From the Department of Cell and Molecular Biology
and the Ludwig Institute for Cancer Research
Karolinska Institutet, Stockholm, Sweden

THE ROLE OF KIF1BBETA IN NEUROBLASTOMA TUMOUR SUPPRESSION DURING SYMPATHETIC NEURON DEVELOPMENT

Stuart Fell

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The Role of KIF1Bβ in Neuroblastoma Tumour Suppression During Sympathetic Neuron Development

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Stuart Fell

Principal Supervisor:
Associate Professor Susanne Schlisio
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology
and Ludwig Institute for Cancer Research

Co-supervisor:
Professor Thomas Perlmann
Karolinska Institutet
Department of Cell and Molecular Biology
and Ludwig Institute for Cancer Research

Opponent:
Professor Ruth Palmer
University of Gothenburg
Sahlgrenska Academy
Department of Medical Biochemistry and Cell biology

Examination Board:
Associate Professor Margareta Wilhelm
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Professor Finn Hallböök
Uppsala University
Department of Neuroscience

Associate Professor Igor Adameyko
Karolinska Institutet
Department of Physiology and Pharmacology

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ABSTRACT

Neuroblastoma, the most common extra-cranial childhood tumour, is believed to arise from precursor cells of the sympathetic nervous system (neuroblasts). Hemizygous loss of chromosome 1p36 strongly correlates with poor prognosis in neuroblastoma and the 1p36 gene KIF1Bβ has been proposed to be a pathogenic target of this deletion. KIF1Bβ is required for normal developmental apoptosis of neuroblasts induced by competition for NGF during embryogenesis. We hypothesised that KIF1Bβ's role in developmental culling of neuroblasts is a molecular mechanism that suppresses neuroblastoma development.

In **Paper I**, we found that KIF1Bβ causes apoptosis through interaction with RNA helicase A (DHX9), promoting accumulation of DHX9 in the nucleus, and up-regulation of proapoptotic XIAP-associated factor 1 (XAF1). We demonstrate that NGF deprivation induced apoptosis requires DHX9 and that loss of KIF1Bβ expression in neuroblastomas with hemizygous deletion of 1p36 impairs DHX9 nuclear localization, implicating loss of DHX9 nuclear activity in neuroblastoma pathogenesis.

In **Paper II**, we discovered that the Ca2+-dependent phosphatase calcineurin (CN) is activated by KIF1Bβ. CN mediates a diversity of cellular responses, and its activity is affected in various diseases. We show that KIF1Bβ effects mitochondrial dynamics through dephosphorylation of Dynamin-related protein 1 (DRP1) by CN, causing mitochondrial fission and apoptosis. We found that KIF1Bβ enables CN recognition of all known substrates, and DHX9, suggesting a general involvement of KIF1Bβ in CN signalling activity. DRP1 dephosphorylation or CN activity could not be stimulated by pathogenic KIF1Bβ mutations previously identified in neuroblastomas and ,0pheochromocytomas. Loss of KIF1Bβ and DRP1 expression in 1p36 hemizygous-deleted neuroblastomas, implies that dysregulation of mitochondrial dynamics and calcineurin activity affect high-risk and poor-prognosis neuroblastoma.

In **Paper III**, we showed that loss of KIF1Bβ in the sympathetic nervous system impairs nervous development and function and dysregulates genes required for sympathoadrenal lineage differentiation. We discovered that KIF1Bβ mediates anterograde transport of the NGF receptor TRKA and is required for NGF-dependent neuronal differentiation. Moreover, TRKA transport is impeded by pathogenic KIF1Bβ mutations identified in neuroblastoma. We observed reduced expression of neuronal differentiation proteins in KIF1Bβ deficient mouse neuroblasts and primary human neuroblastomas that lack KIF1Bβ. Furthermore, transcriptomic analyses revealed that loss of KIF1Bβ in mouse sympathetic neuroblasts causes changes in gene expression similar to those seen in high-risk neuroblastoma, independent of MYCN amplification and the loss of genes neighbouring KIF1B on chromosome 1p36.

Thus, defective precursor cell differentiation, and impaired apoptosis, common traits of aggressive childhood malignancies, are pathogenic effects of KIF1Bβ loss in neuroblastoma.

LIST OF SCIENTIFIC PAPERS

I. Chen ZX, Wallis K, **Fell SM**, Sobrado VR, Hemmer MC, Ramskold D, Hellman U, Sandberg R, Kenchappa RS, Martinson T, Johnsen JI, Kogner, P, Schlisio S
RNA Helicase A Is a Downstream Mediator of KIF1B Tumor-Suppressor Function in Neuroblastoma
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II. Li S, **Fell SM**, Surova O, Smedler E, Wallis K, Chen ZX, Hellman U, Johnsen JI, Martinsson T, Kenchappa RS, Uhlén P, Kogner P, Schlisio S
The 1p36 Tumor Suppressor KIF1Bβ Is Required for Calcineurin Activation, Controlling Mitochondrial Fission and Apoptosis
Developmental Cell (2016) 36, 164–178

III. **Fell SM**, Li S, Wallis K, Kock A, Surova O, Rraklli V, Höfig C, Mittag J, Arsenian Henriksson M, Kenchappa RS, Holmberg J, Kogner P, Schlisio S
KIF1Bβ links neuroblastoma and neurodegenerative disease through anterograde transport of TRKA
Manuscript

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LIST OF ABBREVIATIONS

ALK	Anaplastic lymphoma receptor tyrosine kinase
ARTN	Artemin
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
CALM2	Calmodulin 2
CAMTA1	almodulin binding transcription activator 1
CASZ1	Calmodulin binding transcription activator 1
CHD5	Chromodomain helicase DNA binding protein 5
CMT	Charcot-Marie-Tooth disease
CN	Calcineurin
CNA	Protein phosphatase 3 catalytic subunit alpha (PPP3CA)
CNB	Protein phosphatase 3 regulatory subunit B, alpha (PPP3R1)
CREB	cAMP responsive element binding protein
DBH	Dopamine beta-hydroxylase
DHX9	DExH-box helicase 9
DRG	Dorsal root ganglia
DRP1	Dynamin 1 like (DNM1L)
EGLN3	Egl-9 family hypoxia inducible factor 3
EPAS1	Endothelial PAS domain protein 1
ERK1/2	Mitogen-activated protein kinase 3/1 (MAPK3/1)
FHA	forkhead associated
GATA3	GATA binding protein 3
GDNF	Glial cell derived neurotrophic factor
GFL	GDNF-family ligand
GFRA3	GDNF family receptor alpha 3
HAND2	Heart and neural crest derivatives expressed 2
HRAS	HRas proto-oncogene, GTPase
JNK	JUN n-teminal kinase / Mitogen activated protein kinase

JUN	Jun proto-oncogene, AP-1 transcription factor subunit
KIF1BP	KIF1 binding protein
KIF1Bβ	Kinesin family member 1B isoform beta
MADD	MAP kinase activating death domain
MASH1	achaete-scute family bHLH transcription factor 1 (ASCL1)
MIR34A	microRNA 34a
MYCN	V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
NEFH	Neurofilament heavy
NF1	Neurofibromin 1
NGF	Nerve growth factor
NTF3	Neurotrophin 3
P75NTR	Nerve growth factor receptor (NGFR)
PH	Pleckstrin homology
PHOX2A	Paired like homeobox 2a
PHOX2B	Paired like homeobox 2b
PtdIns(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
RAB3A	RAB3A, member RAS oncogene family
RAS-GAP	Ras family GTPas-activating protein
RET	Ret proto-oncogene
RNA	Ribonucleic acid
RNAi	RNA interference
SCG	Superior cervical ganglia
SDHD	Succinate dehydrogenase complex subunit D
SNS	Sympathetic nervous system
STMN2	Stathmin 2
TH	Tyrosine hydroxylase
TP73	Tumor protein p73
TRKA	Neurotrophic receptor tyrosine kinase 1 (NTRK1)
TRKC	Neurotrophic receptor tyrosine kinase 3 (NTRK3)
VHL	Von Hippel-Lindau tumor suppressor

XAF1	XIAP associated factor 1

1 INTRODUCTION

1.1 WHAT IS CANCER?

Cancer is essentially the unusual and injurious growth of cells that have the ability to spread to and grow in other parts of the body. It is not one disease as such, but the result of cells gaining the capacity to grow when and or where they wouldn't normally, and then invading other parts of the body and growing there. Viral infections, exposure to certain substances, hormonal imbalances, stress, exposure to radiation and even old age can cause cancer. The factors are diverse, but they have something in common. They all act on our genes or the way our genes are expressed. Correct expression of our genes is essential for maintaining the normal identity of a cell, e.g. as a blood cell, muscle cell or neuron.

1.2 MUTATIONS!

Genetic (or genomic) instability is an essential feature of cancer, because it allows cells to transform into something else. Cancer cells must be able to survive, proliferate and spread outside their original environment. Genomic instability, involving mutations and changes in gene expression, creates a permissive environment for the acquisition of new cellular characteristics, and subsequent selection for traits that are advantageous for fledgling cancer cells.

Cancer is predominantly a disease of the elderly, and this is most probably due to accumulation of genetic damage over time. In a large metastudy of cancer associated mutations, Vogelstein and colleagues found that tumours that tend to develop during adult life, such as brain, colon, pancreas or breast cancers, harbour function affecting somatic mutations in between 33 to 66 genes on average (Vogelstein et al., 2013). This number rises to around 200 genes with predicted functional mutations in cancers where a strong mutagen (ie UV light or cigarette smoke) is a driving oncogenic factor, such as in melanoma and lung cancer.

1.3 CHILDHOOD VS ADULT CANCER

The mutational status of paediatric malignancies, contrasts strongly with that observed in adult cancer. Relatively few function-affecting somatic mutations occur in childhood cancers

(Alexandrov et al., 2013; Vogelstein et al., 2013) For example, Vogelstein and colleagues report only 9.6 point mutations on average in paediatric leukaemia and solid tumours (Vogelstein et al., 2013), and similar numbers have been reported in neuroblastoma (Molenaar et al., 2012) and medulloblastoma (Parsons et al., 2008). Over time, cells accumulate mutations and it is logical to expect that as the number of mutations increases so too does the probability that some of the mutations attained will have the capacity to drive cancer development (Beerenwinkel et al., 2007). How then does it take many years, and 10s-100s of protein-changing mutations for a full-blown metastatic colorectal cancer to take hold, while a paediatric cancer with only a handful of amino acid effecting mutations may reach metastasis in a 1 year old child? One potential explanation for this would be that childhood cancers are primarily driven by inherited mutations. However, the presence of predisposing germ line genetic lesions in childhood cancers is generally quite low. Previous studies have estimated that hereditary disease accounts for less than 5% of neuroblastoma (Maris et al., 2002), and around 8.5% of all paediatric cancers (Zhang et al., 2015). Although the actual figures are likely to be higher due to the relatively poor biological understanding of paediatric cancers compared to adult cancer. Nevertheless, hereditary disease is believed to contribute to around 10% of adult cancer cases, suggesting that germ line mutations do not make a special contribution to childhood cancer evolution, however this figure is likely affected by the effects of treatment on the future fertility of childhood cancer survivors (Wallace et al., 2005).

1.4 WHY DO PAEDIATRIC CANCERS HARBOUR FEWER MUTATIONS?

Cancer is a heterogeneous disease. Although a tumour may develop from one single, cancerous cell of origin, tumours are most often complex and contain not just a variety of different tumour cells, but also a range of other recruited cell types that contribute to the structure and ecology within the tumour. Importantly, this diversity is often a factor in disease progression and the failure of treatment (Hanahan and Weinberg, 2011). The structural organisation within tumours can resemble that seen in normal tissues, where a small minority of cells are responsible for tumour growth (Kreso and Dick, 2014). The cancer stem cell hypothesis (Valent et al., 2012) was born out of this realisation that a small fraction of cells within a tumour resemble embryonic stem cells or have the ability to revert to an embryonic character. While these ‘cancer stem cells’ are not identical with their embryonic counterparts, it is clear that they have either retained or regained embryonic features that confer a selective advantage at some stage during tumour development.

Is it possible that it typically takes decades for cancer to develop in certain tissues because the number and type of mutations required to ‘unlock’ the embryonic potential of fully differentiated cells is quite high? On the other hand, cancers that become apparent in young children must have developed quite rapidly. Is this quick evolution a consequence of the

relatively minor transformation required to turn immature or embryonal cells into cancer cells? Or are already mature cells more susceptible to reversion to an embryonic state in a conducive pre and postnatal environment? There is evidence to suggest that many childhood tumours have a prenatal cell of origin. However, it is not clear if there is a unifying model for paediatric oncogenesis, i.e. that one or two oncogenic events affecting key genes can drive rapid tumour development, as proposed for paediatric leukaemia and malignant rhabdoid tumours (Biegel et al., 1999; Schmiegelow et al., 2008; Versteege et al., 1998) or whether a diversity of tissue and cell type dependent mechanisms contribute to oncogenesis. Certainly, the low number of mutations associated with childhood malignancies suggests that only a few genetic hits are required to drive paediatric oncogenesis.

1.5 THE 'REST CELL' HYPOTHESIS

The concept of a cancer stem cell is not new. It is rather a reformulation of an old concept first communicated in the 19th century (summarised in Marshall et al., 2014; Ratajczak et al., 2009). The rest cell hypothesis proposes that tumours arise from embryonic remnant or 'rest' cells that lay dormant in tissues after normal development, but can be reactivated and become cancerous. This scenario is particularly easy to conceive when excess cells are produced during embryogenesis, and mechanisms are required to remove these extra cells during tissue and organ formation. A good example of this is in the peripheral nervous system where terminally differentiating neurons compete for limited amounts of trophic factor upon which they are dependent. Evasion of normal cell death by such a mechanism might be the first step in a cell's journey towards forming a tumour.

The rest cell hypothesis is particularly interesting in relation to childhood cancers, where tumour progression occurs quickly and early in the child's life. The early onset of some malignancies suggests that the first oncogenic events take place during the prenatal period, or, at the latest, not long after birth. Indeed, evidence is beginning to point towards an embryonic cell of origin for several paediatric cancers. Amongst the leukaemias, B-cell lineage acute lymphoblastic leukaemia and myeloid leukaemia- down syndrome both appear to have an embryonic origin, as the diseases present already in newborns (Gruhn et al., 2008; Pine et al., 2007). There is also evidence that the nervous system tumours medulloblastoma and neuroblastoma have an embryonic origin. Together, these four cancers account for around 40% of paediatric cancer cases (Downing et al., 2012) implying that many, if not most, paediatric cancers probably begin in the foetus.

1.6 NEUROBLASTOMA

Neuroblastoma is the most commonly occurring childhood tumour outside of the brain and is the most common cancer diagnosed during the first year of life (Maris, 2010). Although neuroblastoma accounts for about 5-10% of all paediatric cancers (Jiang et al., 2011; Zhang et al., 2015), it is responsible for a disproportionately high number (around 15%) of childhood-cancer related deaths in patients younger than 15 years (Maris et al., 2007). At the time of diagnosis, the disease has most often already reached an advanced, metastatic state, leading to poor clinical outcomes. It arises in the sympathetic nervous system, most commonly in or around the adrenal glands and along the paravertebral ganglia. Neuroblastoma presents as a very variable disease ranging from a large benign primary mass to highly infiltrative and metastatic disease with fatal secondary effects. A particular subtype of neuroblastoma (4s) is often first discovered at a highly metastatic state only to spontaneously regress without any clinical intervention (Maris et al., 2007).

The heterogeneity of neuroblastoma, both biologically and in clinical presentation and response to treatment, leads to frequent failure of therapy, with survival rates of only 30% in high risk patients (Cohn et al., 2009). At the same time, neuroblastoma has one of the highest reported rates of spontaneous regression of all human cancers (Carlsen, 1990; Yamamoto et al., 1998). Despite significant efforts to investigate the molecular mechanisms that promote neuroblastoma development, the disease still presents a clinical challenge. A better understanding of the molecular pathogenesis behind the disease is required to improve treatment strategies and identify potential new chemotherapeutic targets. In particular, increased understanding of the role of cancer stem cells and the developmental biology of the sympathetic nervous system, both of the molecular biochemistry and the types of processes at play should be particularly helpful in understanding and treating neuroblastoma, and perhaps embryonal tumours in general.

1.7 NEUROBLASTOMA CELL OF ORIGIN

Neuroblastoma is believed to arise from sympathetic neuron progenitor cells, neuroblasts. The sympathetic nervous system is part of the autonomic nervous system that coordinates the body's unconscious actions. The main function of the sympathetic nervous system (SNS) is to control the 'flight-or-fight' response, through the release of noradrenalin to peripheral target tissues. However, the SNS also helps to maintain homeostasis through basic levels of activity. As examples, nipple erection, sweating, ejaculation, respiration and pupil dilation are all subject to sympathetic control. The sympathetic nervous system is composed of preganglionic neurons, situated in the spinal cord, and their synaptic targets, postganglionic sympathetic neurons that innervate a diverse range of peripheral targets. The cell bodies of

postganglionic sympathetic neurons reside in ganglia that are found in a paravertebral chain, along with the celiac and mesenteric ganglia and the adrenal medulla (Purves, 2012).The majority of neuroblastomas arise in the adrenal medulla, although they can appear anywhere along the sympathetic chain (Maris, 2010). Gene expression studies have shown that neuroblastoma tumour cells resemble the neuroblast cells present during embryonic development of the SNS (De Preter et al., 2006).

1.8 NEUROBLASTS AND SYMPATHETIC NEURON DEVELOPMENT

Sympathetic neurons originate from migratory neural crest cells, known as neuroblasts, that assemble in the vicinity of the dorsal aorta at around embryonic day 10.5 in mice (Figure 1A). Bone Morphogenetic proteins secreted from the dorsal aorta prompt adjacent parenchymal cells to produce stromal derived factor 1 (SDF1) and Neuregulin 1 (NRG1) (Saito et al., 2012; Schneider et al., 1999) that, in turn, stimulate the expression of PHOX2B and MASH1 in neuroblasts. PHOX2B and MASH1 initiate the expression of genes required for sympathetic neuron differentiation and later noradrenergic function, such as PHOX2A, HAND2, GATA3, TH, DBH, STMN2 and NEFH (Guillemot et al., 1993; Jiang et al., 2011; Pattyn et al., 1999).

Interestingly, and in contrast to the classical models of neurogenesis and differentiation in the central nervous system, sympathetic neuron differentiation does not follow terminal cell cycle exit. Sympathetic neuroblasts already expressing the aforementioned neuronal and noradrenergic proteins undergo a wave of proliferation between E11.5 and E15.5 (Gonsalvez et al., 2013), before they start to extend axons and terminally differentiate. This expansion of the neuroblast population corresponds with transient expression of ALK (Vernersson et al., 2006), and implicitly, MYCN (Edsjö et al., 2004; Umapathy et al., 2014; Wakamatsu et al., 1997). Both genes are strongly linked to neuroblastoma, suggesting that this peculiar proliferative behaviour of sympathetic neuroblasts may have a role in neuroblastoma pathogenesis, and could potentially explain why neuroblastoma does not arise in other parts of the nervous system.

Segregation of the primordial sympathetic chain into distinct ganglia occurs simultaneously (from around embryonic day 11.5-12 for the cervical sympathetic ganglia in mice) and depends upon local secretion of GDNF family ligands (GFLs) by surrounding tissue and subsequent stimulation of the RET tyrosine kinase in sympathetic neuron precursors (Enomoto et al., 2001; Honma et al., 2002; Nishino et al., 1999) (Figure 1B).

Local secretion of ligands from several groups of trophic factors stimulates axon extension, guidance and eventual connection of sympathetic neurons with peripheral targets (Glebova and Ginty, 2005). GFL-RET signalling is also required for initial axon extension, through the actions of the ligand ARTN, on GFRA3 and RET (Enomoto et al., 2001; Honma et al., 2002;

Nishino et al., 1999) (Figure 1B). The neurotrophin NTF3 is also required for initial axon extension, however some evidence suggests that it does not act through it's high-affinity receptor, TRKC, but rather through the related neurotrophin receptor, TRKA (Kuruvilla et al., 2004; Wyatt, 1997). Sympathetic axons extend primarily along arterial vasculature and other signalling factors secreted along the vasculature, both known and unknown, are also required for axonal guidance of specific neuronal subtypes (Glebova and Ginty, 2005; Honma et al., 2002; Makita et al., 2008).

On the other hand, distal axon extension and target innervation occur in response to nerve growth factor (NGF) released by sympathetic target tissues ((Crowley et al., 1994; Fagan et al., 1996; Glebova, 2004). NGF binding to the receptor tyrosine kinase TRKA stimulates a potent, dependence inducing, neurotrophic signalling program that culminates in nascent sympathetic neurons competing for NGF during synaptogenesis (Chao and Ip, 2010; Glebova and Ginty, 2005).

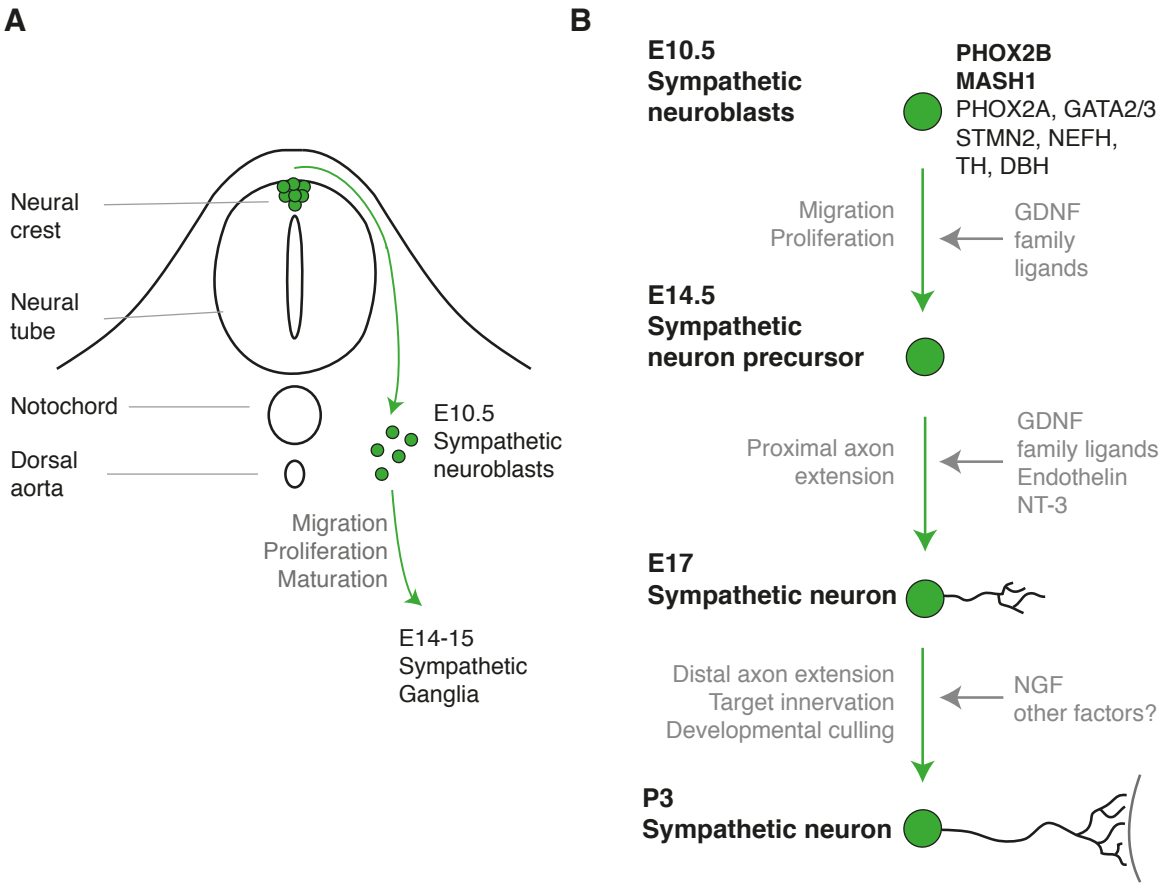

Figure 1: (A) General schema of sympathetic neuroblast mirgation and development with (B) timing of important developmental events and gene expression. Gray arows and proteins indicate the action of locally secreted factors on neuroblast development

1.9 DEVELOPMENTAL CULLING OF EXCESS NEURONS

An excess of sympathetic neuroblasts are produced during embryogenesis and these are removed by programmed cell death during the late prenatal and early postnatal period. Culling of excess neurons occurs via competition for growth factors, such as NGF. The mature peripheral nervous system is essentially sculpted from an embryonic block via this process that kills up to 50% of neuronal precursors.

Over 60 years ago, classic experiments by Levi-Montalcini and Booker revealed that NGF has a critical role in determining the number of neurons in the sympathetic nervous system (Levi-Montalcini and Booker, 1960a, 1960b). NGF secreted by target organs binds TRKA on the surface of nearby sympathetic axons. The amount of NGF secreted by target tissues is important for determining the number of neurons by which they are innervated. Secretion of less NGF allows for the survival of fewer neurons due to increased apoptosis, and subsequent in vivo studies in mice have verified that genetic deletion of NGF or TRKA reduces the number of sympathetic neurons via increased apoptosis during development (Brennan et al., 1999; Crowley et al., 1994; Fagan et al., 1996).

Developing sympathetic neurons in the mouse superior cervical ganglion (SCG) become dependent on the trophic stimulus provided by NGF binding to TRKA from around embryonic day 15 (E15) (Crowley et al., 1994; Fagan et al., 1996). SCG neurons begin to die via apoptosis from around E17 as the amount of NGF available becomes limiting and they effectively 'compete' for NGF. Neurons that are deprived of NGF relative to their competitors die (Deppmann et al., 2008). This NGF deprivation induced apoptosis can be simulated in vitro with primary cultures of sympathetic neurons and has been well studied as a model for neuronal apoptosis.

The molecular mechanisms behind developmental apoptosis of sympathetic neurons have been studied in vitro for over 20 years. It is well established that withdrawal of NGF activates the mitochondrial pathway of apoptosis and that kinase signalling through the JNK-JUN pathway and de novo gene expression are required (Kristiansen and Ham, 2014). Interestingly, this apoptotic pathway is also dependent on oxygen sensing mechanisms. Removal of the oxygen sensing prolyl-hydroxylase EGLN3 in mice results in enlarged sympathetic ganglia due to impaired developmental culling of neurons and this effect is at least partially mitigated by concurrent removal of one allele of the hypoxia inducible transcription factor EPAS1 (Bishop et al., 2008). Yet the precise mechanisms causing these effects on neuronal apoptosis remain elusive.

Despite the large number of studies published on this topic much is still unknown, or a matter of debate. For example, a recent study suggested that TRKA can act as a dependence receptor that causes sympathetic neuron cell death in the absence of NGF (Nikoletopoulou et al., 2010). However, the molecular mechanisms that would explain this activity are yet to be described. Other studies have indicated the involvement of P75NTR and other neurotrophins,

BDNF and NTF3, in NGF deprivation induced apoptosis (Bamji et al., 1998; Brennan et al., 1999; Kenchappa et al., 2006), and provide reasonably compelling data. Yet, generally many findings in this field generated from in vitro data are not clearly reflected in vivo, and different research groups have drawn contrasting conclusions from the same genetic knockouts (Bamji et al., 1998; Brennan et al., 1999; Ernfors et al., 1994, 1995). Undoubtedly, the in vitro model of apoptosis caused by NGF deprivation is and has been a valuable tool for investigating the molecular mechanisms at play during developmental culling of sympathetic neurons. Unfortunately, our ability to draw relevant conclusions from this model is complicated by its relationship to the inherently greater complexity of the developmental processes and molecular mechanisms at play in the in vivo environment occupied by sympathetic neurons during development.

Pheochromocytoma is a cancer related to neuroblastoma that originates in chromaffin cells also derived from the sympathoadrenal cell lineage. Prior studies have suggested that germline mutations in HRAS, RET, VHL, SDHD and KIF1B predispose to pheochromocytoma, and a related tumour, paraganglioma, by impeding developmental apoptosis after NGF withdrawal (Dahia, 2014; Lee et al., 2005; Yeh et al., 2008). Reduced neuronal culling and subsequent retention of excess progenitor cells might set a platform for later tumour development. Presumably, these remnant embryonic cells might have, or acquire, the ability to later form pheochromocytoma, or other related tumours, a la the rest cell hypothesis. Such a conserved mechanism of familiar predisposition through defects in embryonic development could also help explain the relatively high penetrance of heritable paraganglioma and pheochromocytoma (Dahia, 2014). Similarly, several candidate neuroblastoma tumour suppressors and oncogenes are also linked to developmental apoptosis.

1.10 COMMON GENETIC FEATURES OF NEUROBLASTOMA

As in other paediatric cancers, only few recurrent gene-specific mutations have been associated with neuroblastoma and this has led to difficulties in developing targeted treatment strategies for this disease. The most commonly detected gene alterations are amplification of MYCN and activating mutations of ALK, that occur in around 20% and 7% of cases respectively (Molenaar et al., 2012). Instead, oncogenic alteration of DNA in neuroblastoma is dominated by larger chromosomal rearrangements. Loss of chromosomal regions at 1p36 (25-35% of cases) and 11q23 (35-45%) and gain of 17q22 (around 50%) are more prevalent, and are associated with unfavourable prognosis (reviewed in Jiang et al., 2011).

Loss-of-heterozygosity at 1p36 is common in neuroblastoma (Maris et al., 2000; Martinsson et al., 1995) and numerous studies have proposed CAMTA1, CASZ1, CHD5, TP73, MIR34A and KIF1Bβ respectively as 1p36-encoded tumour suppressors (Egan et al., 2013; Henrich et al., 2011; Ichimiya et al., 1999; Liu et al., 2011; Munirajan et al., 2008; Schlisio et

al., 2008; Thompson et al., 2003; Welch et al., 2007). While no bona fide tumour suppressor function has yet been ascribed to any of the these genes individually, the collective loss of these genes that occurs with 1p36 deletion is likely to have a potent tumorigenic effect on the precursor cells of the sympathetic nervous system, from which neuroblastoma is believed to arise.

1.11 KIF1BB, A CANDIDATE 1P36 TUMOUR SUPPRESSOR PROTEIN

The first suggestion that KIF1Bβ might act as a 1p36 tumour suppressor protein came from the report of a neuroblastoma cell line (NB1) with a small 500-kb homozygous deletion at 1p36.2 that harboured at least six genes (Nagai et al., 2000). Of these genes, only KIF1Bβ demonstrated tumour suppressor activity and had altered expression in neuroblastomas. Further in vitro studies have demonstrated that KIF1Bβ is critical for apoptosis in sympathetic neuron progenitors in response to NGF deprivation. In a study investigating the role of oxygen-sensing and EGLN3 in neuronal culling, Schlisio and colleagues used a genome-wide RNAi screen to discover that KIF1Bβ acts downstream of EGLN3 to induce apoptosis (Schlisio et al., 2008). They showed that KIF1Bβ is both necessary and sufficient for NGF deprivation induced apoptosis, and that several inherited missense mutations of KIF1Bβ in neuroblastoma and pheochromocytoma, act by blocking the ability of KIF1Bβ to initiate apoptosis. Although earlier studies had placed KIF1Bβ in the shortest regions of overlap found in 1p36 deletions in neuroblastoma (Bauer et al., 2001; White et al., 2005), these mechanistic insights into the molecular function of KIF1Bβ along with the identification of cancer-associated missense mutations provided the first real evidence that KIF1Bβ might be a haplo-insufficient neuroblastoma tumour suppressor on chromosome 1p36. Additional work showed that, of the potential 1p36 tumour suppressor genes, KIF1Bβ was the only one that suppressed growth of neuroblastoma cell cultures and xenografted tumours (Munirajan et al., 2008). Significantly, these studies by Munirajan and colleagues narrowed down the part of the KIF1Bβ protein required for it to induce apoptosis. These results suggested that KIF1Bβ's ability to transport molecular cargoes is not required for apoptosis, as functionally deleterious mutations in the KIF1Bβ motor domain and fork-head associated domain did not block apoptosis (Munirajan et al., 2008).

These studies argue that KIF1Bβ is a putative neuroblastoma tumour suppressor on 1p36, implying that defective culling of sympathetic neurons during development may contribute to neuroblastoma oncogenesis. Further studies are clearly required to investigate mechanistically how KIF1Bβ regulates apoptosis, and the implications of deregulated sympathetic neuron culling for neuroblastoma pathogenesis in vivo. Future work should also reveal if and how the remaining wild-type allele of KIF1Bβ is silenced in neuroblastoma as the aforementioned studies indicate that the KIF1Bβ promoter is not silenced by DNA hyper-

methylation in 1p36 +/- tumours and that complete ablation of KIF1Bβ expression kills neurons (Munirajan et al., 2008; Schlisio et al., 2008)

1.12 KIF1BB, A MOLECULAR MOTOR PROTEIN

KIF1Bβ is a group 3 kinesin (Lawrence et al., 2004), and is one of several protein isoforms expressed from the KIF1B gene. Kinesins are motor proteins that transport molecular cargoes along microtubules. KIF1Bβ contains three recognised functionally conserved domains; an N-terminal motor ATPase domain that drives ATP- dependent movement along microtubules, a forkhead associated (FHA) domain, that may facilitate interaction with phospho-proteins and a pleckstrin homology (PH) domain that mediates binding to phosphoinositide rich membranes (Hirokawa et al., 2009; Klopfenstein et al., 2002). Between the FHA domain and the PH domain is a unique region that appears to mediate interaction with cargoes and other adaptor proteins (Figure 2). In contrast to other kinesins, the KIF1B proteins are functional monomers and have an unusually compact, globular tertiary structure compared to the typical kinesin architecture with the motor domain anchored to the microtubule and the c-terminal tail sticking up like a stalk (Hirokawa et al., 2009; Nangaku et al., 1994).

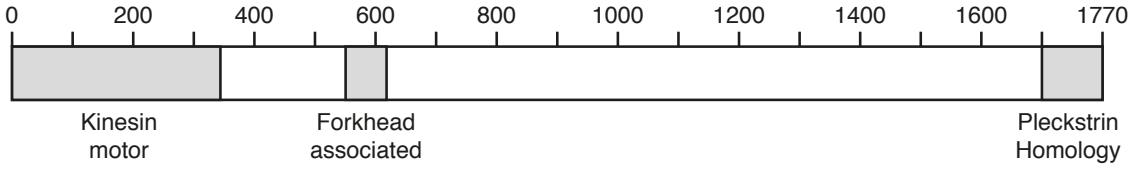

Figure 2: Diagram of KIF1Bbeta protein including functionally conserved domains

Group 3 kinesins, such as KIF1Bβ traffic their cargoes towards the microtubules' plus end, which generally entails movement from the centre of the cell out towards the periphery and cell membrane. Previous studies have demonstrated that KIF1Bβ transports mRNAs in neurons (Charalambous et al., 2013) and synaptic vesicles (Zhao et al., 2001). This transport activity appears to depend upon interactions with proteins such as RAB3A and the adaptor proteins KIF1BP and MADD (Drerup et al., 2016; Niwa et al., 2008). Interactions with specific lipids, such as PtdIns(4,5)P2, also appear to be crucial for regulating interactions with vesicular membranes (Klopfenstein et al., 2002). These studies indicate that KIF1Bβ is required for the transport of several specific cargoes, yet KIF1Bβ most likely transports more, as yet undiscovered, molecules or intracellular objects. Future work should reveal these new

cargoes, and the molecular interactions and adaptor proteins that determine specify of binding to, and transport by KIF1Bβ.

Earlier investigations have also suggested a role for KIF1Bβ in axon development and maintenance in zebrafish (Lyons et al., 2009; Pogoda et al., 2006). Talbot and colleagues demonstrated that a loss of function mutation in the motor domain of KIF1Bβ causes defects in axon development and myelination that mirror those seen in multiple sclerosis. Another mutation in the KIF1Bβ motor domain was also recently associated with amyotrophic lateral sclerosis (Herdewyn et al., 2012). Mutations in the KIF1B gene are also associated with Charcot-Marie-Tooth disease (CMT), a fairly common, hereditary neurological disorder. Pertinently, a specific loss-of-function mutation in the KIF1Bb motor domain carried by a Japanese CMT family was shown to impair axonal protein transport and cause peripheral neuropathy in mice (Zhao et al., 2001). Furthermore, a recent study has implicated KIF1Bβ in the transport of STMN2, a key protein for axon growth in the peripheral nervous system. From these studies it is clear that KIF1Bβ plays an important role in neuronal development and maintenance. However, in vivo studies of KIF1Bβ function and tumour suppressor activity have been hindered by the perinatal death of mice with homozygous loss of KIF1Bβ due to nervous system defects (Zhao et al., 2001).

1.13 SUMMARY

The low number of protein effecting mutations found in childhood cancers suggests that they might originate from cells particularly susceptible to transformation. The many stem cells, expanding cell populations, and growth promoting environmental niches present during embryonic development appear to provide favourable conditions for cancer development, and it seems likely that many paediatric malignancies begin in the embryo. Neuroblastoma is a childhood cancer that appears to arise from sporadic defects during embryogenesis. Failure of either cell differentiation or culling of undifferentiated cells during embryogenesis are processes that could lead to an abnormal accumulation of undesired cells that have, or are predisposed to obtain, cancerous properties. There is considerable evidence that such embryonic ‘pre-cancers’ commonly arise in the sympathetic nervous system, and can precede neuroblastoma. KIF1Bβ is a putative neuroblastoma tumour suppressor protein that is required for apoptosis of sympathetic neurons deprived of NGF. The molecular mechanisms by which KIF1Bβ regulates apoptosis are as yet unknown. Neither has the function and importance of KIF1Bβ in sympathoadrenal development and tumour-suppression been investigated in vivo.

2 AIMS

In **Paper I** we aimed to discover how, mechanistically, KIF1Bβ regulates neuronal apoptosis.

In **Paper II**, we continued to explore the molecular function of KIF1Bβ in apoptosis-induction and neuroblastoma tumour suppression. Specifically, the work in this paper explores a finding made during work on Paper I, that KIF1Bβ physically interacts with CALM2, a protein involved in intracellular calcium dependent signalling that is critical for a myriad of neuronal functions.

Paper III presents an investigation of the *in vivo* function of KIF1Bβ in the sympathetic nervous system using a conditional knock out mouse model.

3 RESULTS AND DISCUSSION

3.1 PAPER I

In this paper we investigated the molecular mechanisms behind KIF1Bβ's involvement in cell death and putative neuroblastoma tumour suppression. We confirmed that the minimal region of KIF1Bβ capable of inducing apoptosis was located between the FHA and PH domains as previously published (Munirajan et al., 2008) and narrowed down this region further (minimal apoptotic domain - amino acid residues 1000-1400 in human KIF1Bβ), clearly demonstrating that the apoptotic activity of KIF1Bβ is independent of its motor activity.

Using immunoprecipitation experiments coupled with mass spectrometry, we discovered that KIF1Bβ interacts with an RNA helicase, DHX9, and that DHX9 binds to the minimal apoptotic domain of KIF1Bβ. DHX9 regulates gene expression through its effects on local chromatin structure and function in transcriptional activation, RNA splicing and translation (Fuller-Pace, 2006). We show that KIF1Bβ promotes translocation of DHX9 into the nucleus of neuroblastoma cells, and that the DHX9 transcriptional activation domain is needed in order for KIF1Bβ to induce apoptosis. Subsequent RNA-seq transcriptome analysis revealed that KIF1Bβ overexpression stimulates a DHX9-dependent transcriptional response that features upregulation of pro-apoptotic proteins, such as XAF1. Importantly, loss of DHX9 expression protects cells from NGF-deprivation induced apoptosis. The nuclear localisation of DHX9, and implicitly it's transcriptional activity, depends upon KIF1Bβ expression in primary human neuroblastoma tumours and in the sympathetic ganglia of mice.

The discovery that DHX9 acts downstream of KIF1Bβ during NGF-deprivation induced apoptosis accords with early reports showing that gene transcription and translation are required for this mode of cell death in sympathetic neurons (Edwards and Tolkovsky, 1994; Martin, 1988). While at first glance the importance of an RNA helicase for a transcription and translation dependent process may seem self-evident, other studies are emerging that describe specific DHX9 mutations and RNA helicase disruption in other cancer settings such as breast cancer and medulloblastoma (Fidaleo et al., 2016; Guénard et al., 2009), suggesting that DHX9 may have tumour-suppressive and oncogenic functions depending on physiological context.

Although we demonstrated that DHX9 is required for apoptosis induced by ectopically expressed KIF1Bβ and that KIF1Bβ is required for DHX9 nuclear translocation, our experiments indicated that DHX9 also regulates the expression of endogenous KIF1Bβ. We did not investigate this in depth in this paper. However, we speculate that DHX9 may exert a post-transcriptional influence on KIF1Bβ expression from outside the nucleus. Insights into the molecular mechanisms controlling KIF1Bβ expression might prove valuable for

understanding neuroblastoma pathogenesis. Loss of the 1p36 locus usually occurs hemizygously in neuroblastoma (Maris et al., 2000), but we (in Paper I, see also paper II) and others (Munirajan et al., 2008) observe that KIF1Bβ protein is most often absent from 1p36 +/- tumours. Hypermethylation of the promoter site does not seem to account for silencing of KIF1Bβ expression on the remaining wild-type allele (Munirajan et al., 2008), and our own unpublished data examining hemizygous loss of 1p36 in pheochromocytoma demonstrate no reduction in KIF1Bβ mRNA in 1p36 +/- tumours, relative to 1p36+/+ tumours or normal adrenal tissue. Similarly, silencing of another putative 1p36 tumour suppressor gene, CHD5, does not depend on promoter hypermethylation in 1p36 +/- neuroblastoma (Koyama et al., 2012). These findings imply that silencing of the remnant wild-type allele in 1p36+/- tumours most likely occurs via post transcriptional regulation of gene expression. Investigation of the biological mechanism behind this is warranted, as experimental data suggests that alleviating loss-of-heterozygosity at 1p36 could suppress neuroblastoma and enhance therapeutic success (Bader et al., 1991). Furthermore, many of the post-transcriptional regulatory enzymes may present attractive targets for future chemotherapeutics that are both targetable with small molecules and associated with cancer-specific gene expression programs.

Curiously, we showed that some NB-associated KIF1Bβ mutants have a reduced ability to affect DHX9 translocation to the nucleus. Those mutants that acted unimpaired on DHX9, were still unable to induce apoptosis (Paper I, Schlisio et al., 2008), implying that additional mechanisms lie behind KIF1Bβ's apoptotic function. Subsequent investigation of these mechanisms is presented in Paper II.

3.2 PAPER II

In this paper we continued our exploration of the molecular mechanism responsible for KIF1Bβ induced apoptosis. Here, we pursued a preliminary finding made in the process of work on Paper I, that KIF1Bβ interacts with CALM2, a calcium-binding protein involved in various calcium-sensitive signalling pathways. Interaction between KIF1Bβ and CALM2 was suggested by a previous study (Charalambous et al., 2013), but the functional implications of the interaction were not explored. We demonstrated that KIF1Bβ stabilises the binding of CALM2 to Calcineurin (CN), a serine/threonine protein phosphatase complex, and facilitates CN phosphatase activity. We found that KIF1Bβ is required for CN-mediated dephosphorylation of serine-637 on the mitochondrial fission protein DRP1, inducing translocation of DRP1 to the mitochondria, mitochondrial fission and apoptosis. Furthermore, DRP1, CALM2 and CNA (PPP3CA), the enzymatic subunit of the CN complex, are required for KIF1Bβ to initiate apoptosis, and for apoptosis of sympathetic neurons deprived of NGF. Previous studies have shown that DRP1 is required for developmentally regulated apoptosis during neural tube formation (Wakabayashi et al., 2009).

In Paper I we found that neuroblastoma tumours with intact chromosome 1p36 express KIF1Bβ protein, in contrast to 1p36 hemizygous deleted tumours that lack KIF1Bβ. Here we extended this observation using a larger set of neuroblastomas. The absence of KIF1Bβ in the 1p36+/- tumours implies that KIF1Bβ might be a pathogenic target of 1p36 loss in neuroblastoma and we showed that indeed low expression of KIF1Bβ is correlated with worse prognosis and more advanced disease. Likewise, neuroblastoma tumours with low expression of DRP1 have worse patient outcome and more advanced stage, indicating that DRP1 may also have tumour suppressive properties in neuroblastoma. Curiously, DRP1 protein was largely absent from 1p36 +/- tumours. In neuroblastoma cell lines we only observed KIF1Bβ dependent changes in DRP1 phosphorylation, rather than expression. The loss of both of these proteins from 1p36+/- tumours suggests that they are not functionally redundant and that they most likely have other functions that effect neuroblastoma pathogenesis that were not explored in this study.

However, it should be noted that other studies have reported additional functions of DRP1 that could be relevant in neuroblastoma. DRP1 is a member of the dynamin family of proteins that execute GTP-dependent fission of membranous structures. Previous studies have shown that DRP1 is involved in various fission activities, including endosomal cleavage from the plasma membrane, peroxisome fission and regulation of the endoplasmic reticulum, in addition to mitochondrial fission (Koch et al., 2003; Li et al., 2013; Pitts et al., 1999; Smirnova et al., 2001). It is highly conceivable that disruption of these other DRP1 functions could have a role in neuroblastoma oncogenesis. In particular, perturbed mitochondrial and peroxisomal dynamics due to loss of DRP1 have also been reported to cause neuronal degeneration through impaired mitochondrial function linked to reactive oxygen species (Chen, 2005; Cho et al., 2009; Wang et al., 2009). Increased ROS and derailed mitochondrial energy metabolism are typical features of cancer cells, so it is also possible that silencing of DRP1 could contribute to neuroblastoma development through such effects.

The molecular motor dependent activity of KIF1Bβ may also figure in neuroblastoma tumour suppression. The interaction that we document between KIF1Bβ and the CN-CALM2 complex in Paper II does not depend on KIF1Bβ motor function. However, DRP1-independent activities of KIF1Bβ could also complement tumour-suppressive DRP1 dependent activity. In this regard, KIF1Bβ cargo transport activity in developing sympathetic neuroblasts is investigated in Paper III.

The work presented in Paper II answered a lingering question posed in Paper I – How does KIF1Bβ affect DHX9 nuclear translocation, and activity during neuronal apoptosis? In Paper II we found that human DHX9 has a CN recognition motif (LxVP) from amino acid residue 647 to 650. Our subsequent experiments demonstrated that in the presence of KIF1Bβ in vitro, CN could dephosphorylate a DHX9 peptide that contained this sequence. We then showed that CNA is required for KIF1Bβ mediated, nuclear translocation of DHX9 in neuroblastoma cell lines, implying that CN enzymatic activity was the mechanistic missing link between DHX9 and KIF1Bβ in Paper I.

The most unexpected finding in Paper II is that KIF1Bβ acts as a critical adaptor protein for the activity of the CN enzymatic complex. We found that KIF1Bβ acts as a scaffold for the CN-CALM2 complex, binding to both CNA and CALM2 (but not CNB) and potentiating CN enzymatic activity. CN is composed of an enzymatic subunit, CNA, and a regulatory subunit, CNB. CALM2 binding to CN in the presence of Ca^{2+} prompts a conformational change in CN, which shifts an auto-inhibitory structural loop in the CNA protein and opens up the enzymatic active site for substrate binding (Li et al., 2011). Our experimental data indicate that KIF1Bβ is required for full calcineurin enzymatic activity because it stabilises the interaction between CN and CALM2, and is required for CN phosphatase activity on a variety of substrates in vitro. We therefore speculated that KIF1Bβ acts as an allosteric regulator of CN activity by relieving enzymatic auto-inhibition.

This hypothesis departs from the prevailing model of calcineurin activity, whereby CALM2 binding to CN in the presence of calcium is sufficient for enzymatic activation (Li et al., 2011; Wang et al., 2008). Our results indicate that while the CN-CALM2 complex is partially active in optimised in vitro conditions, the binding of an adaptor protein such as KIF1Bβ can massively increase CN phosphatase activity. In this paper we demonstrated that this is critical for apoptosis induced by ectopic KIF1Bβ expression and NGF-deprivation in sympathetic neurons.

Surprisingly for us, we also observed that KIF1Bβ was required for dephosphorylation of all the other known calcineurin substrates that we tested in vitro. Calcineurin regulates a broad range of cellular signalling pathways, promoting diverse cell biological responses (Li et al., 2011). We have observed KIF1Bβ expression in the brain and nervous system, and other neural crest derived tissues, and it is also expressed at lower levels in various other tissues including skeletal muscle, blood and in the testis (Lonsdale et al., 2013). It appears unlikely that KIF1Bβ regulates the full range of CN activities in all these tissues in vivo. Interaction between KIF1Bβ and calcineurin is potentially constrained somewhat by intracellular segregation of these proteins in different subcellular compartments or areas. Intriguingly, our findings open the door for discovery of novel CN adaptor proteins that regulate CN signalling in other physiological and pathological contexts. CN signalling is de-regulated in many diseases. Our findings in Paper II indicate that the design of synthetic adaptor peptides could potentially be used to modulate CN activity in a therapeutic context.

3.3 PAPER III

To investigate the role of KIF1Bβ in vivo in sympathoadrenal development and potential neuroblastoma tumour suppression we created mice with a conditional deletion of KIF1Bβ driven by expression of the noradrenergic gene DBH (KIF1Bβ cKO mice). KIF1Bβ

expression is ablated in the sympathetic neuroblasts of these mice. Based on the results observed in Papers I and II, and previous studies, we expected that these mice would develop a hyperplasia in the developing sympathetic nervous system due to defective developmental culling of sympathetic neurons.

Contrary to our expectations, KIF1Bβ cKO mice have smaller sympathetic ganglia than their wild-type littermates. In Paper III, we show that loss of KIF1Bβ causes developmental loss of sympathetic neurons, leading to impaired target innervation and sympathetic nervous system function. We describe misexpression of critical markers of sympathetic identity in KIF1Bβ cKO neurons from the superior cervical ganglia (SCG). Investigating this, we revealed that KIF1Bβ enables NGF induced neuronal differentiation by anterograde trafficking of the NGF receptor, TRKA.

A recent study argues that KIF1A is the main kinesin responsible for anterograde transport of TRKA in sensory neurons of the adult dorsal root ganglia (DRG) and that heterozygous loss of KIF1A leads to sensory neuropathy (Tanaka et al., 2016). Tanaka et al. argue that KIF1A is predominantly expressed in adult DRG, while KIF1Bβ is more highly expressed during embryonic development and in regenerating neurons. Furthermore, immunoprecipitation experiments are presented that suggest TRKA binds to KIF1A in the adult brain, but not to KIF1Bβ. In contrast, we found that TRKA binds to KIF1Bβ in sympathetic neurons and neuroblastoma cell lines, but did not observe any association between KIF1A and TRKA in our experiments despite the abundance of both kinesins. We also observed persistent expression of KIF1Bβ in adult sympathetic neurons, and, somewhat curiously, Tanaka and colleagues also observe KIF1Bβ protein in adult brain. Interestingly, the same authors published an earlier study demonstrating that heterozygous loss of KIF1B, and implicitly KIF1Bβ, causes peripheral neuropathy. This casts some doubt on the developmentally segregated expression and function of these proteins proposed by Tanaka and co. While there are inconsistencies between Paper III and the work presented by Tanaka et al., we demonstrate that, at least in cells of sympathoadrenal origin, KIF1Bβ is the main kinesin responsible for anterograde transport of TRKA.

We showed that the loss of sympathetic neurons in KIF1Bβ cKO mice occurs due to abnormal death of neuronal progenitors during embryogenesis. KIF1Bβ cKO neurons have a reduced capacity to survive when cultured with low concentrations of NGF and display an attenuated NGF neurotrophic signal manifest as reduced phosphorylation of ERK1/2. In agreement with these observations, complete, acute knockdown of KIF1Bβ was previously reported to cause cell death in sympathetic neuron cultures (Schlisio et al., 2008). We hypothesised that loss of KIF1Bβ blocks NGF-TRKA neurotrophic signalling, leading to cell death, and that this could be avoided by introduction of an artificial ERK-mediated survival signal. To this end, we combined conditional knockout of KIF1Bβ with conditional knockout of NF1. NF1 is a RAS-GAP protein that modulates the downstream signal of several groups of trophic factor receptors, including TRKA. We showed that loss of NF1 restored the reduced size and the impaired ERK1/2 phsophorylation in KIF1Bβ cKO sympathetic ganglia,

supporting our hypothesis. Although further experiments are required to verify that loss of NF1 actually ameliorates the elevated apoptosis in KIF1Bβ cKO neurons, or whether the increased size of NF1; KIF1Bβ double cKO ganglia is simply due to increased neuroblast proliferation.

In paper II we demonstrated that KIF1Bβ is required for full activation of calcineurin, CN. CN regulates many calcium sensitive signalling pathways and is important for neuronal development and activity (Graef et al., 2003; Yakel, 1997). In Paper III we found that CN was not required for NGF-mediated neuronal differentiation of neuroblastoma cells or pheochromocytoma cells, implying that the pro-apoptotic motor-independent functions of KIF1Bβ, are likely distinct from KIF1Bβ's motor dependent, TRKA transport activity. It is difficult to dissect these two functions in vivo with a knockout mouse, due to the two-sided nature of TRKA. i.e. neurons that lack TRKA will die due to lack of NGF neurotrophic signal, however neurons that express TRKA, but do not receive NGF will also die due to an incompletely understood, but TRKA dependent pro-apoptotic mechanism (Nikoletopoulou et al., 2010). Consequently, KIF1Bβ-deficient sympathetic ganglia are already reduced in size, due to neuronal apoptosis, before normal NGF-dependent developmental culling of excess sympathetic progenitors has begun. One approach that could be used to distinguish the apparently separate KIF1Bβ functions in sympathetic neuron development would be to add back a motor-deficient mutant KIF1Bβ into KIF1Bβ knockout mice, or to introduce the corresponding specific amino acid substitution into the KIF1B locus of wild type mice using gene-editing technology (Wang et al., 2013).

In Paper III we compared gene expression profiles from KIF1Bβ cKO SCGs with gene expression data from large sets of neuroblastoma tumours. We employed this method to model what the specific effects of KIF1Bβ loss might be in neuroblastoma, since KIF1Bβ is commonly lost along with contiguous genes on 1p36 making it hard to distinguish individual gene specific effects of 1p36 loss in neuroblastoma. This is further complicated by the common co-occurrence of 1p36 loss with MYCN amplification, that has a strong influence over neuroblastoma character and prognosis in its own right. We found that clustering non-MYCN amplified neuroblastoma tumours according to genes differentially expressed in KIF1Bβ cKO SCGs produced prognostically relevant groupings. Specifically, clustering tumours based on their expression of genes down regulated in KIF1Bβ cKO SCGs, gave two clear groups of tumours. The group of tumours that had lower expression of the genes downregulated in KIF1Bβ cKO SCGs, i.e. those tumours that most resembled KIF1Bβ cKO neurons, had worse patient outcome and were associated with high-risk disease and advanced INSS disease stage. Tellingly, the most depleted and enriched genesets in this group of tumours were highly similar to those correlated with KIF1Bβ expression in neuroblastoma and those identified in KIF1Bβ cKO SCGs. This comparative analysis of gene expression implies that loss of KIF1Bβ contributes to neuroblastoma pathogenesis, independent of contiguous genes on 1p36 and MYCN-amplification.

Expression of the NGF receptor TRKA is a strong prognostic factor in MYCN non-amplified neuroblastoma. Furthermore, hemizygous loss of chromosomal locus 1p36 is strongly correlated with poor prognosis. Our finding that the 1p36 candidate tumor suppressor KIF1Bβ is required for NGF mediated neuroblast differentiation by mediating the anterograde transport of TRKA, provides a potential molecular explanation for the reduced expression of neuronal differentiation markers in 1p36+/- neuroblastomas and associated poor prognosis.

At present, the only mouse models of neuroblastoma are the TH-MYCN mouse (Weiss et al., 1997) and the mice lines over-expressing mutant ALK developed by Johannes Schulte and colleagues (Heukamp et al., 2012). These tumour models are based on miss-expression of an oncogene driven by the promoter region of either of the noradrenergic genes TH or DBH. No mouse models of neuroblastoma involving deletion of a tumour suppressor gene have been reported to date. We hypothesised that our KIF1Bβ cKO mice would develop hyperplastic sympathetic ganglia due to impaired developmental culling, and that these mice might also have developed neuroblastoma. However, the phenotype observed in our KIF1Bβ cKO mice diverged from our expectations. This might be due in part to the developmental timing of the knockout and the need for a collaborating oncogenic event. For example, conditional knockout of CREB, a transcription factor required for NGF-dependent sympathetic neuron survival in vitro (Riccio et al., 1999), driven by the DBH promoter increased sympathetic neuronal survival during developmental culling (Parlato et al., 2007). However, germ line ablation of CREB reduced sympathetic neuroblast survival and migration before the acquisition of NGF-dependence. This effect resembled that caused by loss of RET and GFRA3 during neuroblast migration and proliferation (Enomoto et al., 2001; Nishino et al., 1999), and suggested a role for CREB in RET signalling, in addition to its later developmental function in NGF signalling (Parlato et al., 2007). The phenotypic effect of the CREB knockout on sympathetic neurons is determined by developmental timing of the knockout.

Similarly, earlier ablation of KIF1Bβ may affect sympathetic neuroblasts differently to the DBH driven knockout of KIF1Bβ used in Paper III and reveal novel KIF1Bβ functions. In particular, future investigations of the role of KIF1Bβ, and other potential neuroblastoma tumour suppressors may benefit from earlier conditional knockouts. The DBH and TH promoters drive transgene expression in sympathetic neuroblasts from around embryonic day 11. This means that transgenes, such as TH-MYCN, are expressed from around E11 when neuroblasts commence a short period of proliferation from about E11-E15, and are likely most susceptible to oncogenic stimuli. In contrast, TH or DBH driven gene ablation, as used in Paper III, might not remove the protein in question until E13.5 (Paper III, Parlato et al., 2007) when the majority of neuroblast proliferation and, with it, potential sensitivity to oncogenic disruption has passed. Future in vivo studies using different transgenic approaches are required and will undoubtedly reveal more about neuroblastoma tumour suppression by KIF1Bβ and other putative 1p36 tumour suppressors.

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