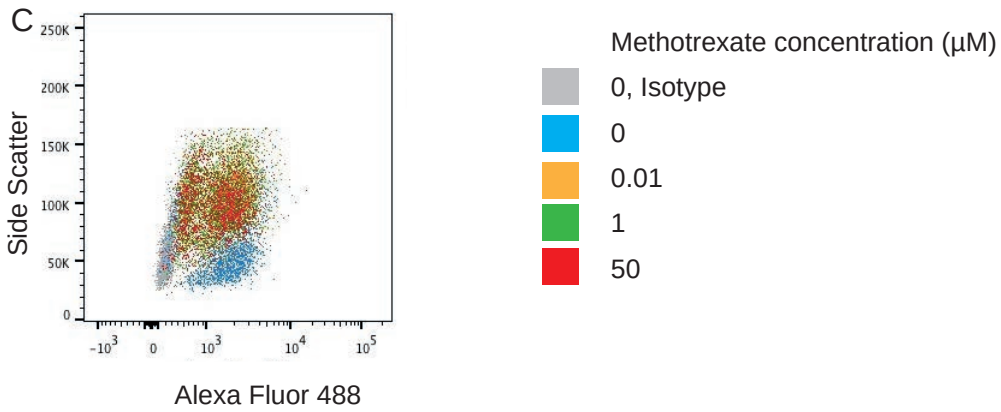
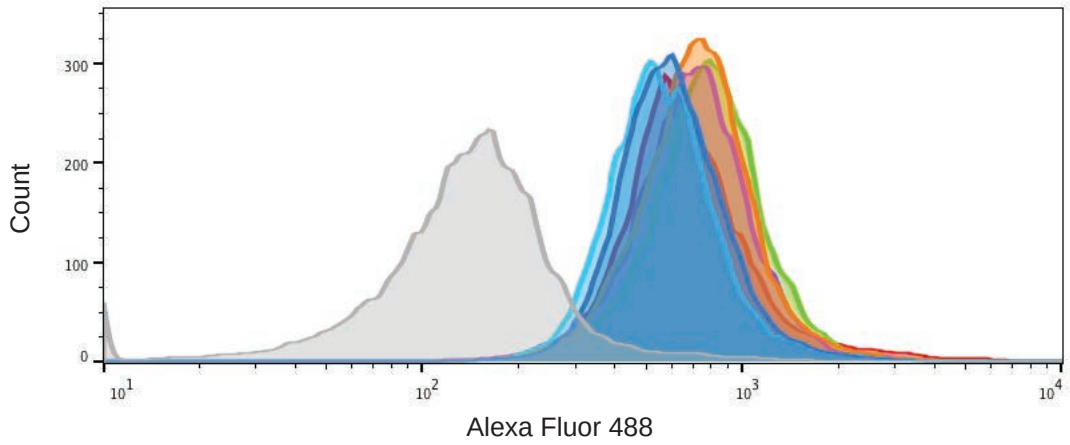


Figure 6-10. Flow cytometry can be used to detect dose-responsive changes in STAT5 phosphorylation in HEL cells treated with methotrexate (see materials and methods for gating strategy).
A. Western blot showing the reductions in pSTAT5 in populations of HEL cells treated with the indicated concentrations of methotrexate, compared to cells treated with DMSO alone.
B. Histograms showing the slight dose-responsive reduction in STAT5 phosphorylation in HEL cells treated with methotrexate that can be detected with flow cytometry. Histograms have been smoothened to facilitate comparison. There is a biphasic pattern of staining that is much more marked than in the ruxolitinib-treated cells. In the cell population examined, the reduction in pSTAT5 detected using flow cytometry is not as marked as that which is detected with western blotting. MFI = median fluorescence intensity.
C. When dot plots of methotrexate-treated HEL cells are superimposed, it is difficult to clearly see the dose-dependent reduction in STAT5 phosphorylation. Note that the number of events displayed has been reduced to facilitate comparison.



	Drug treatment	MFI pSTAT5	Fold change in MFI
	None, Isotype	143	
	None	593	4.1
	DMSO	553	4.1
	0.1uM methotrexate	718	3.8
	1uM methotrexate	703	3.9
	50uM methotrexate	774	4.3
	1uM ruxolitinib	641	4.9

Figure 6-11. Methotrexate treatment does not reduce pSTAT5 in HSCs when examined using flow cytometry. However, in this experiment there was also no reduction in pSTAT5 following treatment with ruxolitinib. MFI = median fluorescence intensity. Methotrexate and ruxolitinib appear to increase pSTAT5 slightly, but this is probably in fact a change in antibody binding, possibly to dead cells, since MFI in these cells is also increased with the isotype control. When the fold-change in MFI (MFI pSTAT5 / MFI isotype) is considered, this apparent effect of methotrexate is lost.

stained cells, suggesting either that the HSCs from these patients did not have phosphorylated STAT5 or that there was a problem with this experimental run (data not shown).

6.2.8 Methotrexate causes apoptosis in HSCs

Compared to DMSO alone, methotrexate causes substantial cell death in haematopoietic stem and progenitor cells (Figure 6-12). There is a significant reduction in the number of live cells following treatment with 1μM methotrexate compared to treatment with DMSO ($P = 0.005$, paired *t* test). This is in contrast to treatment with ruxolitinib, where there is not a significant difference in the number of live cells compared to treatment with DMSO alone ($P = 0.38$). The proportion of live cells following methotrexate or ruxolitinib treatment varies between patients. It should be noted that for patient 2 and patient 3, for which the lowest number of live cells were recorded, drug treatment and analysis were done in the same batch. The differences between patients may be due to experimental conditions.

6.2.9 Changes in STAT5 phosphorylation in HSCs following methotrexate treatment can be detected with western blotting

For patient 1, enough CD34 positive cells were available in a subsequent passage to the flow cytometry experiments for drug treatment experiments to be performed again and effects of methotrexate examined using western blotting. Experimental conditions were slightly different, as by this stage most cells in the population were not CD34+, and the media was not supplemented with factors that promote HSC growth. Constitutive phosphorylation of STAT5 could be detected in these cells. pSTAT5 was reduced by methotrexate treatment in a dose-dependent manner. However, levels of total STAT5 were also reduced to a similar extent (Figure 6-13).

6.3 Discussion

In this study I have investigated the effect of methotrexate on STAT phosphorylation on primary cells from patients with diseases associated with JAK/STAT pathway activation treated *in vitro*. The work has involved development of techniques for HSC isolation and to assess JAK/STAT pathway activation. The results are less conclusive than those obtained from experiments in cell lines, but nonetheless add to

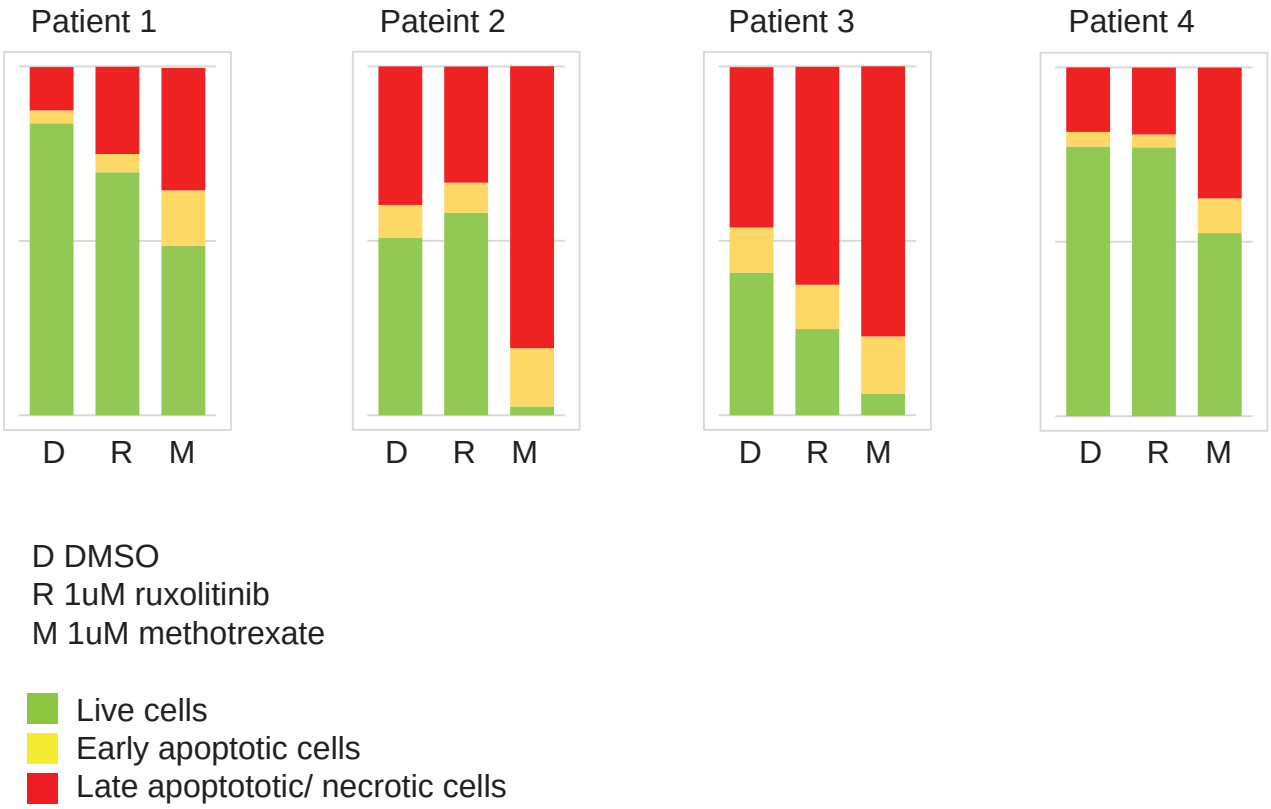


Figure 6-12. Treatment with methotrexate causes substantial apoptosis of HSCs. The effect of methotrexate varies between cells obtained from different patients.

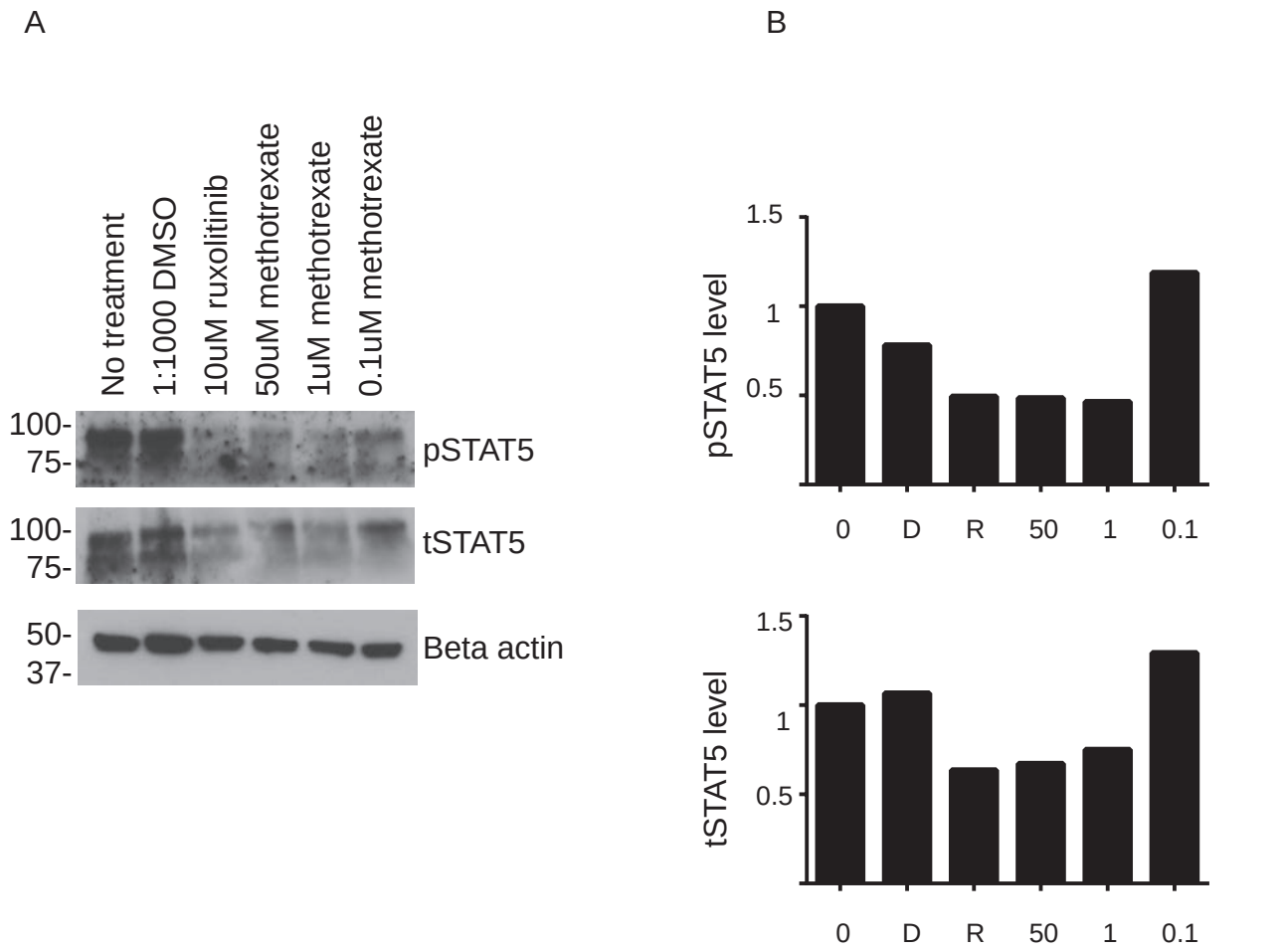


Figure 6-13. Methotrexate produces a dose-responsive reduction in pSTAT5 in primary cells from a patient with MF. However, the reduction in total STAT5 is similar to that seen for pSTAT5. A. Western blot. B. Quantification of band intensities of western blot. Band intensities have been adjusted for loading using the intensity of the actin band, then normalised to the value for the cells with no treatment. 0 no treatment, D DMSO, R 10uM ruxolitinib, 50, 1, 0.1 concentration of methotrexate, uM.

our understanding of the effects of methotrexate, ultimately contributing to consideration of whether these effects are likely to occur in patients.

6.3.1 Development of experimental methods

This work has demonstrated that viable CD34+ HSCs can be isolated from cryopreserved apheresis harvest surplus to requirements for clinical care. This may be of utility for other research examining haematopoietic stem and progenitor cells by providing a source of cells, particularly as it may be possible to use cells which are past their expiry date for transplant and would otherwise be disposed of. It has not been determined, however, whether similar results would be seen with cells from individuals without MPNs.

The protocol established to examine pSTAT5 using flow cytometry could be adapted for a future clinical study. Specifically, pSTAT5 could be examined in leucocytes in whole blood drawn from individuals prior to and following methotrexate treatment. Combining the protocol to examine pSTAT5 with staining using antibodies against cell surface markers that identify specific sub-population of cells would be particularly informative. However, there were problems with the protocol for flow cytometry, and further repetitions are required to determine whether failure to detect pSTAT5 in HSCs from three patients was due to problems with the experimental technique.

6.3.2 JAK/STAT signalling in RA-FLS

Methotrexate appeared to supress STAT1 phosphorylation in RA-FLS from one patient, and have little effect on cells from a second patient. This might suggest that suppression of JAK/STAT activation contributes to the effects of methotrexate in patients with rheumatoid arthritis. No clinical details about the patients from whom the cells were obtained were available, although it is interesting to speculate that these individuals might have differed in their response to methotrexate treatment.

STAT1 was examined because a preliminary profile of JAK/STAT pathway component expression and phosphorylation indicated STAT1 was constitutively phosphorylated in these cells *in vitro*, whereas phosphorylation of other STATs was weak or not detected. However, there is controversy over the role of STAT1 phosphorylation in rheumatoid arthritis, where it may well play a role in suppressing

rather than promoting inflammation (Walker and Smith 2005). This is suggested by a mouse model in which joint inflammation is exacerbated in *STAT1* knockout mice compared to wild-type mice (de Hooge, van de Loo *et al*. 2004). Furthermore, there is evidence that the pattern of JAK/STAT activation in the cytokine-rich microenvironment of the inflamed synovium is very different to that seen in a homogeneous culture of RA-FLS (Kasperkovitz, Verbeet *et al*. 2004).

Finally, an examination of the effects of methotrexate upon JAK/STAT signalling in leucocyte sub-groups in rheumatoid arthritis would be highly relevant. In peripheral blood, 30 minutes incubation in 10μM methotrexate does not have any effect on ligand-stimulated STAT phosphorylation in CD4+ T lymphocytes, B lymphocytes or monocytes when assessed with flow cytometry (Prof. Olli Silvennoinen and Dr. Pia Isomäki, personal communication). Given that my experiments in cell lines indicated that the effects of methotrexate take around 24 hours to become apparent, it would be interesting to examine whether methotrexate has effects on leucocytes following a longer exposure.

6.3.3 JAK/STAT signalling in HSCs from patients with myelofibrosis

Examination of methotrexate effects in primary cells from patients with MPNs adds substantially to the data from cell lines when considering whether suppression of STAT phosphorylation by methotrexate may be clinically relevant. It is disappointing that, due to challenges with working with very small numbers of cells and potential problems with flow cytometry, data on the effects of methotrexate on STAT5 phosphorylation is only available for one of the four patients.

Interpretation of the data is not straightforward. For this patient, western blotting suggested that methotrexate suppressed STAT5 phosphorylation, whilst also lowering levels of total STAT5, in a late passage of cells. Flow cytometry on methotrexate-treated cells from the same patient from an earlier passage, however, showed no effect of methotrexate or ruxolitinib on pSTAT5 but indicated that approximately 50% of the methotrexate-treated cells were dead. This disparity could relate to experimental techniques – western blotting examines mixed cell population of live and dead cells. If the rate of turnover of pSTAT5, or indeed tSTAT5, in dead cells was faster than that of actin this would appear as a reduction in STAT5

phosphorylation on a western blot. It was notable that in other patients in whom pSTAT5 was not detected with flow cytometry (although it is not clear whether this was a true feature of the cells or an experimental problem) the proportion of live cells was much lower. One approach to address this would be to develop a multi-colour flow cytometery protocol that included a viability marker alongside staining for pSTAT5, with the caveat that the approach to assess viability would have to be suitable for permeabilised cells.

Differences in culture conditions could also contribute to the conflicting results, since the early passage cells were treated in the presence of a cytokine supplement to favour HSC expansion that included TPO, a JAK/STAT pathway ligand. Given that experiments in HEL cells suggested that JAK/STAT pathway stimulation with EPO could overcome pathway suppression by methotrexate this may have led to the effect of methotrexate being masked.

The cell populations in the early and late passage will have been different, as no CD34+ cells remained after 14 days in culture. It is not clear whether this is due to differentiation leading to downregulation of CD34 expression or due to selective expansion of the, initially small, population of CD34 negative cells in this population. It is interesting that others have observed CD34 positive cells to be relatively resistant to JAK inhibition, whereas CD34 negative cells, representing a more differentiated myeloid population, did show a reduction in STAT5 phosphorylation following treatment with a JAK inhibitor (Anand, Stedham *et al*. 2011).

Finally, the extent to which these primary cells reflect what methotrexate might do in patients with myelofibrosis needs to be considered. Specifically, it is not known what proportion of cells in the CD34 positive population are from the malignant clone, and what proportion are normal HSCs. Although of little relevance in my patient group, which included only one individual with the JAK2V617F mutation, it is interesting that others have been able to examine STAT phosphorylation (Chen, Beer *et al*. 2010) and drug effects (Wyspianska, Bannister *et al*. 2014) in JAK2 mutant and wild-type cells from the same patient, by performing genotyping of individual colonies in colony formation assays.

6.4 Conclusions

This data tentatively supports the idea that effects of methotrexate as a suppressor of JAK/STAT signalling in cell lines also apply in primary patient cells, although it highlights problems with western blotting as a technique to examine drug effects. It suggests methotrexate may suppress JAK/STAT phosphorylation in patients. Strategies to examine JAK/STAT phosphorylation in patients taking methotrexate as part of their clinical care would be a possible next step to determine this.

Chapter 7: Conclusions and future directions

7.1 Overview

Activation of the JAK/STAT signalling pathway is observed in haematological malignancies and solid tumours. JAK/STAT activation contributes to the malignant phenotype of cancer cells and there is an association between activation of certain pathway components and prognosis in many types of cancer (Bowman, Garcia *et al.* 2000, Chen, Staudt *et al*. 2012). There is evidence of the success of targeting JAK/STAT signalling as a therapeutic strategy from clinical trials in myeloproliferative neoplasms (Harrison, Kiladjian *et al*. 2012, Vannucchi, Kiladjian *et al*. 2015), and pre-clinical studies suggest that targeting the pathway may be beneficial in solid tumours (Rath, Naidu *et al.* 2014).

Some of the molecular events leading to aberrant JAK/STAT pathway activation in cancer are understood. However, in many cases the causes of pathway activation, and the factors which modulate activation of the pathway, are not well defined. A better understanding of the regulation of JAK/STAT pathway activation in cancer may ultimately lead to improved treatments for patients, if it helps to refine diagnostic classifications and predict prognosis, and predict responses to therapies. Furthermore, patients would also benefit from access to a wider range of therapies which target the pathway.

The aim of this work was to improve our understanding of genetic and chemical modulators of JAK/STAT signalling. The role of the gene *ANKHD1*, known to positively regulate JAK/STAT signalling in *Drosophila* and cell lines, was investigated in haematological malignancies and the skin cancer malignant melanoma. The effect of methotrexate on JAK/STAT signalling in human cell lines and primary cells was examined, to build on the identification of methotrexate as a suppressor of JAK/STAT pathway activation in a high-throughput screen.

7.2 Summary of findings

This work has established the expression patterns of ANKHD1 in normal blood cell types, where it is found in monocytes and lymphocytes but absent from neutrophils. ANKHD1 also appears to be variably expressed in CD34+ haematopoietic stem

cells, and expressed in blasts in acute myeloid leukaemia and acute lymphocytic leukaemia.

In melanoma, ANKHD1 can be found in the nucleus and the cytoplasm and is expressed in all the histological sub-types of melanoma examined. The level of nuclear and cytoplasmic expression is variable, although there is no clear relationship between expression patterns and histological sub-type or melanoma thickness.

The study of methotrexate shows that methotrexate suppresses STAT phosphorylation in human cell lines, with some effect on JAK phosphorylation. This effect occurs at concentrations of methotrexate equivalent to those measured in patients receiving high-dose intravenous methotrexate as a chemotherapy drug, and low dose oral methotrexate for the treatment of rheumatoid arthritis. Methotrexate suppresses STAT phosphorylation due to JAK2V617F, and has an effect comparable to the JAK2 inhibitor ruxolitinib which is currently in use clinically. The suppression of STAT phosphorylation is partially independent of the effects of methotrexate on folate metabolism, and can be overcome with ligand stimulation. There is some evidence that the effect of methotrexate on STAT phosphorylation in cell lines also occurs in disease-relevant primary cells from patients with rheumatoid arthritis and myelofibrosis.

7.3 Future work

ANKHD1

The work on ANKHD1 expression in blood cells, haematological malignancies and melanoma was carried out in a relatively small number of samples. A more robust indication of ANKHD1 expression would be obtained from examination of a larger number of samples, and could enable examination of the relationship between ANKHD1 expression and disease features. However, to justify profiling ANKHD1 expression in a larger number of patient samples a better understanding of the role ANKHD in normal cells and in these diseases is needed.

To investigate the function of ANKHD1 in normal blood a mouse model could be created. Knockout of ANKHD1 appears to cause embryonic lethality (S.Constantinescu, unpublished observation). Generating a model in which ANKHD1 was conditionally knocked down only in certain blood cell lineages might

overcome this, however. An alternative approach would be to attempt to reconstitute haematopoiesis in lethally irradiated mice using stem cells in which ANKHD1 is knocked down with shRNA from a lentiviral vector.

The rationale for investigating ANKHD1 expression in haematological malignancies and melanoma is based on evidence that it affects Ras/Raf/MEK/ERK, Hippo and JAK/STAT signalling. Further data would be useful to validate these findings, particularly for the effects on Ras/Raf/MEK/ERK and JAK/STAT signalling. Determining the effects of ANKHD1 knockdown and overexpression upon constitutive and ligand-stimulated STAT and ERK phosphorylation in cell lines, which could be done with immunofluorescence and western blotting, may be informative here. There is also the potential to identify which domains of ANKHD1 are responsible for the signalling effects as I have plasmids encoding tagged fragments of the protein containing only certain domains. These could also be used for co-immunoprecipitation experiments to examine which regions of the protein mediate the known physical interactions with Hippo and JAK/STAT pathway components.

Methotrexate

There are two questions to be addressed in future work on the effects of methotrexate on JAK/STAT signalling: establishing the mechanism through which methotrexate suppresses the signalling pathway, and determining whether methotrexate may be a suitable treatment for patients with JAK/STAT associated malignancies, in particular MPNs.

There is some evidence to suggest methotrexate might be binding to and inhibiting JAKs. Methotrexate appeared to reduce JAK1 phosphorylation in HDLM-2 cells. Furthermore, *in silico* modelling suggested that methotrexate could bind the kinase pocket of JAK2 and that the energy involved in this binding was physiologically realistic. Equipment to directly measure protein-substrate interactions is available in the department of biomedical science. A caveat to this approach is the impact that ATP concentration is likely to have on these binding interactions. Although the screen for effects on *in vitro* kinase assays did not show an effect on JAK activity, it may nonetheless be relevant to examine methotrexate

effects in *in vitro* kinase assays further, particularly if it was possible to examine polyglutamated forms of methotrexate in these assays.

To consider the mechanism through which methotrexate may be affecting JAK/STAT signalling, a more conclusive determination is needed of whether the effects are secondary to inhibition of DHFR. This could be addressed by examining the effects of methotrexate on JAK/STAT signalling in the presence of hypoxanthine, which should rescue the effects of methotrexate on nucleotide synthesis.

Several approaches may be used to generate further data needed to determine whether investigating methotrexate as a treatment for MPNs in a clinical trial would be worthwhile. Further investigation of the *in vitro* effects of methotrexate in CD34+ and CD34- cell fractions from patients with MPNs is needed. Approaches to isolate these cells from peripheral blood might enable a larger number of patients to be examined than was possible using apheresis samples. It would also be of interest to examine STAT phosphorylation in peripheral blood cells of patients taking methotrexate as part of their routine clinical care (both patients with inflammatory conditions and those receiving methotrexate as part of a chemotherapy regime). This would allow the pharmacokinetics of methotrexate to be taken into account. Basal levels of JAK/STAT pathway activation in blood cell types would need to be determined to establish whether this approach would be informative.

Finally, the effect of methotrexate could be examined in mouse models of MPNs. Effects on disease features such as haematocrit and spleen weight could be examined. However, some direct examination of effects on JAK/STAT pathway activation would be needed. Methotrexate toxicity, due to effects on folate metabolism, would lead to cytopaenias and it would be important to distinguish this from a specific effect on JAK/STAT signalling.

7.4 Impact

The impact of this work arises from the study of methotrexate as a suppressor of JAK/STAT signalling. If subsequent experiments suggest pursuing an investigation of methotrexate as a treatment for patients with MPNs is worthwhile, it has the potential to improve quality of life and potentially prolong life in this group of patients. Furthermore, the relatively low cost of methotrexate means that its use as a

treatment for MPNs could bring financial benefits by reducing the cost of care for these patients.

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