Metastasis is the spread of cancer cells from the primary site to other organs in the body. Although metastasis is responsible for 90% of all cancer-related deaths, the molecular mechanisms involved and tools to study metastasis are not well established in pediatric solid tumours. Two such tumours are osteosarcoma (OS) and neuroblastoma (NB), in which metastatic disease predicts poor survival (<20%). Here, I identify Special AT-rich Binding Protein 2 (SATB2) as a protein that enhances invasion of Osteosarcoma (OS) cells. High level of SATB2 expression was detected in OS cells and metastases, and knockdown of SATB2 resulted in decreased invasion without affecting proliferation. Moreover, SATB2 knockdown induced global changes in gene expression including up-regulation of a protein, epithelial protein lost in neoplasm (EPLIN), and increased stress fiber formation. These results indicate that SATB2 functions as a pro-metastatic protein by modulating gene expression to regulate cytoskeleton remodeling. In addition to studying the role of SATB2 in OS invasion in vitro, in order to study molecular mechanisms that promote metastases in vivo, I also developed a novel metastatic mouse model of Neuroblastoma (NB). Intra-cardiac and in vivo selection were used to isolate metastatic subpopulations with enhanced metastatic capability to bone and central nervous system (CNS). Gene expression profiling revealed two subtypes, parental and metastatic, which
had 412 genes and multiple signaling pathways that were differentially regulated. I functionally validated novel genes including *GJA1, CADM1, SPHK1*, and *YAP/TAZ* using shRNA and over-expression, which rescued metastatic phenotype *in vitro* and *in vivo*. Treatment with SKI II and Verteporfin that target SPHK1 and YAP/TAZ, respectively, inhibited NB metastasis *in vivo*. In addition, we identified a metastatic gene signature (MET-75) that predicts NB patient survival. Together, my work has identified novel genes and molecular mechanisms involved in osteosarcoma and neuroblastoma metastasis.
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**ABI3**: Abscisic Acid Insensitive 3  
**ABI3BP**: ABI3 Binding Protein  
**ALK**: Anaplastic Lymphoma Kinase  
**ALP**: Alkaline Phosphatase  
**AMOTL2**: Angiomoitin-Like 2  
**APOE**: Apolipoprotein  
**ATAC**: Assay for Transposase-Accessible Chromatin  
**ATF4**: Activating Transcription Factor 4  
**ATRX**: Alpha Thalassemia/Mental Retardation Syndrome X-Linked  
**BEC**: Brain Endothelial Cells  
**BM**: Bone Marrow  
**BrdU**: 5-bromo-2'-deoxyuridine  
**CADM1**: Cell Adhesion Molecule 1  
**CAF**: Cancer-associated Fibroblast  
**cAMP**: cyclic Adenosine Monophosphate  
**CD**: Cluster of Differentiation  
**CDC42**: Cell Division Cycle 42  
**ChIP-seq**: Chromatin Immunoprecipitation Sequencing  
**CNS**: Central Nervous System  
**CNV**: Copy Number Variations  
**COG**: Children’s Oncology Group  
**COL4A1**: Collagen, Type IV, Alpha 1  
**COL4A2**: Collagen, Type IV, Alpha 2  
**CRISPR**: Clustered Regularly Interspaced Short Palindromic Repeats  
**CT**: Computed Tomography  
**DMSO**: Dimethyl Sulfoxide  
**DNA**: Deoxyribonucleic Acid  
**ECM**: Extracellular Matrix  
**EFEMP1**: EGF Containing Fibulin-Like Extracellular Matrix Protein 1  
**EGF**: Epidermal Growth Factor  
**EGFR**: Epidermal Growth Factor Receptor  
**EML4**: Echinoderm Microtubule Associated Protein Like 4  
**EMT**: Epithelial-to-Mesenchymal Transition  
**EPLIN**: Epithelial Protein Lost In Neoplasm  
**EURAMOS**: European and American Osteosarcoma Study Group  
**FACS**: Fluorescence-activated Cell Sorting  
**FAK**: Focal Adhesion Kinase  
**FBLN2**: Fibulin 2  
**FBS**: Fetal Bovine Serum  
**FDA**: Food and Drug Administration  
**FGF**: Fibroblast Growth Factor  
**GD2**: Disialoganglioside 2  
**GDNF**: Glia cell line-derived Neurotrophic Factor  
**GDP**: Guanosine diphosphate  
**GEM**: Genetically Engineered Mouse
GJA1: Gap Junction protein, Alpha 1
GJIC: Gap Junction Intercellular Communication
GPCR: G-Protein Coupled Receptor
GPR126: G-Protein coupled Receptor 126
GSEA: Gene Set Enrichment Analysis
GSN: Gelsolin
GTP: Guanosine-5’-triphosphate
GWAS: Genome-Wide Association Studies
HAS2: Hyaluronan Synthase 2
HNSCC: Head and Neck Squamous Cell Carcinoma
hOB: human Osteoblast
HOXA2: Homeobox A2
HVA: Homovanillic Acid
IGF: Insulin Growth Factor
IHC: Immunohistochemistry
INRG: International Neuroblastoma Risk Group
INSS: International Neuroblastoma Staging System
INV: Invasion
ITGB3: Integrin Beta 3
JAK: Janus Kinase
KEGG: Kyoto Encyclopedia of Genes and Genomes
LAMC2: Laminin, Gamma 2
LATS: Large Tumour Suppressor Kinase
LDH: Lactate Dehydrogenase
LEC: Lymphatic Endothelial Cells
LIFR: Leukemia Inhibitory Factor Receptor
LIM1: LIM domain and Actin Binding 1
LOH: Loss-of-Heterozygosity
LOXL3: Lysyl Oxidase-Like 3
LPA: Lysophosphatidic Acid
MALAT1: Metastasis-associated Lung Adenocarcinoma Transcript 1
MAP: Methotrexate, doxorubicin/Adriamycin, Cisplatin
MAPK: Mitogen-activated Protein Kinase
MDM2: Mouse Double Minute 2
MIBG: Meta-iodobenzylguanidine
MIG: Migration
miRNA: microRNA
MMP16: Matrix Metallopeptidase 16
MRI: Magnetic Resonance Imaging
MST: Mammalian Ste2-Like Kinase
MTBP: MDM2 Binding Protein
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUM1L1: Mutated Melanoma-Associated Antigen 1-Like Protein 1
MYC: V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
MYCN: V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma Derived Homolog
NB: Neuroblastoma
NCAM: Neural Cell Adhesion Molecule
NET: Norepinephrine Transporter
**NFkB**: Nuclear Factor Kappa-light-chain-enhancer of activated B cells  
**NGS**: Next-generation Sequencing  
**NOD/SCID**: Non-obese diabetic/Severe combined immunodeficiency  
**OB**: Osteoblast  
**OPN**: Osteoprotegrin  
**OS**: Osteosarcoma  
**Osx1**: Osterix 1  
**PARP**: Poly ADP Ribose Polymerase  
**PCR**: Polymerase Chain Reaction  
**PDX**: Patient-derived Xenograft  
**PDZ**: PSD-95, Dlg, and ZO-1/2  
**PI3K**: Phosphatidylinositol-4,5-bisphosphate 3-kinase  
**PKA**: Protein Kinase A  
**PRKAR1A**: Protein Kinase, cAMP-Dependent, Regulatory, Type I, Alpha  
**Rac1**: Ras-related C3 botulinum toxin substrate 1  
**RANK**: Receptor Activator of Nuclear factor Kappa-B  
**RANKL**: RANK-ligand  
**RB1**: Retinoblastoma 1  
**RECQL4**: RecQ Helicase-Like 4  
**RhoA**: Ras homolog gene family, member A  
**RNA**: Ribonucleic Acid  
**ROCK**: Rho-associated Kinase  
**RT-PCR**: Real-Time Polymerase Chain Reaction  
**RUNX2**: Runt-Related Transcription Factor 2  
**S1P**: Sphingosine-1-Phosphate  
**S1PR**: S1P Receptor  
**SATB1**: Special AT-rich Binding protein 1  
**SATB2**: Special AT-rich Binding protein 2  
**SDC2**: Syndecan 2  
**sgRNA**: single guide RNA  
**shRNA**: short-hairpin RNA  
**SMAD**: Sma- And Mad-Related  
**SPHK1**: Sphingosine Kinase 1  
**STAT3**: Signal Transducer and Activator of Transcription 3  
**TAZ**: Transcriptional Co-activator with PDZ-Binding Motif  
**TEAD**: TEA Domain Family proteins  
**TGF-β**: Transforming Growth Factor β  
**TP53**: Tumour Protein 53  
**TR**: Triple Reporter  
**TRAP**: Tartrate-Resistant Acid Phosphatase  
**TSLC1**: Tumour Suppressor in Lung Cancer 1  
**VEGFR1**: Vascular Endothelial Growth Factor Receptor 1  
**VMA**: Vanillylmandelic Acid  
**VP**: Verteporfin  
**WASH1**: WAS Protein Family Homolog 1  
**WGS**: Whole-Genome Sequencing  
**WNT**: Wingless-Type  
**YAP**: Yes-associated Protein
Chapter 1
Introduction

1 Introduction

1.1 Metastasis

Metastasis is responsible for more than 90% of cancer-related deaths. For most solid tumours, metastatic disease predicts an inferior outcome compared to patients with non-metastatic disease. Thus, it is of great interest to understand and target metastases to improve the survival of cancer patients.

Metastasis is the spread of a primary tumour to secondary sites in the body and involves a complex cascade of biological processes. First, cells from the primary tumour migrate and invade the surrounding extracellular matrix to intravasate into the blood vessels. Once in circulation, tumour cells survive in an anchorage-independent environment. Tumour cells then adhere to blood vessels and extravasate into the parenchyma of a secondary tissue where they colonize and form metastases (Figure 1.1). These multi-step processes involved in metastasis can be studied both in vitro and in vivo through various assays (see Figure 1.1). To successfully complete the metastatic cascade, metastatic cells often require changes in gene expression. For example, epithelial cells express Twist1 to activate the epithelial-to-mesenchymal transition (EMT) program to promote dissemination from the primary tumour, however, turning off Twist1 is required for extravasation into and colonization at the metastatic site (Tsai, Donaher, Murphy, Chau, & Yang, 2012).

Metastasis is not a random process but involves specific interaction between selected tumour cells and the secondary tissue as proposed in ‘seed and soil’ hypothesis by Stephen Paget in 1889. In fact, a study by Kang et al. on breast cancer metastasis revealed the existence of subpopulation of cells within the primary that had enhanced capacity to form metastasis. In addition, gene expression profiling of metastatic subpopulations resulted in the discovery of specific genes and pathways involved in metastasis to bone, lung, and brain (Bos et al., 2010; Kang et al., 2003; Minn et al., 2005). For example, metastatic subpopulations with enhanced metastasis to the bone had increased levels of CTCF and IL11 involved in angiogenesis and osteolysis, respectively, which promote tumour growth (Kang et al., 2003).
Metastasis is a multi-step process involving various biological processes. Cells from the primary tumour proliferate and acquire phenotypic traits that promote invasion and angiogenesis. The formation of blood vessels around the tumour allows cells to intravasate into the systemic circulation where they survive via anchorage-independent survival mechanisms. Tumour cells in circulation adhere to endothelial cells of the blood vessel and extravasate into the parenchyma of the secondary tissue. Finally, tumour cells colonize the secondary tissue to become overt metastases. Listed are examples of assays that can be used to monitor the specific steps of the metastatic cascade.

Researchers have previously focused on studying the genes and pathways within metastatic cells that regulate metastasis. Recent findings, however, suggest the importance of the microenvironment in promoting metastasis including reports demonstrating a formation of pre-metastatic niche that support the survival and growth of metastatic tumours (Costa-Silva et al., 2015; Kaplan et al., 2005). For example, VEGFR1-positive bone marrow-derived progenitor cells colonize the lung before tumour cells and produce fibronectin that forms a permissive niche for incoming tumour cells in the lung (Kaplan et al., 2005). Furthermore, immune cells including T cells, neutrophils, and natural killer cells have been implicated in both promoting and
suppressing metastasis (DeNardo et al., 2009; S. Kim et al., 2008; Malladi et al., 2016). For example, neutrophils promote breast cancer lung metastasis by releasing leukotrienes that promote tumorigenecity and increase metastasis-initiating cells (Wculek & Malanchi, 2015).

Regulation of the cytoskeleton is critical during metastasis. The actin cytoskeleton forms filopodia and lamellapodia at the leading edge of migrating cells and mediates extracellular signals into the cell via integrins and other cell surface receptors (Olson & Sahai, 2008). Dynamics of the actin cytoskeleton are regulated by small GTPases including RhoA and Rac1, and actin-binding proteins such as epithelial protein lost in neoplasm (EPLIN) and cofilin. For example, activation of Rac1 promotes membrane ruffling and lamellapodia formation, whereas RhoA regulates stress fiber formation and adhesion via activation of downstream kinases (for example, Rho-associated kinase, ROCK) (Moyano, Maqueda, Casanova, & Garcia-Pardo, 2003; Worthylake & Burridge, 2003). EPLIN, a cytoskeleton-associated protein that binds to and stabilizes actin filaments, is down-regulated in many cancers such as prostate and esophageal, and has been shown to negatively regulate growth and invasion by inhibiting epithelial-to-mesenchymal transition (Zhang et al., 2011). In epithelial cells, EPLIN links cadherin-catenin complexes to F-actin, stabilizing apical adhesion belts and also inhibits Rac1-induced ruffling by cross-linking and forming stabilized F-actin structures such as stress fibers at the expense of more dynamic actin filament structures (Abe & Takeichi, 2007; Maul & Chang, 1999). The role of EPLIN and how it is regulated in OS has not been previously described. In chapter 2 of my thesis, we demonstrate that EPLIN regulates OS invasion by modulating adhesion and stress fiber formation.

1.2 Osteosarcoma

1.2.1 Diagnosis, Prognosis, and Treatment

Osteosarcoma (OS) is a bone tumour, characterized histologically by the presence of the osteoid, which is produced by malignant cells. OS is the most common malignant bone tumour in children and adolescents, and patients clinically present with swelling and pain at the site of disease (Anderson, 2016). Common sites of OS include the femur, tibia and the humerus, and
occasionally the jaw and skull. For proper diagnosis, several imaging modalities and laboratory test are used. Radiographs and magnetic resonance imaging (MRI) of the involved bone and joints demonstrate lytic lesions and soft tissue extensions, respectively. Chest CTs and technetium bone scans are used to determine if metastatic spread to lung and other bones, respectively. Laboratory-based test are sometimes used to assess levels of alkaline phosphatase (ALP) and lactate dehydrogenase (LDH), which are elevated in 30% of OS patients (Geller & Gorlick, 2010). There are many factors that determine the prognosis of OS patients including presence of metastatic disease, response to chemotherapy, and histologic subtypes. For example, patients that present with metastatic disease and have lower histologic response after chemotherapy have poor prognosis compared patients with localized and highly chemoresponsive disease (Anderson, 2016). Despite advances in surgery and chemotherapy, survival rates remain 65-70% and for patients with metastases, most commonly pulmonary, survival is <20% (Anderson, 2016).

Currently, most OS patients are given neoadjuvant chemotherapy followed by a surgical procedure such as limb-salvage surgery. After surgery, patients receive maintenance chemotherapy and this leads to cure for approximately 70% of OS patients with localized disease (Geller & Gorlick, 2010). The most commonly used systemic chemotherapy combination includes high-dose Methotrexate, doxorubicin/Adriamycin, cisPlatin (MAP). Recent studies have added new combinations of chemotherapeutic agent including, for patients with poor response. However, efforts are underway to test addition and new combination of chemotherapeutic agents in patients with poor response. The European and American Osteosarcoma Study Group (EURAMOS) is currently conducting a clinical trial in which patients are randomized to receive MAP or MAP with ifosfamide and etoposide (Whelan et al., 2015).

1.2.2 Genetics, Etiology, and Models of Osteosarcoma

OS is a bone tumour and is thought to originate from mesenchymal cells of osteoblast (OB) origin that share several characteristics with immature osteoblasts. OS cells are characterized by genomic instability and high frequency of chromothripsis, which leads to chromosomal deletions, duplications, copy number variations (CNVs), and somatic structural variations (Chen et al., 2014; Perry et al., 2014; Savage et al., 2013). Genome-wide association studies (GWAS) and
whole-genome sequencing (WGS) studies have revealed mutations, loss-of-heterozygosity (LOH), and CNVs associated with OS (Chen et al., 2014; Perry et al., 2014; Savage et al., 2013). Patients with Li-Fraumeni and retinoblastoma syndrome harbouring mutations in TP53 and RB1, respectively, are predisposed to OS. In fact, TP53 (80-90%) and RB1 (10-39%) were the most frequently mutated genes, along with mutations in RecQ DNA helicase family members such as RECQL4, which may in part be responsible for high genomic instability (Kansara, Teng, Smyth, & Thomas, 2014; Nishijo et al., 2004). In addition, genomic aberrations occur in RUNX2, a master regulator of bone development, as well as in known oncogenes (MDM2, MYC, MET) were identified (Kansara et al., 2014).

To study the etiology of OS, various models have been developed including canine, xenotransplantation, and genetically engineered mouse (GEM) models (Guijarro, 2014). GEM models have been most insightful in terms of understanding development and biology of OS. For example, two different groups created mouse OS models by inactivating TP53 and Rb in the osteoblast lineage (Berman et al., 2008; Walkley et al., 2008). Specifically, these investigators generated conditional deletions of TP53 and Rb in osteogenic precursor cells by using a Cre recombinase driven by the promoter of Osterix1 (Osx1), a master regulator of bone differentiation that is expressed in mesenchymal cells committed to osteoblast-lineage. Although Rb deletions alone did not result in OS, both heterozygous (TP53+/−) and homozygous (TP53−/−) deletions of TP53 resulted in OS. These results indicate that TP53 is a driver of OS. Moreover, Rb deletion potentiated OS development and TP53−/−;Rb−/−;Osx-Cre+ mice developed OS with complete penetrance and short latency (Berman et al., 2008; Walkley et al., 2008). OS developed in these mice displayed similarities with human OS in terms of histology, genetic complexity, and metastatic behavior. For example, a significant number of mice developed metastases in the lung, which is the most common site of metastasis in human OS (Kansara et al., 2014). In addition to TP53 and Rb, other genes implicated in the development of OS has been found using GEM models including the tumour suppressor Prkar1a, a cAMP-dependent protein kinase type I-alpha regulatory chain. Deletion of Prkar1a in osteoblastic-lineage cells results in the development of OS characterized by RANKL over-expression (Molyneux et al., 2010). Given the long latency to the development of OS in GEM models, xenotransplantation models whereby OS cell lines are injected directly into the bone of mice via intra-femoral and/or intra-tibial injections are also used to study OS (Guijarro, 2014; Khanna, Prehn, et al., 2001b). More
recently, orthotopic patient-derived xenograft (PDX) models are being used to better model patients’ own tumours, which likely represents the most accurate pre-clinical modeling of the disease.

1.2.3 Regulators of Osteosarcoma Invasion and Metastasis

The survival rates for OS patients with localized disease is over 70%, however, patients with metastatic disease have survival rates of <20%. The most frequent site of metastasis in OS patients is the lung (~80%) but also less commonly include other distant bones, liver, and brain. Although germline and somatic mutations associated with the development of OS are well studied, the genes and pathways that regulate OS invasion and metastasis are less well understood. Despite the formation of metastases in OS GEM models, the long latency supports use of complementary models. Thus, orthotopic tumour models, which gives rise to metastases at a higher and faster rate, are widely used as well as in vitro models.

One of the first studies investigating the genes and pathways involved in OS metastasis utilized two clonally related cell lines, K7M2 and K12, derived from a spontaneously occurring murine OS (Khanna, Khan, et al., 2001a). Implantation of these two cell lines in an orthotopic primary tumour growth model showed differential capabilities for pulmonary metastatic potential with K7M2 cell line giving rise to lung metastases in over 90% of mice compared to K12 cell line, which only gave rise to metastases in 33% of mice (Khanna, Prehn, et al., 2001b). Subsequently, microarray analysis on K7M2 and K12 cell lines led to the identification of genes and pathways that were differentially regulated between the two cell lines. Notably, differentially regulated genes were enriched for those involved in motility, adherence, and angiogenesis (Khanna, Khan, et al., 2001a). One of the most functionally characterized genes involved in OS metastasis is ezrin. Ezrin is a linker protein that links the cell membrane to actin cytoskeleton and provides scaffolding of signaling proteins for intracellular signaling transduction. In OS, ezrin promotes lung metastasis by providing survival advantage of OS cells at the lung (Khanna et al., 2004). Consistent with this data, OS patients with high expression of ezrin had poor outcome. Moreover, using other models, other genes including CD99 and MTBP were found to regulate OS invasion by modulating rho-associated kinase 2 (ROCK2) activity and filopodia formation, respectively, both of which involve cytoskeletal remodeling (Agarwal et al., 2012;
Furthermore, numerous gene expression profiling studies using \textit{in vitro} and \textit{in vivo} models identified both cytoskeleton-remodeling and focal adhesion as metastatic pathways in OS (Flores et al., 2012; Jones, Salah, Del Mare, Galasso, Gaudio, Nuovo, Lovat, LeBlanc, Palatini, Randall, Volinia, Stein, Croce, Lian, & Aqeilan, 2012a; Khanna, Prehn, et al., 2001b; ODonoghue et al., 2010). These data suggests the importance of actin cytoskeleton dynamics in OS invasion and metastasis.

1.3 Neuroblastoma

1.3.1 Diagnosis, Prognosis, and Treatment

Neuroblastoma (NB) is the most common extra-cranial solid tumor of childhood and the most frequent cause of cancer-related death in children (Maris, 2010). NB is the most common tumour of infancy with average age of ~18months. NB patients clinically present with a variety of symptoms depending on tumour location, including abdominal pain, respiratory distress, signs of spinal cord compression. If metastatic, symptoms may include weight loss, bone pain, and anemia. For diagnosis, several procedures are used including imaging, and biochemical tests. The level of urinary catecholamine metabolites, vanillylmandelic acid (VMA) and homovanillic acid (HVA), are measured as NB tumours release high levels of catecholamines (Irwin & Park, 2015). In addition, an $^{123}$I radioactive compound meta-iodobenzylguanidine (MIBG), a norepinephrine analogue that binds to the NET receptor on NB cells, is used for imaging to detect NB tumours and metastases in the body. High doses of MIBG are also used as a therapy for relapses (Irwin & Park, 2015). For more direct confirmation of the disease, biopsies from primary tumours or metastases are taken for histopathological analysis including immunohistochemistry (IHC), and genomic studies.

NB has a complex staging and risk group stratification system that utilizes clinical and biological factors. Although there is currently a new system that will be incorporated into future North American trials, the International Neuroblastoma Risk Group (INRG) and the International Neuroblastoma Staging System (INSS) has been widely used to stage NB, which mostly assess size and spread of the tumour (Irwin & Park, 2015). It ranges from INSS Stage 1 tumours, which are usually tumours that can be completely removed by surgery, to INSS Stage 4 tumours, which
are metastatic tumours with distant spread to bone, bone marrow, lymph nodes, and liver. NB patients are stratified into low, intermediate, and high risk groups by the Children’s Oncology Group (COG) based on stage and several prognostic indicators including age, tumour histology, DNA ploidy, MYCN gene amplification, and metastasis (Irwin & Park, 2015). For example, the low risk group includes patients with Stage 1 tumours, <12 months old, and without MYCN amplification, whereas the high risk group includes older (>18 months) patients with more aggressive cancer characterized by MYCN amplification and/or metastases, chromosomal abnormalities, and unfavourable histology.

Survival rates for low and intermediate risk NB patients are over 90% (Maris, 2010). These patients can usually be cured with surgery and chemotherapy. Interestingly, patients with Stage 4S special disease, asymptomatic <12 months old with metastatic disease, and favourable biology (histology, MYCN non-amplified) do not receive any therapies but rather are monitored carefully as these tumours often spontaneously resolve or differentiate. In contrast to non-high risk, High risk patients have an inferior outcome with 5-year survival of 40-50% (Maris, 2010). These patients require more intensive therapy including high-dose chemotherapy, surgery, stem cell transplant, and differentiation and/or immunotherapy. Recently, the FDA approved Unituxin (dinutuximab), a monoclocal antibody for GD2, as part of first-line therapy for high-risk NB (Yu et al., 2010). Unfortunately, patients with metastatic relapse have a dismal outcome with 5-year survival rates of less than 10%. Currently, there are no therapies that target specifically metastatic NB suggesting the urgent need for new treatment options for NB patients with metastatic relapse disease.

1.3.2 Genetics, Etiology, and Models of Neuroblastoma

Many cancers have recurrent mutations in oncogenes and tumour suppressor genes that may be exploited for targeted therapies in the clinic. For example, EGFR gene is mutated in 15-20% lung adenocarcinoma patients and these patients with EGFR alterations are treated with Gefitinib, an EGFR inhibitor (Janne et al., 2005). Similarly, melanoma patients with tumours harbouring BRAF mutations (~50%) are treated with BRAF inhibitors including Vemurafinib (Chapman et al., 2012). Unlike these cancers, targetable recurrent mutations are rare in NB with the most frequent alteration, missense mutations of ALK, detected in only 10% of patients
(George et al., 2008; Mossé et al., 2008). \textit{ALK} encodes the anaplastic lymphoma kinase (ALK) and activating mutations lead to constitutive activation of the kinase, enhanced anti-apoptotic program, and MAPK signaling leading to proliferation of NB cells \textit{in vitro} and \textit{in vivo} (Y. Chen et al., 2008; Eleveld et al., 2015; George et al., 2012). In lung cancer, \textit{ALK} gene can be fused to \textit{EML4} (EML4-ALK) or other partners to promote malignant phenotype and can be targeted with ALK inhibitors including Crizotinib (Kwak et al., 2010). Currently, clinical trials are being conducted to study the efficacy of Crizotinib for NB patients with ALK mutations (Mossé et al., 2013).

One of the most important genetic aberrations of NB is \textit{MYCN} amplification, which is observed in about 25% of patients and predicts poor survival (Maris, 2010). \textit{MYCN} is a member of the MYC family of transcription factors that regulate gene expression involved in various biological processes including cell cycle progression, differentiation, and apoptosis. MYC proteins are well known oncogenes that promote tumorigenesis and are one of the most frequently activated gene in human cancers. Although MYC proteins, like other transcription factors, are difficult to target pharmacologically, there are efforts to indirectly target MYCN proteins in NB. For example, the aurora kinase inhibitor (Alisertib) and BET bromodomain inhibitor (JQ1) are used to indirectly down-regulate MYCN expression and activity (Gustafson et al., 2014; Puissant et al., 2013). Both inhibitors showed efficacy in pre-clinical models of NB and are in clinical trials for further development into the clinic (DuBois et al., 2016; Krytska et al., 2016).

To study and understand the etiology of NB, various GEM models have been developed. The first mouse model was generated by transgenic expression of \textit{MYCN} oncogene in neural crest cells using sympathoadrenal lineage specific tyrosine hydroxlase (TH) promoter (Weiss, Aldape, Mohapatra, Feuerstein, & Bishop, 1997). These mice develop NB that shares both histological and chromosomal abnormalities detected in human NB tumours. This study supported \textit{MYCN} to be a driver of NB and validates the notion that NB originates from sympathoadrenal progenitors of neural crest cells. Unlike \textit{MYCN} models, introducing \textit{ALK} mutation (F1174L) into sympathoadrenal progenitors alone did not induce NB tumorigenesis in both mouse and zebrafish models (George et al., 2012; Zhu et al., 2012). \textit{ALK} mutation, however, potentiated tumorigenesis in \textit{MYCN}-driven NB models suggesting that ALK plays an important role in promoting NB progression (George et al., 2012; Zhu et al., 2012).
1.3.3 Models and Regulators of Neuroblastoma Metastasis

More than half of NB patients present with bone and/or bone marrow (BM) metastases, and long-term survival is less than 40%. At the time of recurrence, the majority of patients have evidence of metastatic spread to the bones, bone marrow, and increasingly, the central nervous system (CNS) (Kramer, Kushner, Heller, & Cheung, 2001). Survival after metastatic relapse is < 5% (Maris, 2010; Modak & Cheung, 2010). Although there has been significant progress using next-generation sequencing (NGS) approaches to identify of genetic alterations such as MYCN, ALK, ATRX (Molenaar et al., 2013; Mossé et al., 2008; Pugh et al., 2013; W. A. Weiss et al., 1997) in primary NB tumors, recurrent driver mutations are uncommon, and specific alterations associated with NB metastasis have not been well studied. Recent studies have identified MAPK signaling pathway to be highly up-regulated in relapsed NB tumours (Eleveld et al., 2015; Schramm et al., 2015).

Although limited, several regulators of NB invasion and metastasis have been identified. For example, caspase-8 has been shown to regulate NB metastasis without affecting primary tumour growth (Stupack et al., 2006). Mechanistically, caspase-8 induced integrin-mediated apoptosis in NB cells invading the collagenous stromal microenvironment and loss of caspase-8 promotes survival of NB cells for metastatic outgrowth in chick chorioallantoic membrane (CAM) model (Stupack et al., 2006). Moreover, genetic knockout of caspase-8 in TH-MYCN mouse model increased the incidence of NB bone-marrow metastases, further supporting the important role of caspase-8 in NB metastasis (Teitz et al., 2013).

To date, there are no in vivo models that faithfully recapitulate metastatic NB, both in terms of tissue tropism and burden of disease. Furthermore, the genetic heterogeneity and lack of frequent recurrent genetic alterations make it challenging to generate GEM models that reflect the spectrum of mutations. Current NB models rely on the use of tail-vein and intra-femoral cell injections and xenograft, orthotopic, or genetically engineered mouse (GEM) models of MYCN and/or ALK alterations, which although informative, have several limitations (George et al., 2012; Iwakawa, Ando, Ohkawa, Koike, & Chen, 2005; Nevo et al., 2008; Teitz et al., 2011; W. A. Weiss et al., 1997). First, the incidence and pattern of metastatic spread in these models is low, with only rare osteolytic bone lesions. Second, tail-vein injections rarely result in BM
metastases and instead, lead to frequent lung lesions, which are rare in NB patients at diagnosis, and intra-femoral injections produce secondary outgrowths (George et al., 2012; Teitz et al., 2011; W. A. Weiss et al., 1997) that only recapitulate metastatic colonization, which a later step in the metastatic cascade. Third, most current models lack the capacity to image and quantify metastases in vivo. Finally, most models tend to give rise to micrometastatic lesions with low frequency and no significant incidence of brain metastases (Iwakawa et al., 2005; Sohara et al., 2003; Teitz et al., 2011). Hence, more faithful, reproducible and quantifiable models of metastatic NB are urgently required. In chapter 3 of my thesis, we demonstrate the development of a novel metastatic mouse model of NB as well as the identification of novel regulators of NB metastasis. Background on the genes and pathways identified and studied in OS and NB are described below.

1.4 Special AT-rich Binding Protein 2 (SATB2)

SATB2 is a member of the SATB family of transcription factors, which share structural homology consisting of PDZ, CUT, and homeobox domains. They bind to AT-rich DNA sequences in nuclear matrix attachment regions to regulate gene expression by orchestrating chromatin organization and remodeling (Dobreva, Dambacher, & Grosschedl, 2003). In addition, SATB2 can directly bind transcription factors acting as a co-activator or repressor. Knockout mouse models have demonstrated roles for SATB2 in craniofacial morphogenesis and osteoblast differentiation in part via SATB2-mediated repression of Hoxa2 and cooperation with ATF4 and Runx2 to promote osteoblast differentiation (Dobreva et al., 2006). SATB2 also regulates neuronal specification and migration by recruiting chromatin-remodeling complexes to the Ctip2 locus for repression (Britanova et al., 2008). In mice, SATB2 mutant cortical neurons acquire Ctip2 expression and project axons along the corticospinal tract instead of axon extension along the corpus collosum (Alcamo et al., 2008). However, the role of SATB2 in cancer is less well understood. Interestingly, the SATB2 homologue SATB1, promotes breast tumor growth and metastasis by reprogramming global gene expression (Han, Russo, Kohwi, & Kohwi-Shigematsu, 2008). Pathway analyses of SATB1-depleted breast cancer cells showed enrichment in genes involved in growth, including cell cycle and phosphatidylinositol signaling, and metastasis, such as cell adhesion, extra-cellular matrix, and focal adhesions (Han et al., 2008).
Both SATB1 and SATB2 proteins comprise a SATB domain (PDZ-like), 2 CUT domains, and 1 homeodomain. The SATB domain has been implicated in dimerization of SATB1, which is required for high-activity DNA binding (Purbey et al., 2008). All four domains have been implicated in high affinity DNA-binding (Dickinson & Kohwi-Shigematsu, 1995; Nakagomi, Dickinson, & Kohwi-Shigematsu, 1994; B. Wang, Dickinson, Koivunen, Ruoslahti, & Kohwi-Shigematsu, 1995).

Our group previously reported the first role for SATB2 in cancer, identifying it as a novel binding partner for the p53 paralogues p63 and p73, but not p53. In head and neck squamous cell carcinoma (HNSCC), SATB2 augments the activity of the N-terminally truncated p63 isoform ΔNp63α to promote chemoresistance. In primary HNSCC tumours, SATB2 protein expression was associated with more advanced stage HNSCC (Chung et al., 2010). SATB2 has also been reported to be upregulated in cancer associated fibroblasts mediating migration of endometrial cancer cells (Aprikolova et al., 2014), and higher SATB2 mRNA levels were detected in breast cancer tissue compared to normal matched tissue (Patani, Jiang, Mansel, Newbold, & Mokbel, 2009). In contrast, SATB2 expression is associated with more favorable outcome in colon carcinoma (Eberhard et al., 2012), suggesting that like many other proteins involved in tumorigenesis, the function(s) of SATB2 in cancer may be cell or context dependent and further studies are required to dissect the molecular mechanisms by which SATB2 may promote tumorigenesis and/or invasion and metastasis. In chapter 2 of my thesis, we demonstrate that SATB2 is highly expressed in and is a specific marker for OS, and that SATB2 regulates the expression of genes involved in actin cytoskeleton dynamics to promote OS invasion and migration, including EPLIN, which is a key mediator by which SATB2 regulates OS invasion.
1.5 Gene Expression Signatures in Neuroblastoma

Recent advances in sequencing and high-throughput technologies have revolutionized cancer research. For example, high-throughput gene expression analysis can easily be performed to help understand fundamental questions in cancer biology. One of the applications of gene expression arrays is molecular subtyping/classification of patient tumours. For example, combined use of gene expression profiling, DNA copy number aberrations, and clinical characteristics led to the identification of distinct molecular variants in medulloblastoma, which correlate with different clinical outcomes (Northcott et al., 2011).

In NB, numerous gene signatures have been identified through various strategies including gene sets representing specific biological pathways (Asgharzadeh et al., 2012), experimental interventions (Barbieri et al., 2013; Fardin et al., 2015; Valentijn et al., 2012), and comparison of expression profile derived from high and low risk NB patients (Asgharzadeh et al., 2006; De Preter et al., 2010; Oberthuer et al., 2006; Schramm et al., 2005; Vermeulen, De Preter, Laureys, Speleman, & Vandesompele, 2009). For example, Valentijn and colleagues have generated a functional MYCN signature by profiling NB cell lines in absence and presence of shRNA-mediated knockdown of MYCN (Valentijn et al., 2012). Their functional gene signature was comprised of 157 genes that were differentially regulated between shMYCN and control NB cell lines and predicted overall survival of NB patients.

These gene signatures are useful in identifying high and low risk patient subsets and thus can help tailor treatment strategies. One of the limitations of current gene signatures is their inability to predict outcome for patients within the high-risk group, and to specifically distinguish between those who experience rapid progression and/or metastatic relapse from those who will be long-term survivors. In chapter 3 of my thesis, we identify metastatic gene signature (MET-75) that has predictive power within high-risk subsets, which potentially can be applied to further stratify this poor prognosis group of patients.

1.6 Regulators of Neuroblastoma Metastasis

Although genetic aberrations involved in NB tumourigenesis are well-studied, the genes and pathways that regulate NB metastasis are largely unknown. In chapter 3 of my thesis, we
demonstrate the development of our metastatic NB mouse model and the identification of novel regulators of NB metastasis including CADM1, GJA1, the Hippo pathway, and SPHK1. Here, I provide brief background to each of the novel regulators of NB metastasis.

1.6.1 Cell adhesion molecule 1 (CADM1)

*CADM1* encodes the cell adhesion molecule 1 (CADM1) protein, which is also widely known as tumor suppressor in lung cancer 1 (TSLC1). CADM1 is a transmembrane glycoprotein with multiple immunoglobulin domains in the extracellular domain and mediates intracellular adhesion via hemophilic interactions in a Ca$^{2+}/\text{Mg}^{2+}$-independent manner (Masuda et al., 2002). For example, CADM1 mediates the interaction between the nerve and mast cells that forms a functional neuro-immune mechanism (Hagiyama et al., 2011). CADM1 was first identified as a tumour suppressor by mapping of genes located on chromosome 11q23, which is a region that is frequently lost in lung cancer (Kuramochi et al., 2001). Experiments restoring CADM1 expression in the CADM1 deficient A549 lung cancer cell line suppressed tumour formation in mice, confirming functional importance of CADM1 as a potential tumour suppressor. Subsequent studies reported multiple mechanisms that down-regulate CADM1 expression in tumours including promoter hypermethylation, miRNA-mediated knockdown, and hypoxia (Fukami et al., 2003; Momose et al., 2013). In addition to the role of CADM1 as a tumour suppressor due to its role in inhibiting growth, recent studies have shown that CADM1 also inhibits metastasis (Faraji et al., 2012; Qiu et al., 2014; Wikman et al., 2014). For example, Faraji and colleagues found that CADM1 inhibits metastasis in tumour non-autonomous manner by sensitizing tumour cells to CD8$^+$ T cell surveillance mechanism (Faraji et al., 2012).

Loss-of-heterozygosity (LOH) at chromosome 11q23 region is commonly detected in NB and is associated with poor outcome (Attiyeh et al., 2005). Similar to its role in lung cancer, CADM1 acts as a tumour suppressor in NB. Low expression of *CADM1* is associated with poor outcome and over-expression of CADM1 inhibits survival of NB cell lines (Nowacki et al., 2007). The role of CADM1 in NB metastasis, however, has not been studied. In chapter 3 of my thesis, we demonstrate that CADM1 expression is down-regulated in metastatic subpopulations of NB cells and that CADM1 over-expression inhibits NB metastatic phenotype both *in vitro* and *in vivo*. 
1.6.2 Gap junction, alpha 1 (GJA1)

*GJA1* encodes for a gap junction alpha 1 (GJA1) protein, which is also widely known as connexin-43. GJA1 is a member of the connexin family of proteins that make up gap junctions, which acts as an intercellular channel that allows for diffusion of various low molecular weight molecules from cell to cell. In addition to low molecular weight molecules, gap junction also enables rapid transmission of electrical signals in the heart and neuronal tissues. In fact, GJA1 is expressed in both ventricular and atrial cardiomyocytes and is critical in mediating electric coupling for proper impulse propagation (Michela, Velia, Aldo, & Ada, 2015). Inhibition of GJA1 expression by shRNA knockdown results in decreased conduction velocity and the organization of gap junctions were disordered in myocardial ischemia and infarction, which are accompanied by arrhythmia (Michela et al., 2015).

In cancer, the role of GJA1 is less well defined and has been described to have roles as both tumour suppressor and oncogene, suggesting its function is cell or context dependent. A potential tumour suppressor-like role of GJA1 is suggested by the findings that observed low GJA1 expression in various cancer and cancer stem cells (Hitomi et al., 2015; McLachlan, Shao, Wang, Langlois, & Laird, 2006). In breast cancer, overexpression of GJA1 reverts EMT phenotype and inhibits tumour growth *in vivo* in a gap junctional intercellular communication (GJIC)-independent manner (McLachlan et al., 2006). On the other hand, GJA1 promotes tumour progression in multiple cancers including glioma, melanoma, bladder and prostate cancers (Poyet et al., 2015; Sin et al., 2015; Villares et al., 2009; A. Zhang et al., 2015). For example, high expression of GJA1 was detected in prostate cancer cell lines with increased metastatic potential. Moreover, shRNA-mediated knockdown down of GJA expression decreased migration of prostate cancer cells (Zhang et al., 2015).

Although limited, the expressions of several gap junction proteins have previously been implicated in NB (Arnold, Phipps, Chen, & Phipps, 2005; Carystinos, Alaoui-Jamali, Phipps, Yen, & Batist, 2001; Morley et al., 2010; Srinivas et al., 1999). For example, upregulation of GJA1 expression was associated with cAMP-induced chemosensitivity in NB cells (Carystinos et al., 2001). Moreover, activation of cAMP-dependent protein kinase (PKA) restored the abnormal nuclear localization of GJA1 to the cell membrane and decreased proliferation of the IMR-32 NB cell line (Arnold et al., 2005). These studies suggest that GJA1 may have tumour
suppressor-like roles in NB. In chapter 3 of my thesis, we demonstrate that GJA1 knock down inhibits metastatic phenotypes in vitro and suggest that unlike previous reports, GJA1 functions to promote tumour progression by increasing migration of NB cells.

### 1.6.3 The Hippo Pathway

The Hippo Pathway is an evolutionally conserved signaling pathway that regulates numerous biological processes including cell growth, differentiation, and organ size control. It was initially identified and characterized in *Drosophila melanogaster* as a tumour suppressor pathway because loss-of-function mutations in several components of the pathway resulted in an overgrowth phenotype (Huang, Wu, Barrera, Matthews, & Pan, 2005; S. Wu, Huang, Dong, & Pan, 2003). In mammals, the Hippo pathway consists of multiple kinases including MST1/2 (mammalian Ste2-like kinases) and LATS1/2 (large tumour suppressor kinase) and effector proteins YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ binding motif). Upon activation of the pathway, MST1/2 phosphorylates LATS1/2, which in turn, phosphorylates the effector proteins YAP and TAZ. The phosphorylation of YAP and TAZ results in cytoplasmic retention and inhibits transcriptional activity of the effector proteins. When the pathway is disrupted, YAP and TAZ are dephosphorylated and enter the nucleus to promote transcriptional activity through interacting with transcription factors such as TEAD family transcription factors (Huang et al., 2005; S. Wu, Liu, Zheng, Dong, & Pan, 2008) (Figure 1.3).
Figure 1.3. The regulation of the Hippo pathway.
The Hippo pathway is activated mainly by the presence of cell-cell contact. Once activated (ON state), kinase cascade involving MST1/2, and LATS1/2 results in phosphorylation of YAP/TAZ at multiple sites. This leads to inactivation of YAP/TAZ activity by cytoplasmic retention of proteosomal degradation. The Hippo pathway is inactivated (OFF state) by the absence of cell-cell contact and activated growth signals including receptor kinase and G-protein coupled receptor activation. This leads to inhibition of MST1/2 and LATS1/2 kinases resulting in unphosphorylated YAP/TAZ. The unphosphorylated YAP/TAZ translocates into the nucleus where they bind to transcription factors (i.e. TEAD transcription factors) to regulate downstream genes involved in proliferation, anti-apoptosis, and invasion.
Although mutations in the components of the Hippo pathway are rare, the pathway is deregulated and high expression of the effectors YAP and TAZ are detected in many tumours. One mechanism by which this occurs is through changes in structural architecture of the cell. For example, depletion of Scribble and Par3 polarity complex in polarized epithelial cells increases the activation of YAP by suppressing RASSF, a negative regulator of the Hippo pathway (Grzeschik, Parsons, Allott, Harvey, & Richardson, 2010). In addition, YAP and TAZ can be regulated by both mechanical and hormonal cues that result in cytoskeleton remodeling. For example, extracellular mitogens such as lysophosphatidic acid (LPA) can induce YAP nuclear localization and activity through G-protein coupled receptors (GPCRs) signaling. More specifically, GPCRs activate Rho GTPases to induce actin stress fiber formation, which in turn inhibits LATS1, and thus leading to YAP activation (Yu et al., 2012). Indeed, mutations in GNAQ encoding for GPCR in uveal melanoma induces YAP activation to promote tumorigenesis (Yu et al., 2014). In addition, YAP can also be activated by Wnt signaling and act in concert with β-catenin to induce a transcriptional program that promotes intestinal crypt hyperplasia (Azzolin et al., 2012). Furthermore, YAP signaling has been implicated in promoting metastasis through transcriptional reprogramming of various cancers including breast, lung, and melanoma (Chen et al., 2012; Lamar et al., 2012; Lau, Curtis, Fillmore, Rowbotham, Mohseni, Wagner, Beede, Montoro, Sinkevicius, Walton, Barrios, Weiss, Camargo, Wong, & Kim, 2014a; Z. Wang et al., 2013).

Despite recent findings on the role of Hippo signaling in many tumours, its role in NB has not been well studied. Although limited, there are only a few studies examining a role for the Hippo signaling in NB. First, the Hippo pathway effector TAZ was shown to promote proliferation and tumorigenicity of NB cells in vitro and in vivo by upregulating CTGF and PDGF-β expressions. In addition, pathway analysis of relapsed NB tumours revealed YAP activation as the only functional nodule enriched in relapsed NB tumours compared to the matched-primary tumours (Schramm et al., 2015). To date, however, the role of the Hippo pathway in NB metastasis is not known. In chapter 3 of my thesis, we demonstrate that YAP and TAZ are over-expressed in subpopulations of NB cells with enhanced metastatic capability. Furthermore, we demonstrate that increased YAP and TAZ is, in part, responsible for increased invasion of metastatic cells and that knocking down YAP and TAZ suppresses NB metastasis in vivo.
1.6.4 Sphingosine kinase 1 (SPHK1)

*SPHK1* encodes the sphingosine kinase 1 (SPHK1) protein, a lipid kinase that phosphorylates sphingosine to generate sphingosine-1-phosphate (S1P). S1P is a bioactive lipid that regulates various biological functions including cellular proliferation, migration, differentiation, and angiogenesis (Kunkel, Maceyka, Milstien, & Spiegel, 2013). S1P generated by SPHK1 at the plasma membrane can either stay inside the cytoplasm or exported out of the cells by the ABC transporters. S1P inside the cells can activate multiple signaling pathways including the NFκB pathway and secreted S1P binds to S1P receptors in both autocrine and paracrine manner (Lee et al., 2010; Liang et al., 2013) (Figure 1.4). One of the most prominent roles of S1P is its role in immune trafficking. During immune response, S1P concentration gradient is responsible for the egress of T and B lymphocytes from lymphoid organs. In fact, lymphocyte egress is inhibited by the treatment with Fingolimod, a S1P receptor blocker, which causes immune suppression (Brinkmann, 2002; Mandala et al., 2002).

Recent studies have indicated a role for SPHK1 in tumorigenesis. For example, in colitis-associated cancer, SPHK1 expression is upregulated and S1P activates NFκB-STAT3 signaling pathway via S1P receptor 1 (S1PR1) for colonic epithelial cell proliferation. The S1P-S1PR1-STAT3 signaling axis has also been implicated in other cancers including breast (Lee et al., 2010) and lymphoma (Liu et al., 2012b), as well as in the formation of a pre-metastatic niche by myeloid cells in prostate and melanoma (Deng et al., 2012). In addition, SPHK1 also play a key role in angiogenesis, chemoresistance, and invasion (Brizuela et al., 2014; J. Li et al., 2016; Long, Xie, Yin, Lu, & Fang, 2015; Nagahashi et al., 2012). For example, inhibition of SPHK1 decreased the number of both blood endothelial cells (BECs) and lymphatic endothelial cells (LECs) in the tumour and inhibited lymph node metastasis of breast cancer cells by suppressing hemangiogenesis and lymphangiogenesis *in vivo* (Nagahashi et al., 2012).
Figure 1.4. The sphingosine kinase (SPHK1)-sphingosine-1-phosphate (S1P) pathway.
The sphingosine is generated from ceramide by ceramidases. Sphingosine can also be converted into a ceramide by ceramidase synthase, both of which regulate apoptosis, cell-cycle arrest, and senescence programs. Sphingosine kinases (SPHK1/2) phosphorylates sphingosine to generate sphingosine-1-phosphate (S1P), which plays critical roles in regulating proliferation, invasion, inflammation, and immune trafficking. Mechanistically, S1P can bind to S1P receptors (S1PRs) and/or activate NFκB signaling to regulate cytoskeleton dynamics and transcriptional activation. There are several approaches that have been developed to inhibit SPHK1-S1P signaling. First, sphingosine analogues such as Fingolimod have been implicated to inhibit S1PR signaling. In addition, monoclonal antibody to S1P, Sphingomab, is used to inhibit S1P-S1PR binding. Both Fingolimod and Sphingomab are being tested in clinical trials. Furthermore, small-molecule kinase inhibitors targeting SPHK1/2 are being tested in preclinical models for inhibition of SPHK1-S1P signaling.

Although the oncogenic role of SPHK1 has been well documented in many cancers, the role of SPHK1 is limited in NB. In one study, SPHK1 was shown to mediate glial cell line-derived neurotrophic factor (GDNF)-dependent cell proliferation and neuronal differentiation of NB cells (Murakami et al., 2007). Moreover, amyloid β-induced death was mediated by inhibition of SPHK1 activity in a NB cell line SH-SY5Y (Gomez-Brouchet et al., 2007). In addition, SPHK2 induces VEGF expression through S1P-S1PR2 signaling, which was abrogated by S1PR2 antagonist JTE-013 to inhibit growth of NB xenograft (Li, Hla, & Ferrer, 2011). Nevertheless, the role of SPHK1 in NB metastasis has not been studied. In chapter 3 of my thesis, we demonstrate that SPHK1 is upregulated in metastatic subpopulations of NB cell lines.
Furthermore, we demonstrate that pharmacological inhibitors for SPHK1, SKI II and PF-543, rescue metastatic phenotype \textit{in vitro} and that SKI II treatment \textit{in vivo} suppress NB metastasis.

### 1.7 Thesis objectives

There are two main objectives of my thesis that will further our knowledge in molecular determinants of metastasis in pediatric cancers. The first objective is to investigate the role of SATB2 in mediating OS invasion. In Chapter 2, I will present data supporting a role for SATB2 in promoting the invasion of OS cells by regulating the expression of cytoskeletal genes including EPLIN. The second objective is to develop a metastatic mouse model of NB and to identify novel regulators of NB metastasis. In Chapter 3, I will describe how a novel metastatic mouse model of NB is developed using intra-cardiac and \textit{in vivo} selection techniques. In addition, I will present data on the isolation of metastatic subpopulations of NB cells and the role of novel regulators of NB metastasis including CADM1, GJA1, the Hippo pathway, and SPHK1. The work in my thesis sheds light into how SATB2 may promote OS metastasis and how our metastatic mouse model of NB can be used to study the biology of NB metastasis and to test putative anti-metastatic drugs.
Chapter 2
The Role of SATB2 in Osteosarcoma Invasion

This work has been published:


I performed all experiments in this paper except immunohistochemistry, microarray analysis and real-time PCR validation, which were performed with help from other authors.

2 The Role of SATB2 in Osteosarcoma Invasion

2.1 Background

Our lab had previously identified SATB2 as an interacting protein for p63 and p73 (Chung et al., 2010). SATB2 is a DNA-binding protein capable of modulating gene expression by regulating chromatin remodeling and transcription (Dobreva et al., 2003). In addition, SATB2 plays a crucial role in normal osteoblast development and its deletion results in craniofacial abnormalities and defects in osteoblast differentiation and function (Dobreva et al., 2006). SATB1, a SATB2 homolog, has been reported to promote breast tumour growth and metastasis by reprogramming gene expression (Han et al., 2008). The role of SATB2 in cancer, however, is controversial as studies reported both oncogenic and tumour suppressive-like functions for SATB2 (Aprelikova et al., 2014; Chung et al., 2010; Eberhard et al., 2012; Mansour et al., 2015; S. Wang et al., 2009). For example, SATB2 plays a oncogenic role in HNSCC by promoting chemoresistance whereas SATB2 suppress proliferation of colorectal cancer cells (Chung et al., 2010; Mansour et al., 2015). This supports the idea that function of SATB2 may be context-dependent. EPLIN is a cytoskeleton protein that links focal adhesion complexes to actin filaments and stabilize actin stress fibers (Abe & Takeichi, 2007; Maul & Chang, 1999). In
breast and prostate cancers, EPLIN inhibits the growth and invasion of cancer cells (Jiang et al., 2008; Sanders, Martin, Ye, Mason, & Jiang, 2011; S. Zhang et al., 2011). However, the role of EPLIN in OS has not been investigated.

We detected highest SATB2 protein expression in Osteosarcoma cells compared to other cancer cell lines. This raises the possibility that SATB2 may have oncogenic roles in Osteosarcoma. The goal of this chapter is to determine the role of SATB2 in Osteosarcoma.

2.2 Hypothesis/Rationale

I hypothesize that increased SATB2 expression in OS cells leads to increase migration and invasion by regulating expressions of its target genes.

2.3 Results

2.3.1 SATB2 is highly expressed in OS

We had previously reported that high levels of SATB2 are detected in advanced stage HNSCC primary tumors and cell lines (Chung et al., 2010). We screened more than 20 cancer cell lines from different tumors and noted high SATB2 protein levels in OS cell lines. SATB2, but not SATB1, was detected in all five human OS cell lines tested at levels significantly higher than those detected in non-transformed hOBs (human OBs), the putative cell of origin for OS (Figure 2.1a). The increased expression in OS cell lines in comparison with non-transformed OBs suggests that SATB2 may be involved in transformation of OBs to OS cells. We also examined SATB2 expression in murine OS cells that were derived from the MOTO mouse model of OS in which expression of the simian virus T-antigen transgene is driven by an OB-specific promoter (Molyneux et al., 2010). SATB2 was expressed in both primary bone tumors and lung metastases. Interestingly, SATB2 expression was higher in cells derived from metastases compared to matched primary tumors (Figure 2.1b), suggesting that SATB2 may play a role in promoting OS metastasis in vivo. We next asked whether SATB2 is expressed in primary human OS tumors. Immunostaining of a tissue microarray of primary pediatric tumors showed nuclear SATB2 protein expression in 41/44 (93%) OS samples. In contrast, SATB2 expression was
undetectable in 76/77 of non-OS sarcomas (Figure 2.1c).

Figure 2.1. SATB2 expression in Osteosarcoma (OS).

(A) Lysates from human osteoblast (OB) and OS cell lines were immunoblotted for SATB1 and SATB2. HaCat cells transduced with adenovirus encoding SATB1, or SATB2 were used as positive controls. (B) Lysates of Murine OS cell lines derived from primary tumors and pulmonary metastases of MOTO mouse were immunoblotted for SATB2. Note: hOB lysate was resolved in the same gel as murine OS cells. (C) OS tumour biopsy samples were immunostained with SATB2 antibody. Shown are examples of positive (brown nuclear staining) and negative tumors. Chart: Results of SATB2 immunostaining of tissue microarray with different histologic types of non-OS sarcomas.
2.3.2 SATB2 knockdown decreases migration and invasion of OS cells

To determine the role of SATB2 in OS, we generated OS cell lines with stable knockdown of SATB2 (KHOS- shSATB2-1, and KHOS- shSATB2-2) and control (KHOS- shGFP). shSATB2-1 and -2 target different SATB2 sequences (Chung et al., 2010). Knockdown was confirmed by immunoblot (Figure 2.2a). Since the level of SATB2 was higher in murine pulmonary metastases in comparison with matched primary OS tumors we predicted that SATB2 might be involved in metastatic spread via effects on migration and/or invasion. Significant decreases in migration and invasion were observed in three KHOS- shSATB2 cell lines compared with control cells, as detected using Boyden chambers (Figure 2.2a and Figure 2.3a). To exclude the possibility that decreased invasion in shSATB2 knockdown cells may reflect differences in proliferation, we performed BrdU and MTT assays. We did not detect any differences in proliferation or viability between KHOS- shSATB2 cells, -sh-control and parental control cells suggesting that the effects observed in response to SATB2 knockdown were due to migration and invasion (Figure 2.2b). Similarly, we also performed scratch-wound migration assay to look at collective cell migration and detected decreased migration in KHOS- shSATB2 cells compared with control (Figure 2.2c). To exclude cell line- or clone-specific effects, we performed transient SATB2 knockdown in MNNG and U2OS cells and observed that lower levels of SATB2 were associated with decreased migration and invasion (Figure 2.2a). Since knockdown led to decreased invasion we examined whether SATB2 over-expression would result in increased invasion. KHOS cells were transfected with a plasmid encoding T7-tagged SATB2 to generate two cell lines with stable SATB2 over-expression: KHOS-T7-SATB2-1 and KHOS-T7-SATB2-2. As predicted, overproduction of SATB2 led to increased migration and invasion compared with the control cells (Figure 2.2d). Furthermore, the relative level of migration and invasion observed in OS cell lines correlated with endogenous SATB2 expression (Figure 2.4 and Figure 1a).
**Figure 2.2. SATB2 KD decreases OS invasion and migration.**

(A) SATB2 immunoblot of lysates from KHOS cell lines with stable knockdown with the indicated lentishRNAs (shGFP control, shSATB2-1 (bp 506), shSATB2-2 (bp 1335)). Transwell migration (MIG) assay using Boyden chambers. shSATB2 and control KHOS cells (left panel), and MNNG cells transiently infected with indicated shRNAs (right panel) were assessed for migration using FBS gradient 20 hours after seeding. Matrigel was used for invasion (INV) assays. Cells were lysed and immunoblotted with anti-SATB2 for SATB KD. The data represent fold change normalized to the control ± SD (N=3; *p<0.05).

(B) MTT and BrdU assays were performed for sh-SATB2 and controls cells (in triplicate) for the indicated times. Data represent mean ± SD (N=3).

(C) Scratch-wound assay was performed on confluent monolayers of sh-SATB2 and control cells. Shown are representative images at indicated timepoints. The data represent mean (N=3). Graph demonstrates % recovery of wounds in cells with sh-SATB2 (N=3).

(D) T-7 immunoblot of lysates of KHOS cells that were transfected and selected for SATB2 overexpression (transfected with plasmid encoding T7-SATB2). KHOS- T7-SATB2 and parental cells were subjected to transwell migration and invasion assays described above. Data represent fold change normalized to the control ± SD (N=3; *p<0.05).
A

B

KHOS-shControl

KHOS-shSATB2-3

C

Adhesion

Abs @ 570 nm

KHOS-shControl

KHOS-shSATB2-3

*
Figure 2.3. SATB2 knockdown affects cytoskeleton.

(A) Validation of decreased invasion phenotype of KHOS-shSATB2-3 cells. (B) KHOS-shSATB2-3 cells display more stress fibers compared to the control. KHOS-shSATB2-3 and control cells were plated on fibronectin-coated slides, fixed and stained with AlexaFluor-568 Phalloidin antibody. Shown are representative images of N=3 experiments. (C) Decreased adhesion in shSATB2-3 cells. KHOS-shSATB2-3 and control cells were seeded for 30 mins, washed, fixed, and stained with crystal violet. Data represents absorbance at 570 nm ± SD (N=2; *p<0.05). (D) Increased phosphorylation of FAK and paxillin in KHOS-shSATB2-3 cells. KHOS-shSATB2-3 and control cells were serum-starved, induced with serum, lysed, and immunoblotted with indicated antibodies. N=2. (E) Cells lacking SATB2 display more focal adhesions. KHOS-shSATB2-3 and control cells were plated on fibronectin-coated slides, fixed and stained with p-FAK and p-Paxillin antibodies. Shown are representative images of N=2 experiments. (F) Increased RhoA activation in shSATB2 cells. Serum starved KHOS-shSATB2 and control cells induced with serum were lysed and Rhotekin beads were used to pull down RhoA-GTP. Lysates were immunoblotted with anti-RhoA. N=3.
Figure 2.4. High SATB2 expression correlates with increased migration OS cells. Four different OS cells were assessed for relative migration and invasion using boyden chamber assay. Shown are representative of N=3 experiments.
2.3.3 Identification of genes regulated by SATB2

Given the role of SATB family proteins in chromatin remodeling and as transcription factors, we asked whether SATB2 might regulate the expression of genes involved in migration and invasion. We performed a microarray analysis with KHOS control shRNA, shSATB2-1, and shSATB2-2 cells using the Affymetrix Human Gene platform. Approximately 425 genes were significantly differentially regulated between KHOS-shSATB2 and control cells. The most differentially expressed genes based on fold changes in two different clones of sh-SATB2 cells in comparison with control cells include *LIMA1, MMP16, GPR126, EFEMP1, MUM1L1*, and *NOX4* (Figure 2.5a).

To validate our microarray data, we performed qRT-PCR and immunoblot analyses to confirm expression of candidate SATB2-target genes that we prioritized based on (1) fold difference between control and SATB2 knockdown cells, (2) similar results in both knockdown lines, (3) potential role in OS or metastases (Figure 2.5c). To determine whether differentially expressed genes had functional relationships in similar signaling pathways, we performed pathway-based analyses using GSEA (Gene Set Enrichment Analysis) tool and Partek software (KEGG-based). GSEA revealed enrichment of genes in pathways such as cytoskeleton organization, cell leading edge, and regulation of small GTPase signaling, all of which are associated with cell migration (Figure 2.5d). In agreement with roles in chromatin remodeling, the SATB differentially expressed genes were also enriched for pathways involved in chromosome and chromatin organization and modification (Figure 2.5d). The KEGG-based pathway enrichment analysis of genes that were differentially expressed by >1.5 fold revealed that the greatest proportion of genes were associated with regulation of actin cytoskeleton followed by focal adhesion, and extracellular matrix-receptor interaction (Figure 2.5e), all of which are also known to regulate cytoskeleton dynamics required for cell migration. For example, *ABI3, ABI3BP, GSN*, and *WASH1* genes, which promote actin polymerization and branching that is crucial for cell migration (Latini, Hemerly, Oler, Riggins, & Cerutti, 2008; Yuan et al., 2013; Zech et al., 2011) were down-regulated in KHOS-shSATB2 cells.
### A

**Upregulated Genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>KHOS-shSATB2-1</th>
<th>KHOS-shSATB2-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL10</td>
<td>6.733</td>
<td>9.697</td>
</tr>
<tr>
<td>KRT8</td>
<td>6.366</td>
<td>4.645</td>
</tr>
<tr>
<td>FAM38B</td>
<td>6.000</td>
<td>4.183</td>
</tr>
<tr>
<td>EFEMP1</td>
<td>5.958</td>
<td>3.815</td>
</tr>
<tr>
<td>MMP16</td>
<td>5.853</td>
<td>3.572</td>
</tr>
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<td>CDH6</td>
<td>5.401</td>
<td>3.519</td>
</tr>
<tr>
<td>NNMT</td>
<td>5.229</td>
<td>3.504</td>
</tr>
<tr>
<td>PRF126</td>
<td>4.822</td>
<td>3.409</td>
</tr>
<tr>
<td>HBE1</td>
<td>4.092</td>
<td>3.149</td>
</tr>
<tr>
<td>LIMA1</td>
<td>3.881</td>
<td>3.125</td>
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</table>

**Downregulated Genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>KHOS-shSATB2-1</th>
<th>KHOS-shSATB2-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUM1L1</td>
<td>-4.226</td>
<td>-10.43</td>
</tr>
<tr>
<td>ENPP1</td>
<td>-4.193</td>
<td>-6.873</td>
</tr>
<tr>
<td>NOX4</td>
<td>-3.588</td>
<td>-6.580</td>
</tr>
<tr>
<td>ITGA8</td>
<td>-3.400</td>
<td>-5.572</td>
</tr>
<tr>
<td>MFAP2</td>
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<td>-5.112</td>
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<tr>
<td>PLXNA2</td>
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<tr>
<td>ESAM</td>
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<td>-4.576</td>
</tr>
<tr>
<td>PRRX1</td>
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<td>-4.415</td>
</tr>
<tr>
<td>PDE3A</td>
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<td>-3.684</td>
</tr>
<tr>
<td>ROBO4</td>
<td>-2.605</td>
<td>-3.663</td>
</tr>
</tbody>
</table>

### B

![Bar graph showing gene expression levels](image)

**Gene Expression**

- ADAM23
- CDH6
- E2F5
- EFEMP1
- EGFR
- GPR126
- KRT8
- LIMA1
- MMP16
- NNMT
- ESAM
- ENPP1
- NOX4
- ROBO4

**Legend**

- KHOS-shSATB2-1
- KHOS-shSATB2-2
- Array shSATB2-1
- Array shSATB2-2

### C

![Western blot images](image)

**Western Blot**

- **ENPP1**
- **SATB2**
- **α-Tubulin**
- **MMP16**
- **KRT8**
- **NOX4**
- **Vinculin**
- **SATB2**
Figure 2.5. SATB2 changes global gene expression.

(A) Ten genes with the most significant differential expression in sh-SATB2-1 and -2 cells compared to sh-Control. (B) Validation of the microarray by real-time PCR. Values normalized to shGFP control. The data represent average fold change (ddCT) ± SD (N=3). (C) Validation of the microarray by immunoblot analysis. shSATB2 and shControl cells were lysed and immunoblotted for proteins that showed differential mRNA expression in shSATB2 cells compared to shControl. (D) Cytoscape view of the top enriched pathways in shSATB2 cells compared to shControl using GSEA analysis (p<0.05; q<0.1). (E) List of top 5 enriched pathways in shSATB2 cells compared to the control revealed by KEGG-based pathway enrichment analysis performed with the list of genes filtered for fold change +/- 1.5 and P<0.05.
2.3.4 The SATB2 regulated gene EPLIN mediates the decreased migration and invasion in SATB2 knockdown cells

*Lima1* was one of the most highly upregulated genes in SATB2-knockdown cells. *Lima1* encodes EPLIN, which has two actin-binding domains flanking a central LIM domain. Microarray analysis showing upregulated EPLIN expression in sh-SATB2 was validated at both the RNA and protein levels using real-time PCR (RT-PCR), immunoblot, and immunofluorescence assays (*Figure 2.5b and 2.6a*). To exclude cell line- and shRNA- specific effects, we transiently transfected U2OS cells with siRNA targeting SATB2 and observed increased EPLIN expression (*Figure 2.6b*). This inverse relationship between SATB2 and EPLIN expression was also observed at the endogenous level (*Figure 2.6b*). Lysates from cells with high SATB2 levels had lower levels of EPLIN. To determine whether SATB2 directly regulates EPLIN expression, we examined whether the EPLIN promoter contains consensus SATB-binding site. BLAST analysis revealed no SATB-binding sites in promoters of both α and β isoforms of EPLIN, suggesting that the regulation of EPLIN expression by SATB2 is not due to direct SATB binding to the EPLIN promoter (data not shown).

The role of EPLIN in regulating actin cytoskeleton, together with our data supporting the role of SATB2 in OS cell invasion, led us to investigate the role of EPLIN in SATB2-mediated invasion. Given that EPLIN is upregulated in sh-SATB2 cells, we asked whether inhibiting EPLIN expression in sh-SATB2 cells, which already have increased EPLIN expression, can rescue the decreased invasion phenotype. To this end, sh-SATB2 cells transfected with siRNA targeting EPLIN were subjected to migration and invasion assays. Down-regulating EPLIN expression resulted in increased migration and invasion of sh-SATB2 cells compared with the control cells, partially rescuing the sh-SATB2 decreased invasion phenotype (*Figure 2.6c*), suggesting that SATB2-mediated invasion is at least in part due to effects on EPLIN expression.
Figure 2.6. EPLIN is downregulated by SATB2 and EPLIN knockdown partially rescues shSATB2 MIG/INV phenotype.

(A) Lysates of KHOS- shSATB2, KHOS- T7-SATB2 clones and control cells immunoblotted with antibodies to SATB2 and EPLIN (Left panel). KHOS- shSATB2-2 and control cells were plated on fibronectin-coated slides, fixed and stained with EPLIN antibody. Shown are representative images of N=3 experiments (Right Panel).

(B) Lysates of U2OS cells transiently transfected (48 hours) with indicated siRNAs (Left Panel) and lysates of various OS cell lines (Right Panel) immunoblotted with antibodies to SATB2 and EPLIN.

(C) KHOS- shSATB2-2 stable cells were transiently transfected with control siRNA or siRNA targeting EPLIN and subjected to transwell migration and invasion assay. Data represent fold change normalized to KHOS- shGFP control ± SD (N=3; *p<0.05).
2.3.5 SATB2 modulates actin cytoskeleton and effects are mediated by EPLIN

Our pathway analyses revealed enrichment of genes that regulate the actin cytoskeleton, which is essential for cell migration, and knockdown of EPLIN, an actin binding protein, was able to partially rescue the decreased invasion observed in sh-SATB2 cells. Thus, we asked whether the expression of SATB2 was associated with global changes in OS cell cytoskeleton. Immunofluorescence with phalloidin staining of actin filaments revealed increased stress fibers in sh-SATB2 cells compared with the control cells, (Figure 2.7a, Figure 2.8 and Figure 2.3b). In contrast, we did not observe any differences in microtubule structures as detected by tubulin staining (Figure 2.9). The actin cytoskeleton is highly regulated by the activities of small GTPases including RhoA, Rac1, and Cdc42, which act as molecular switches controlling multiple signaling pathways. These proteins are activated by GTP binding and are inactivated by the hydrolysis of GTP to GDP. Since it is well known that RhoA activity regulates stress fiber formation and adhesion (Parri & Chiarugi, 2010; Sahai & Marshall, 2002), we asked whether sh-SATB2 cells with increased stress fibers have altered RhoA activation. GTPase activation assay using rhotekin-RBD beads to pull-down GTP-bound RhoA demonstrated increased RhoA activation in sh-SATB2 cells compared with the control (Figure 2.7b and Figure 2.3f). Under the same conditions we observed decreased Rac1 activation in sh-SATB2 cells (Figure 2.7b). To further determine which effector of RhoA may mediate the migration phenotype, we treated sh-SATB2 cells with a pan-ROCK (Y-27632) and a ROCK2-specific inhibitor. Pharmacologic inhibition of ROCK1, but not ROCK2, rescued the decreased migration observed in sh-SATB2 cells without affecting proliferation (Figure 2.10). This suggests that ROCK1 is an effector of RhoA that regulates migration in OS cells.

We next examined cell adhesion by staining adherent cells at different time points and observed decreased adhesion in sh-SATB2 cells compared with the control (Figure 2.7c and Figure 2.3c). Consistent with the role of RhoA in driving maturation of focal adhesions, we observed increased phosphorylation of focal adhesion kinase (FAK) and paxillin in sh-SATB2 cells upon serum-induction (Figure 2.7d and Figure 2.3d). As confirmation, immunofluorescence showed increased focal adhesions in sh-SATB2 cells compared to the control as measured by p-FAK and p-paxillin staining (Figure 2.7e and Figure 2.3e). It has previously been shown that EPLIN inhibits Rac-1 induced ruffling and increases stress fibers by
cross-linking and bundling actin filaments (Maul et al., 2003), however, the functional link between EPLIN and adhesion is poorly understood. Thus, we examined whether EPLIN also regulates SATB2-dependent adhesion in OS cells. EPLIN knockdown in sh-SATB2 cells resulted in increased adhesion, partially rescuing the decreased adhesion detected in sh-SATB2 cells (Figure 2.11a). In addition, EPLIN knockdown resulted in decreased stress fiber formation in the sh-SATB2 cells (Figure 2.11b). To further elucidate the mechanism by which EPLIN regulates adhesion, we examined focal adhesion proteins. Transient knockdown of EPLIN expression in sh-SATB2 cells decreased paxillin expression and serum-induced phosphorylation of paxillin (Figure 2.11c). In addition, EPLIN overexpression was associated with increased phosphorylation of paxillin and decreased invasion (Figure 2.11d,e). This finding suggests that EPLIN regulates both the phosphorylation and turnover of paxillin and invasion.

Taken together, our data supports a model whereby high levels of SATB2 in OS lead to EPLIN downregulation, which in turn results in decreased phosphorylated paxillin, increased adhesion, and decreased stress fibers, resulting in increased OS cell invasion (Figure 2.12).
Figure 2.7. SATB2 knockdown affects cytoskeleton.
(A) KHOS-shSATB2-2 cells display more stress fibers and stable actin structures compared to the control. KHOS-shSATB2-2 and control cells were plated on fibronectin-coated slides, fixed and stained with AlexaFluor-568 Phalloidin antibody. Shown are representative images of N=3 experiments. (B) GTPase activation assay demonstrates alterations in RhoA/Rac1 in shSATB2 cells. Serum starved KHOS-shSATB2 and control cells induced with serum were lysed and Rhotekin and PAK beads were used to pull down RhoA-GTP and Rac1-GTP, respectively. Lysates were immunoblotted with anti-RhoA and anti-Rac1. N=3. (C) Diminished adhesion in shSATB2 cells. KHOS-shSATB2-2 and control cells were seeded, washed, fixed at indicated time-points, and stained with crystal violet dye. Data represent absorbance at 570 nm ± SD (N=3; *p<0.05). (D) Increased phosphorylation of FAK and paxillin in shSATB2 cells. KHOS-shSATB2-2 and control cells were serum-starved overnight, induced with serum, lysed, and immunoblotted with indicated antibodies. N=3. (E) Cells lacking SATB2 display more focal adhesions compared to control cells. KHOS-shSATB2-2 and sh-control cells were plated on fibronectin-coated slides, fixed and stained with p-FAK and p-Paxillin antibodies. Shown are representative images of N=3 experiments. KHOS-shSATB2-2 cells display more focal adhesions compared to the control.
Figure 2.8. SATB2 knockdown affects actin cytoskeleton.
KHOS- shSATB2-2 cells display more stress fibers compared to the control. KHOS- shSATB2-2 and control cells were plated on both collagen and poly-D-lysine-coated slides, fixed and stained with AlexaFluor-568 Phalloidin antibody. Shown are representative images of N=3 experiments.

Figure 2.9. Knockdown of SATB2 does not affect microtubule structure.
KHOS- shSATB2-2 and control cells were plated on fibronectin-coated slides, fixed and stained with α-Tubulin antibody. Shown are representative images of N=2 experiments. No changes in tubulin structure or pattern was observed in shSATB2-2 cells compared to the control.
Figure 2.10. Inhibition of ROCK1, but not ROCK2, increases migration of shSATB2 cells. 
(A) shSATB2 and control cells were assessed for migration by Boyden chamber assay in the presence of ROCK pan-inhibitor (Y-27632) and (B) ROCK2-specific inhibitor. (C) ROCK inhibitors do not affect cell viability. MTT assay was performed for shSATB2 and control cells in the presence of Y-27632. Data represent mean ± SD (N=3).
Figure 2.11. **EPLIN** knockdown rescues shSATB2 adhesion phenotype and regulates paxillin.  
(A) Increased adhesion in sh-SATB2 cells with EPLIN knockdown. KHOS- shSATB2 cells in which EPLIN expression was inhibited with EPLIN siRNA were seeded, washed, fixed at indicated time-points, and stained with crystal violet dye. Data represent absorbance at 570 nm ± SD (N=3; *p<0.05).  
(B) Decreased stress fibers in sh-SATB2 cells with EPLIN knockdown. KHOS-shSATB2 cells in which EPLIN expression was inhibited with EPLIN siRNA were seeded, washed, and fixed on fibronectin-coated slides, and stained with phalloidin. Shown are representative images of N=2 experiments.  
(C) Decreased stability and phosphorylation of paxillin in shSATB2 cells EPLIN knockdown. KHOS-shSATB2 cells in which EPLIN expression was transiently inhibited with EPLIN siRNA were serum-starved overnight, induced with serum, lysed, and immunoblotted with indicated antibodies. N=3.  
(D) EPLIN over-expression increases phosphorylation of paxillin. KHOS- shGFP control cells were transiently transfected with increasing amounts of a plasmid encoding EPLIN, lysed, and immunoblotted with indicated antibodies. N=3.  
(E) EPLIN over-expression increases migration/invasion of KHOS-shGFP control cells. KHOS-shGFP control cells transiently transfected with a plasmid encoding EPLIN were subjected to boyden chamber migration/invasion assays. Data represent fold change normalized to KHOS- shGFP control cells transfected with pcDNA3 plasmid ± SD (N=3; *p<0.05).
Figure 2.12. SATB2 model for OS invasion.
In OS high SATB2 regulates expression of genes to increase adhesion and decrease stress fibers, resulting in increased OS invasion and migration. SATB2 downregulates the expression of its target EPLIN and inhibits invasion by increasing and decreasing phosphorylation of paxillin and adhesion, respectively. In OS, SATB2 suppresses the inhibitory effect of EPLIN to increase invasion. Modulation of SATB2 levels also affects Rho/Rac, which have previously been reported to be regulated by EPLIN.
2.4 Discussion

The SATB family proteins have diverse functions due to their abilities to regulate gene expression and higher order chromatin structure. Until recently, the majority of reports on SATB1 and SATB2 were limited to roles in development of the immune and nervous systems, respectively, (Satoh et al., 2013; Zhao et al., 2014) and roles for SATB proteins in cancer had not been well described. Han et al. demonstrated that SATB1 is highly expressed in aggressive breast tumors and reprograms gene expression to promote growth and metastases (Han et al., 2008). We recently showed that SATB2 is highly expressed in advanced HNSCC where it promotes survival and chemoresistance (Chung et al., 2010). We now have identified a novel role for SATB2 in promoting OS invasion and migration, but not OS proliferation, by regulating the expression of EPLIN and other genes involved in motility, cytoskeletal organization and adhesion.

Previous reports of patients and mice with mutant SATB2 demonstrate a critical role in osteoblast differentiation (Britanova et al., 2006; Dobreva et al., 2006; Leoyklang et al., 2007; Wei et al., 2012). SATB2 is required for early OB differentiation and its expression is turned off during terminal stages of differentiation by miR-34b/c and 23a~27a~24-2 clusters (Hassan et al., 2010; Wei et al., 2012). Here, we demonstrate that the expression of SATB2 is higher in OS cells and tumors compared to normal OBs, the putative cell of origin for OS. This suggests that OB progenitors expressing high levels of SATB2 may have either failed to undergo normal differentiation or may have undergone additional genetic events leading to malignant transformation, both of which may partially be due to high SATB2 expression. We also showed that SATB2 is a highly specific marker for OS (93% of OS tumors vs. 1% of non-OS pediatric sarcomas). The use of SATB2 as an immunohistochemical maker for OS has also recently been reported by Conner and Hornick who, using a different SATB2 antibody, concluded that SATB2 positivity can distinguish tumors containing cartilage from those with osteoid (Conner & Hornick, 2013). OS histologic diagnosis is based on morphology and the presence of malignant osteoid. The addition of SATB2 immunostaining may facilitate diagnosis for OS tumors with minimal osteoid formation or other atypical features. Although the molecular mechanism by which SATB2 is upregulated in OS has not been determined, both miR-31 and 34b/c have been shown to target SATB2 transcripts and these miRNA clusters have been implicated in metastases and bone differentiation (Aprelikova et al., 2014; Wei et al., 2012). Thus, further studies to
elucidate SATB2 upstream regulation may provide insights into miRNA pathways involved in the progression of osteosarcoma.

Our findings of increased SATB2 in murine OS lung metastases and demonstration that modulating SATB2 levels by overexpression and shRNA led to increased and decreased invasion, respectively, suggest that, like SATB1 in breast cancer, SATB2 has pro-metastatic functions in OS. Cancer cells exploit many different biological pathways to adapt to and survive during different parts of the metastatic cascade such invasion and colonization. Among many pathways, cytoskeleton-remodeling and focal adhesion pathways have previously been shown to be critical in cancer metastasis (Hall, 2009). However, the molecular mechanisms by which cytoskeletal-related genes are regulated in OS are largely unknown. Previous studies have reported that the cytoskeleton linker ezrin provides an early survival advantage for OS cells colonizing the lung (Khanna et al., 2004), and CD99 suppresses OS cell migration by inhibiting Rho-associated kinase 2 (ROCK2) (Zucchini et al., 2013), a critical effector of RhoA signaling. In our study, we demonstrated that SATB2 promotes OS invasion by, in part downregulating the expression of the cytoskeleton-associated actin-binding protein EPLIN. Inhibition of EPLIN in cells without SATB2 partially rescued the decreased invasion phenotype of sh-SATB2 OS cells. Interestingly, EPLIN expression has been previously inversely correlated with cancer metastasis. For example, downregulation of EPLIN has been reported to be associated with epithelial-mesenchymal transition and was correlated with lymph node metastasis in prostate cancer (Zhang et al., 2011), however, the mechanisms by which EPLIN expression is regulated were not identified. Our study suggests that SATB2 regulation of EPLIN may occur in prostate and other non-OS tumors. Although we did not detect SATB2 binding sites in the EPLIN promoter, a recent publication by Steder et al. provides a possible mechanism by which SATB2 may indirectly regulate EPLIN (Steder et al., 2013). These investigators showed that the truncated p73 isoform ΔNp73 directly inhibits EPLIN expression by binding to the EPLIN promoter and that this leads to enhanced invasion of melanoma cells. We have previously reported that SATB2 binds to the closely related ΔNp63 and in HNSCC, augmenting ΔNp63 binding to p53-family consensus promoter sites in target genes. Thus, similar to the role of SATB2 binding to ΔNp63 in HNSCCs, it is possible that SATB2 modulates the activity of p53 family proteins to repress EPLIN expression in OS.
In addition to EPLIN, we demonstrated that SATB2 regulates the expression of additional genes associated with cytoskeletal dynamics and adhesion such as ABI3, GSN, and FMN2. The pathways altered by SATB2 are similar to those identified in breast cancer cells lacking SATB1 (Han et al., 2008). This is likely due to conserved DNA-binding consensus sites in target genes that are recognized by SATB family proteins. Moreover, our finding is also in agreement with previous gene expression profiling studies using both in vitro and in vivo models that identified both cytoskeleton-remodeling and focal adhesion as metastatic pathways in OS (Flores et al., 2012; Jones, Salah, Del Mare, Galasso, Gaudio, Nuovo, Lovat, LeBlanc, Palatini, Randall, Volinia, Stein, Croce, Lian, & Aqeilan, 2012b; Khanna, Khan, et al., 2001a; ODonoghue et al., 2010). Consistent with our pathway analyses, OS cells with SATB2 knockdown were characterized by morphological differences in the actin cytoskeleton. Sh-SATB2 cells were more elongated with increased stress fiber formation. Biochemical experiments showed increased RhoA activation, and decreased Rac1 activation suggesting that the highly regulated balance between RhoA and Rac1 signaling is altered, resulting in decreased migration of sh-SATB2 cells. The activity of RhoA in cell adhesion exhibits a biphasic relationship whereby RhoA activity is inhibited during initial cell adhesion and spreading. In the later phases of cell adhesion RhoA activity gradually increases (Huveneers & Danen, 2009). Our observations are in agreement with this idea in that sh-SATB2 cells, which have increased RhoA activity, exhibit decreased adhesion in early phases of spreading (within 30 minutes) and increased phosphorylation of FAK and paxillin at later phases (>16 hours post-plating).

Considering the role of phosphorylated FAK and paxillin in focal adhesion turnover, the increase in the phosphorylation of FAK and paxillin suggest that sh-SATB2 cells may have altered turnover rate of focal adhesions compared to control cells that express high levels of SATB2.

Previous reports demonstrate that EPLIN regulates cell adhesion; however, the molecular mechanism is poorly understood (Sanders, Ye, Mason, & Jiang, 2010; Tsurumi et al., 2014). We have now connected this important role for EPLIN to its regulation by SATB2 demonstrating that EPLIN rescues the decreased adhesion, invasion, and increased stress fiber phenotypes detected in sh-SATB2 cells. Furthermore, we report that EPLIN regulates the level and phosphorylation of paxillin. This novel finding together with known roles for phosphorylated paxillin affecting focal adhesion turnover (Abou Zeid, Vallés, & Boyer, 2006; Quizi et al., 2012; Zaidel-Bar, Milo, Kam, & Geiger, 2006) suggests that EPLIN may regulate OS invasion by
modulating cell adhesion and focal adhesion turnover. Thus, our model suggests that SATB2 promotes OS migration and invasion by reprogramming gene expression to modulate and prevent hyperactivation of focal adhesion proteins and RhoA signaling.

Taken together, our findings demonstrate that SATB2 is highly expressed in OS cells, is a specific marker to distinguish OS from other sarcoma tumors, and that SATB2 modulates the actin cytoskeleton and focal adhesion pathways to promote OS invasion, in part via its regulation of EPLIN. Future directions for studies related to SATB2 signaling and in vivo analysis are discussed in section 4.1.

2.5 Materials and Methods

2.5.1 Cell Culture, Viability, Proliferation and Tissue Microarray

Human osteosarcoma U2OS and SAOS-2, and keratinocyte HaCat cells were purchased from ATCC (Rockville, MD). HOS, KHOS, and MNNG human OS cells were provided by Dr. David Malkin. Murine MOTO OS cells were provided by Dr. Rama Khokha (Molyneux et al., 2010). Primary human osteoblasts obtained from Promocell (Heidelberg, Germany) were cultured in complete Osteoblast Growth Medium (Promocell). Other cells were cultured in DMEM (Gibco; Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone; Logan, UT). A tissue microarray was generated at the Hospital for Sick Children from biopsies of osteosarcoma, rhabdomyosarcoma, Ewing’s sarcoma, synovial sarcoma, and neurofibrosarcoma tumors. Cells seeded in 96-well plates in triplicate were subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Applied Science; Branford, CT) according to manufacturer’s instructions and absorbance at 575nm was measured. BrdU cell proliferation assay (Cell Signaling, MA) was performed according to manufacturer’s instructions. Following BrdU incubation for indicated time-points absorbance at 450nm was determined.
2.5.2 Antibodies and immunoblot analyses

Cells were lysed in EBC buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40) supplemented with complete protease inhibitors (Roche; Indianapolis, IN) and protein concentration determined by Bradford method (Bio-Rad; Hercules, CA.). For immunoblots, proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose (Bio-rad), blocked in TBS (10 mM Tris [pH 8.0], 150 mM NaCl) with 5% (wt/vol) milk/BSA and incubated with primary antibodies @ 4°C followed by horseradish peroxidase-conjugated secondary antibodies. Bound antibodies were detected by chemiluminescence with SuperSignal West Pico (Thermo Scientific, IL). The antibodies used included anti-SATB2 (Chung et al., EMBO Rep 2010), SATB2 (Cat. 611182), FAK (Cat. 610087), p-FAK (Tyr-297) (Cat. 611722) (BD Biosciences), EPLIN (NB100-2305), MMP16 (NB100-91874) (Novus Biologicals), Paxillin (Cat. 2542), p-Paxillin (Tyr-118) (Cat. 2541), E-cadherin (Cat. 3195) (Cell Signaling), NOX (SC-30141), ENPP1 (SC-33813), KRT8 (SC-101459) (Santa Cruz), Vinculin (Clone V284, EMD Millipore), T7 (Cat. 69522-3, Novagen), a-tubulin (Cat. T5168, Sigma), RhoA (Cat. ARH03), and Rac1 (Cat. ARC03) (Cytoskeleton, Inc).

2.5.3 shRNA knockdown and transfections

KHOS cells were infected with lentivirus:DMEM (1:1) with 10% FBS containing 8mg/mL polybrene (hexa-dimethrine bromide; Sigma-Aldrich; Oakville, ON). The short hairpin RNA (shRNA) sequences directed against different regions of SATB2 (shSATB2-1 and shSATB2-2) and shGFP negative controls were previously described (Chung et al., EMBO Rep 2010). A third shRNA for SATB2 (shSATB2-3) with a sequence 5’ CCAATTACAAAGTTGTTCA 3’ and non-targeting shRNA control was purchased from Thermo Scientific, IL. Puromycin-resistant clones were isolated and screened for SATB2 knockdown by immunoblotting (IB). Cells stably expressing shSATB2-1, shSATB2-2, shSATB2-3, shGFP and shControl were designated KHOS-shSATB2-1, KHOS-shSATB2-2, KHOS-shSATB2-3, KHOS-shGFP, and KHOS-shControl, respectively. Knockdown efficiency was confirmed by IB for each experiment. For transient siRNA- knockdown, oligofectamine was used according to the manufacturer’s instructions. siRNAs (Dharmacon Incorporated, Lafayette, CO) targeted the EPLIN sequence: - 5’ GCAGUGAUGAUAGUGCGUAA 3’ and 5’ AGGUUAAGAGUGAGGUUCA 3’.

Cells were
transfected with plasmids using polyethylenimine (PEI; Polysciences; Warrington, PA) method (Chung et al., 2010). The plasmid encoding EPLIN was purchased from Addgene (plasmid #40929). Viral work was performed in accordance with the Hospital for Sick Children (Toronto, ON, Canada) safety guidelines.

2.5.4 Microarray analysis and Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted with TRIZOL (Invitrogen; Carlsbad, CA) from KHOS-shSATB2 and control cells. Analysis of GeneChip Human Gene 1.0 ST Arrays (Affymetrix; Santa Clara, CA) was performed by the Microarray Analysis and Gene Expression Facility at The Hospital for Sick Children. Pathway analyses were conducted using Partek software (KEGG-based) and Gene Set Enrichment Analysis (GSEA) tool (Broad Institute) with a p-value cut-off of 0.05. The differentially regulated pathway networks were visualized using Enrichment Map Plug-in in Cytoscape (Merico, Isserlin, Stueker, Emili, & Bader, 2010) with p-value and q-value cut-off of 0.05 and 0.1, respectively. For RT-PCR, RNA was reverse transcribed using an Omniscript RT Kit (Qiagen) with random hexamers (ABI). cDNA was used as template for qRT-PCR using SYBR Green Master Mix (Roche) and 0.5µM gene-specific primers (Sigma Genosys) on a Lightcycler480 (Roche). To generate standard curves, cDNA templates from all cell lines were pooled, serially diluted, and subjected to qRT-PCR amplification (40 cycles) for GAPDH and genes of interest. Each primer pair amplification efficiency was determined by plotting the slope of CT versus log cDNA dilution. If efficiencies agreed within 10% amplification from individual cDNA samples was compared using the comparative CT method (2- ΔΔCT). Melt curve analysis and resolution using agarose gel electrophoresis were used to verify PCR products.

2.5.5 Migration and invasion assays

Migration was analyzed using Boyden Chambers assay with 8-µm pore inserts (BD Biosciences). Cells in 0.1 % FBS growth media (100 µL) were seeded into the upper chamber and the lower chamber contained 10 % FBS containing growth media. For invasion, 6% matrigel (BD Biosciences; Mississauga, ON) in serum free media was added to the upper chamber.
Following 16-24 hour incubation (37°C, 5% CO₂), cells that migrated or invaded into the bottom chamber were stained with crystal violet and detected by Northern Eclipse, Empix Imaging Inc and ImageJ analyses (Schneider, Rasband, & Eliceiri, 2012) of images of 5 random fields of view per chamber. Values were normalized to control, and expressed as fold changes with standard deviations of triplicates. For scratch-wound assays, cells were grown to confluent monolayers. A line was scratched through cells in 3 replicates and low-serum media (DMEM/0.5% FBS) was added. Images were captured at 0, 6, 12 and 24 h, and wound widths were measured, normalized to control, and expressed as percent recovery. Experiments were performed ≥ 3 times unless otherwise indicated in legends.

2.5.6 GTPase activation, adhesion and immunofluorescence assays

For GTPase activation assay, cells were seeded (30% confluence), serum-starved overnight and induced with 10% FBS-containing growth media for the indicated durations. Pull-downs using rhotekin-RBD and PAK1-PBD beads were performed according to manufacturer’s instructions (Cytoskeleton, Inc). For adhesion assays, cells were seeded in triplicate in 96-well plates and allowed to adhere and spread. At indicated time-points, non-adherent cells were washed and adherent cells were fixed with 4% paraformaldehyde (PFA), washed, stained with crystal violet, and the absorbance at 570nm was read. Experiments were performed >3 times unless otherwise indicated in legends. For immunofluorescence, cells were seeded on fibronectin (FN)-coated culture slides, fixed with 4% PFA, and permeabilized with 0.2% Triton X-100. Slides were blocked with 6% normal goat serum (NGS) and 0.5% bovine serum albumin (BSA), incubated with the indicated primary and secondary antibodies. Images were taken with Olympus IX81 Quorum Spinning Disk Confocal Microscope and analyzed with Perkin Elmer Volocity software.
Chapter 3

Modeling Metastatic Neuroblastoma and Identification of Genes and Pathways to Target Neuroblastoma Metastasis

The manuscript for this work is under review.


I performed all the experiments except in vivo isolation and initial characterization of metastatic cells, microarray analysis, and gene signature analysis.

3 Modeling Metastatic Neuroblastoma and Identification of Genes and Pathways to Target Neuroblastoma Metastasis

3.1 Background

Neuroblastoma (NB) is the most common extra-cranial solid tumor and the most frequent cause of cancer-related death in children (Maris, NEJM 2010). NB is thought to arise from neural crest-derived sympathoprogenitors and most often originates in the adrenal gland. More than half of NB patients present with bone and/or bone marrow (BM) metastases, and long-term survival is less than 40%. At the time of recurrence, the majority of patients have evidence of metastatic spread to the bones, bone marrow, and increasingly, the central nervous system (CNS) (Kramer et al., 2001). Survival after metastatic relapse is < 5% (Maris, 2010; Modak & Cheung, 2010). Although there has been significant progress in the identification of genetic alterations in primary NB tumors, including MYCN amplification and mutations in genes such as ALK and ATRX, (Molenaar et al., 2013; Mossé et al., 2008; Pugh et al., 2013; W. A. Weiss et al., 1997), recurrent driver mutations are uncommon, and specific alterations associated with NB metastasis have not been well-studied.
Metastasis is the leading cause of death in cancer, and involves a multi-step process in which cells from the primary tumor often acquire specific biological properties including invasion, intravasation into and extravasation from blood vessels, migration, and colonization of and survival in secondary sites for metastatic outgrowth (Figure 1.1). Various in vitro and in vivo models have been developed to study the role of specific genes mediating metastasis (Kang et al., 2003; Minn et al., 2005). To date, however, there are no in vivo models that faithfully recapitulate metastatic NB, both in terms of tissue tropism and burden of disease. Current NB models rely on the use of tail-vein and intra-femoral cell injections and xenograft, orthotopic, or genetically engineered mouse (GEM) models of MYCN and/or ALK alterations, which although informative, have several limitations (George et al., 2012; Iwakawa et al., 2005; Nevo et al., 2008; Teitz et al., 2011; W. A. Weiss et al., 1997). First, the incidence and pattern of metastatic spread in these models is low, with only rare osteolytic bone lesions and brain metastases. Second, tail-vein injections rarely result in BM metastases and instead, lead to frequent lung lesions, which are rare in NB patients at diagnosis, and intra-femoral injections produce secondary outgrowths (George et al., 2012; Teitz et al., 2011; W. A. Weiss et al., 1997) that only recapitulate metastatic colonization, which is a late step in the metastatic cascade. Third, most current models lack the capacity to image and quantify metastases in vivo. Finally, most models tend to give rise to micrometastatic lesions with low frequency and no significant brain metastases (Iwakawa et al., 2005; Sohara et al., 2003; Teitz et al., 2011). Hence, more faithful, reproducible and quantifiable models of metastatic NB are required.

To generate a model to better understand the biology and molecular and genetic mechanisms involved in NB metastasis, we used intra-cardiac injection and in vivo selection, a method previously used to model breast cancer metastasis (Kang et al., 2003; Minn et al., 2005). Using this model, Kang et al. have identified distinct set of genes that mediate the bone metastasis including CTGF and FGF5 involved in angiogenesis and IL11 and OPN that promote osteolysis (Kang et al., 2003). Subsequently, Bos et al. identified ST6GALNAC5 that specifically mediate brain metastasis by enhancing cancer cell adhesion to brain endothelial cells for passaging through blood-brain barrier (Bos et al., 2010). Green-fluorescent protein and luciferase-expressing NB cells with low to moderate metastatic capability were injected into the heart to facilitate the distribution of cells. Cells from metastatic sites were isolated, cell lines were generated and re-injected back into the heart, and cells from secondary metastases again
isolated and cell lines generated (Figure 3.1). Using quantitative in vivo imaging, we show that the in vivo selected cells gave rise to osteolytic bone metastases with 100% frequency and to CNS metastases at higher frequency, with decreased latency and shorter overall survival as compared to the parental line. We identify genes and pathways that are differentially regulated in the enhanced metastatic NB cells. Functional validation of several of these genes in vitro and in vivo, including CADM1, GJA1, SPHK1 and the YAP/TAZ Hippo pathway effectors, revealed important roles in NB metastasis. In addition, we describe a novel metastatic gene signature (MET-75) developed from our mouse model that predicts human NB patient outcome.

Figure 3.1. The in vivo selection model of NB metastasis.
First, SK-N-AS cells are labeled with the triple reporter (TR), which allows for GFP sorting and bioluminescence imaging. Labeled cells are injected into mice via intra-cardiac injection and allowed to form metastases. Once metastases are detected by bioluminescence imaging, cells from different metastatic sites are isolated in vivo, expanded in culture (~1-2 weeks), GFP-sorted, and re-injected back into mice via intra-cardiac injection. Multiple rounds of injections give rise to subpopulations of cells with enhanced metastatic capability.
3.2 Hypothesis/Rationale

I hypothesize that developing a metastatic mouse model of NB that better recapitulates human metastatic NB will provide a platform to study the biology and to identify novel regulators of NB metastasis.

3.3 Results

3.3.1 Metastatic subpopulations show enhanced metastatic burden, a higher propensity for bone and CNS metastases, and increased lethality when injected into mice as compared to the parental population

To better understand NB metastasis, we sought to develop a novel metastatic mouse model of NB to the bone and CNS. Towards this goal, the human NB cell line SK-N-AS was infected with a retrovirus encoding a triple reporter (TR) consisting of thymidine kinase, GFP, and luciferase, which allows for nuclear imaging, FACS analysis and whole body bioluminescence, respectively (Ponomarev et al., 2004). The transduced SK-N-AS-Triple Reporter (TR) cells were introduced into NOD/SCID immunodeficient mice through intra-cardiac injection and mice were imaged for bioluminescence bi-weekly to monitor metastatic outgrowth. Metastatic outgrowth of the parental SK-N-AS cells was observed in the bone/bone marrow (71%, 15/21 animals), adrenal gland (17/21, 81%), and CNS (2/21, 9%) of recipient mice as early as 14 days post-injection (Table 1). At endpoint (median = 69 days), metastatic lesions were confirmed by necropsy, ex vivo bioluminescence, 3D bioluminescence, MRI, or micro-CT (Figure 3.2a and Figure 3.3a). Metastatic cells were isolated from bone, CNS, and adrenal gland, expanded in culture for 1-2 weeks, GFP-sorted, and re-injected into a second cohort of mice. The metastatic subpopulations isolated after a single cycle of in vivo selection demonstrated varying degrees of metastatic capability (Table 1). For example, the bone- derived cell line, B1, showed a significant increase in metastatic burden relative to the parental line, with mice injected with B1 exhibiting 100% metastasis to bone and 60% to the CNS as determined by micro-CT and MRI, and decreased overall survival (Figure 3.2a-c, f and Figure 3.3b). Bone and CNS lesions generated from mice injected with B1 were isolated and expanded in culture to confirm that the enhanced metastatic capability was retained after in vitro culturing. Indeed, B5, a subpopulation derived from a bone
lesion of mice injected with B1, behaved in a similar manner to its predecessor, indicating that the enhanced metastatic potential is retained after culturing. Mice injected with B1 and B5 exhibited enhanced metastatic burden relative to the parental cell line, as determined by bioluminescence and overall survival (Figure 3.2a, b and Figure 3.3b) and demonstrated bone degradation, when assessed by micro-CT (Figure 3.2c). Previous studies have illustrated that osteoclast activation is required for NB-mediated osteolytic bone degradation (Sohara et al., 2003). Bone lesions derived from B1 and B5 incorporated numerous activated osteoclasts into the periphery of the lesions, as determined by tartrate-resistant acid phosphatase (TRAP) staining, while there were fewer TRAP-positive cells within the parental cell line-derived lesions. These results suggest that B1 and B5 lines derived from metastatic lesions show an increased ability to degrade the bone (Figure 3.3c).

Similar to B1 and B5, metastatic subpopulations derived from the CNS (BR1, BR2) demonstrated enhanced metastatic burden, as determined by bioluminescence, with a high propensity to form CNS metastases relative to the parental cells (Figure 3.2d and Figure 3.3d). Metastases arising within the CNS occurred within the brain parenchyma, the leptomeninges, and in the pituitary gland (Figure 3.2f, and Figure 3.3e). Kaplan-Meier survival analysis showed that the animals within the BR1 and BR2 cohorts that developed CNS metastases had significantly lower probability of survival relative to the parental and B1 and B5 bone-derived cohorts that in addition to their bone metastases, also developed CNS metastases (Figure 3.2e).
Figure 3.2. The metastatic subpopulations isolated from bone and brain display enhanced metastatic ability relative to the SK-N-AS-TR parental cell line in vivo and in vitro.

(a) Metastatic outgrowth of the Parental and B1 and B5 enhanced metastatic subpopulations was measured by bioluminescence. Representative luciferase images from day 49 post-injection are shown (left panel). Body-wide bioluminescence was quantified as photon flux per second over time. Averages from each cell line were plotted on a log scale +/- SEM; ** represents p<0.01, *** represents p<0.001 (right panel). (b) Kaplan-Meier curves comparing the overall survival of mice introduced by intra-cardiac injection with parental, B1, and B5 cells. (c) Representative microCT 3D reconstruction images of bone...
metastases from mice injected with parental, B1 or B5 cells, or not injected with cells at 49 days post-injection. White arrows indicate areas of osteolysis. (d) Metastatic outgrowth of the parental and BR1 and BR2 enhanced metastatic subpopulations was measured by bioluminescence. Representative luciferase images from day 42 post-injection are shown (left panel). Averages of body-wide bioluminescence from each cell line from each cell line were plotted on a log scale +/- SEM; ** represents p<0.01, *** represents p<0.001 (right panel). (e) Kaplan-Meier curves comparing overall survival in animals exhibiting CNS metastases injected with the parental, B1, B5, BR1 or BR2 enhanced metastatic subpopulations. (f) CNS metastatic lesions (highlighted in yellow) were confirmed by MRI. (g) The B1 and B5 enhanced metastatic subpopulations exhibit increased migration and invasion (MIG/INV). Parental (P), B1 and B5 cells were assessed (in triplicates) for migration using transwells. Matrigel coated membranes were used for invasion assays. Data represent mean +/- SEM (n=3); ** represents p<0.01, *** represents p<0.001. The significance was measured relative to the parental. (h) The B1 and B5 enhanced metastatic subpopulations do not proliferate faster compared to the parental both in vitro and in vivo. Cell proliferation was measured (in triplicates) by BrdU labeling. Data represent average +/- SEM for each cell line (n=4). (i) Subcutaneous outgrowth was measured and the average volume of tumors in mice injected with parental, B1 and B5 cell lines (n=10 per cell line) were plotted over time +/- SEM; ** represents p<0.01, *** represents p<0.001.
Figure 3.3. The metastatic subpopulations display enhanced metastatic burden relative to the SK-N-AS-TR parental cell line in vivo and increased chemoresistance in vitro.

(a) Metastatic localization in the jaw was confirmed using DLIT and ex vivo bioluminescence. (b) Box plots of bioluminescence readings of mice injected with parental, B1, and B5 cells at Day 49. Data represent mean +/- SEM; ** represent p<0.01, *** represent p<0.001. (c) Metastatic bone lesions are osteolytic in vivo. Representative histological image of H&E staining of a bone metastatic lesion (i) as well as positive TRAP staining (ii, iii, iv) for activated osteoclasts (indicated by black arrows) are shown. (d) Box plots of bioluminescence readings of mice injected with parental, BR1, and BR2 cells at Day 42. Data represent mean +/- SEM; *** represent p<0.001. (e) Representative histological images (10x) of various CNS metastases, including lesions within the brain parenchyma (i), pituitary gland (ii). (f) IC50 curves and values (in parentheses) for the parental and metastatic subpopulations (B1, B5, BR2) for the active metabolite of irinotecan, SN38 (n=3).
Table 1 - Summary of metastatic lesions in mice injected with the parental and enhanced metastatic subpopulations of SK-N-AS-TR.
A summary of the metastatic incidence for the parental and enhanced metastatic subpopulations that were isolated from the bone (B), adrenal gland (AG), and CNS (BR). Sixty days (indicated by *) indicates the designated endpoint of the secondary and tertiary injections.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Source</th>
<th>Adrenal Gland</th>
<th>Bone</th>
<th>Brain</th>
<th>Time til death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>ATCC</td>
<td>23/28 (82%)</td>
<td>22/28 (78%)</td>
<td>5/28 (18%)</td>
<td>69 days</td>
</tr>
<tr>
<td>B1</td>
<td>Parental</td>
<td>17/18 (94%)</td>
<td>18/18 (100%)</td>
<td>10/18 (56%)</td>
<td>48 days</td>
</tr>
<tr>
<td>B2</td>
<td>Parental</td>
<td>4/4 (100%)</td>
<td>2/4 (50%)</td>
<td>0/4 (0%)</td>
<td>60 days*</td>
</tr>
<tr>
<td>B3</td>
<td>Parental</td>
<td>3/6 (50%)</td>
<td>4/6 (67%)</td>
<td>0/6 (0%)</td>
<td>60 days*</td>
</tr>
<tr>
<td>B4</td>
<td>Parental</td>
<td>0/5 (0%)</td>
<td>3/5 (60%)</td>
<td>0/5 (0%)</td>
<td>60 days*</td>
</tr>
<tr>
<td>AG1</td>
<td>Parental</td>
<td>6/7 (86%)</td>
<td>6/7 (86%)</td>
<td>3/7 (43%)</td>
<td>60 days*</td>
</tr>
<tr>
<td>B5</td>
<td>B1</td>
<td>9/10 (90%)</td>
<td>10/10 (100%)</td>
<td>5/10 (50%)</td>
<td>56 days</td>
</tr>
<tr>
<td>BR1</td>
<td>B1</td>
<td>16/16 (100%)</td>
<td>14/16 (88%)</td>
<td>7/16 (44%)</td>
<td>55 days*</td>
</tr>
<tr>
<td>BR2</td>
<td>B1</td>
<td>6/6 (100%)</td>
<td>6/6 (100%)</td>
<td>5/6 (83%)</td>
<td>45 days</td>
</tr>
<tr>
<td>BR3</td>
<td>B1</td>
<td>1/4 (25%)</td>
<td>3/4 (75%)</td>
<td>3/4 (75%)</td>
<td>60 days*</td>
</tr>
<tr>
<td>BR4</td>
<td>B1</td>
<td>0/5 (0%)</td>
<td>2/5 (40%)</td>
<td>0/5 (0%)</td>
<td>60 days*</td>
</tr>
<tr>
<td>BR5</td>
<td>B1</td>
<td>3/3 (100%)</td>
<td>3/3 (100%)</td>
<td>1/3 (33%)</td>
<td>60 days*</td>
</tr>
<tr>
<td>BR6</td>
<td>B1</td>
<td>5/5 (100%)</td>
<td>5/5 (100%)</td>
<td>3/5 (60%)</td>
<td>60 days*</td>
</tr>
</tbody>
</table>
3.3.2 Metastatic subpopulations exhibit increased migration and invasion without changes in proliferation

To understand how these metastatic subpopulations (also referred to as “enhanced metastatic cell lines”) acquired the ability for increased metastatic burden in vivo, we assessed the ability of the B1 and B5 cell lines to migrate and invade and proliferate in vitro. Migration and invasion were determined using transwell assays, where cells were assessed for migration through a permeable membrane, or invasion through the membrane when pores were coated with matrigel. The level of migration and invasion was higher in the B1 and B5 cells as compared to the parental cells (Figure 3.2g). There were, however, no significant differences in their capacity to proliferate relative to the parental cell line, as measured by BrdU labeling in vitro and the rate of tumor growth in subcutaneous xenografts in vivo (Figure 3.2h and i). This suggests that the enhanced metastatic burden observed in vivo in mice injected with B1 and B5 cells is not due to a faster growth rate of the cells, but to a selection of metastasis-promoting functions. One of the hallmarks of metastatic relapsed NB is resistance to chemotherapy. We therefore examined the relative chemotherapy sensitivity of the parental and enhanced metastatic cells. We observed increased resistance in vitro to the irinotecan metabolite SN-38 in B1, B5, and BR2 cells as compared to the parental cells (Figure 3.3f).

3.3.3 Gene expression profiling identifies parental and metastatic subtypes of in vivo-selected cell populations

Previous studies examining gene expression profiles of metastatic breast cancer subpopulations isolated from the bone, lung, and brain have identified distinct sets of genes that mediate organ-specific metastasis (Kang et al., 2003; Minn et al., 2005). However, genes that regulate metastasis to bone or the CNS in NB have not been identified. To identify genes and pathways that regulate NB metastasis, microarray analysis was performed on the SK-N-AS-TR parental line and 15 cell populations isolated from bone and CNS metastases and adrenal gland. Unbiased clustering of gene expression profiles based on the top 1000 most variably-expressed genes demonstrated the existence of two dominant subtypes; “primary” subtype, which comprised the parental cell line and subpopulations isolated from the adrenal gland, and “metastatic” subtype, which comprised subpopulations isolated from the bone and CNS (Figure 3.4a). Comparison of the parental and metastatic subtypes revealed differential regulation of 412 genes (FDR<0.05).
Network analysis of differentially-expressed genes or GSEA based on parental/metastatic subtype highlighted pathways and processes that were differentially regulated in cell lines derived from NB metastases. These included the Hippo, integrin, IGF, PI3K, NOTCH, EGF, and JAK/STAT signaling pathways, as well as gene sets associated with hypoxia, angiogenesis, EMT, transformation and NCAM signaling (Figure 3.4b, c and Figure 3.5). Some of these pathways such as ECM-integrin signaling have been implicated in NB metastasis (Meyer, van Golen, Kim, van Golen, & Feldman, 2004; Stupack et al., 2006), however, pathways such as Hippo, Zinc finger, and SPHK1 have not been previously described.

To begin to determine whether our model would have clinical utility and share characteristics with human NB, we examined whether differentially regulated genes in the metastatic subtype had prognostic value. Briefly, we performed univariate Cox-regression with each of the 412 parental/metastatic genes in a relatively large and publically available gene expression cohort of NB patients (E-MTAB-179, n=479). We identified 75 genes that were either parental subtype-enriched and associated with favorable outcome or metastatic subtype-enriched and associated with poor outcome (p<0.05, Wald test) (Figure 3.4d). Among these were genes that belong to differentially-regulated pathways in metastatic cells such as integrin beta 3 (ITGB3) and angiomotin-like 2 (AMOTL2) from the integrin and hippo pathways, respectively. In addition to this data, we used multiple published clinical NB expression datasets (R2 database: http://r2.amc.nl) (Asgharzadeh et al., 2006; Molenaar et al., 2013) to prioritize genes for further validation studies based on their association with metastasis in adult cancers and NB patient outcome. High or low expression of CADM1 and SPHK1 independently predicted NB patient outcome in all datasets, whereas the high or low expression of GJA1 and YAP/TAZ were prognostic in a subset of the patient datasets (Figure 3.4e and Figure 3.6). While high or low expression of YAP or TAZ alone did not independently predict survival, a YAP pathway signature (Zanconato et al., 2015) did indeed predict patient outcome (Figure 3.4e and data not shown).
A Parental Adrenal Metastasis

B

ECM/Integrin/FAK

P53/E2F signalling

EGF/GPCR

Notch

Hippo

Zn finger

Jak/STAT

RAP1/INSR

PI3K

EGF/SPHK1
C

Primary
Metastatic

Hypoxia
RhoA transformation
Dental caries/Synovia
Angiogenesis
Integrin signalling
DNA replication and transcription
Integrin signalling
DNA replication and transcription
ECM organization
Glycosammoniglycan metabolism
Integrin signalling
DNA replication and transcription
EMT
NCAM signalling
Primary
Metastatic

D

Predict good outcome
Predict poor outcome

P-value

10^-20
10^-15
10^-10
10^-5
10^0

Hazard Ratio

-4
-3
-2
-1
0
1
2
3
4

CADM1
SPHK1
JTGB3
AMOTL2
Figure 3.4. Gene expression profiling reveals differences in primary and metastatic models that are associated with biological pathways.

(a) Semi-supervised analysis reveals 2 distinct gene expression subtypes of the profiled SK-N-AS sublines, which indicate either primary or metastatic expression subtype. (b) Network analysis of 412 significantly differentially expressed genes between primary and metastatic models cluster into distinct modules with predicted enrichment in signaling pathways. (c) Enrichment maps of enriched genes sets from GSEA analysis of expression subtype reveals biological processes associated with either metastatic or primary expression subtypes. (d) Survival analysis using the 412 differentially expressed genes identifies 75 genes (MET-75 signature) that are either associated with favourable outcome when down-regulated or associated with poor outcome when over-expressed in the metastatic models, using the E-TABM-179 cohort. (e) Kaplan-Meier survival curves showing overall survival by the expression of CADM1, GJA1, YAP signature, and SPHK1 in E-TABM-179 cohort.
Figure 3.5. GSEA enrichment map analysis shown in figure 2, annotated by gene set ID from mysigDB.
Figure 3.6. Genes identified in enhanced metastatic subpopulations predict survival of human NB patients in multiple datasets. 

(a) Cumulative overall survival curves for 88 patients in Versteeg dataset, and (b) relapse-free survival curves for 102 patients in Seeger database, for indicated genes. The cut-off is the median expression level. Kaplan-Meier curves and Box blots were generated from the R2: microarray analysis and visualization platform (http://r2.amc.nl) (Asgharzadeh et al., 2006; Molenaar et al., 2013). (c) Kaplan-Meier survival curves showing event-free survival by the expression of CADM1, GJA1, YAP signature, and SPHK1 in E-TABM-179 cohort (Oberthuer et al., 2010).
3.3.4 CADM1 overexpression suppresses the metastatic phenotype in vitro and in vivo

*CADM1* has been reported to have tumor suppressor properties in NB and localizes to chromosome 11q23, which is a region that is frequently lost in tumours from poor prognosis patients (Attiyeh et al., 2005). While CADM1 inhibits the growth of some cultured NB cell lines (Nowacki et al., 2007), the function of CADM1 in NB metastasis has not been examined. In the microarray analysis, the expression of *CADM1* was lower in the metastatic subpopulations as compared to the parental cell lines. The lower levels in CADM1 protein in the B1, B5 and BR2 lines as compared to the parental cell line was confirmed by immunoblotting cell lysates with anti-CADM1 (Figure 3.7a). As CADM1 is expressed at lower levels in the enhanced metastatic cell lines, we asked whether overexpressing CADM1 in these cells could suppress the metastatic phenotype. B1 cells were infected with a lentiviral vector encoding LacZ (control) or CADM1 (Figure 3.7b). Cells were subjected to BrdU cell proliferation and migration and invasion assays in culture. 24 hours post-seeding, we detected a significant decrease in migration and invasion in cells with exogenous CADM1 overexpression. Importantly, there was no significant decrease in cell proliferation at this time point (Figure 3.7c and d). To determine whether overexpression of CADM1 rescues the metastatic phenotype in vivo, CADM1-overexpressing B1 cells were introduced into mice via intra-cardiac injection. Significant inhibition of metastasis and increased disease-free survival was observed in mice injected with the CADM1 overexpressing B1 cells (Figure 3.7e and f). The mice injected with CADM1-overexpressing B1 cells had no detectable metastases by bioluminescence (Figure 3.7e). This result, along with the in vitro invasion data, suggests that CADM1 plays a role in the metastatic cascade, perhaps by preventing the seeding of metastases in vivo.
**Figure 3.7.** CADM1 expression is reduced in enhanced metastatic subpopulations and its overexpression decreases the metastatic phenotype *in vitro* and *in vivo*.

(a) Differential expression of CADM1 in primary and enhanced metastatic subtypes from the microarray is shown (left). The decreased expression of CADM1 was confirmed at the protein level by immunoblot analysis with CADM1 antibody (right). (b) CADM1 overexpression inhibits MIG/INV without affecting proliferation *in vitro*. B1 cells were infected with lentivirus encoding LacZ (control) or CADM1-V5. Over-expression of CADM1 was confirmed by immunoblotting with V5-tag antibody. (c) BrdU labeling and (d) MIG/INV assay were performed on B1 cells over-expressing LacZ or CADM1 (in triplicates). Data represent the average fold change relative to control +/- SEM (n=2) for BrdU and mean +/- SEM (n=3) for migration and invasion; ** represents p<0.01, *** represents p<0.001. (e) Over-expression of CADM1 rescues the metastatic phenotype *in vivo*. B1 cells infected with LacZ (control) or CADM1 were introduced by intra-cardiac injection into mice. Averages of body-wide bioluminescence from each cell line, control (n=10) and CADM1 (n=7), were plotted on a log$_2$ scale +/- SEM; ** represents p<0.01, *** represents p<0.001 (left panel). The data is representative of 3 independent experiments. Representative luciferase images from day 35 post-injection are shown (right panel). (f) Kaplan-Meier curve showing the disease-free survival for mice injected with control or CADM1 over-expressing B1 cells. The threshold for the disease was calculated as average signal at day 7 +/- 3 S.D. for each condition.

### 3.3.5 Expression of the gap junction protein GJA1 is increased in the metastatic subpopulations, and knockdown of GJA1 expression inhibits their migration and invasion *in vitro*

*GJA1*, which encodes connexin-43, a major structural and functional component of gap junctions, was one of the most highly up-regulated genes in the metastatic subpopulations. Although *GJA1* was not among the 75 genes in our prognostic signature (Figure 3.2d), recent evidence on the role of GJA1 in cancer cell invasion in other tumors (Sin et al., 2015; A. Zhang et al., 2015) and novelty regarding its role in NB led us to investigate the role of this gene in NB metastasis. Western blot analysis confirmed the increased expression of GJA1 in the B1 and B5 enhanced metastatic cell lines (*Figure 3.8a*) as compared to the parental cells. To ask whether GJA1 has a functional role in metastatic NB cells, GJA1 expression was reduced by expressing a lentiviral vector encoding GJA1 shRNA in the B1, B5 and BR2 enhanced metastatic and parental cell lines (*Figure 3.8b*), and invasion, migration and growth assays were performed. Decreasing the expression of GJA1 resulted in a reduction in the levels of migration and invasion of the enhanced metastatic cell lines without affecting cell growth (*Figure 3.8c and d*). In contrast, we did not detect significant changes in the migration and invasion of parental (P) cells with GJA1 knockdown, although there was a modest increase in cell growth, suggesting that GJA1 may have different roles in the enhanced metastatic and parental cells. To determine whether
knocking down GJA1 can rescue the metastatic phenotype in vivo, B1 cells with reduced GJA1 expression were introduced into mice via intra-cardiac injection. No significant differences were observed in metastatic outgrowth between mice injected with GJA1 knockdown cells as compared to control vector-transfected cells (Figure 3.8e). We therefore asked whether the other prioritized genes, YAP/TAZ and SPHK1, had functional roles in NB metastasis.
Figure 3.8. GJA1 expression levels are elevated in the enhanced metastatic subpopulations and knockdown of GJA1 inhibits migration and invasion in vitro. 

(a) Differential expression of GJA1 in primary and metastatic subtypes from the microarray is shown (left). Overexpression of GJA1 was confirmed at the protein level by immunoblot analysis with GJA1 antibody (right). (b) GJA1 knockdown selectively inhibits migration and invasion (MIG/INV) of the enhanced metastatic subpopulations without affecting proliferation. Parental, B1, B5, and BR2 cells were infected with lentivirus encoding shControl or shGJA1. Stable knockdown of GJA1 was confirmed by immunoblot analysis. (c) BrdU labeling and (d) migration (left) and invasion (right) assays were performed for parental, B1, B5, and BR2 cells exhibiting stable knockdown of GJA1 expression. Data represent average fold change relative to control +/- SEM (n=3) for BrdU and mean +/- SEM (n=3) for migration and invasion; * represents p<0.05, ** represents p<0.01, *** represents p<0.001. (e) GJA1 knockdown does not rescue the metastatic phenotype in vivo. B1 cells infected with shControl or shGJA1 were introduced by intra-cardiac injection into mice. Averages of body-wide bioluminescence from each cell line expressing shControl (n=5) or shGJA1 (n=5) were plotted on a log2 scale +/- SEM. The data is representative of 3 independent experiments.
3.3.6 Inhibition of the expression of the Hippo pathway effectors YAP and TAZ rescues the metastatic phenotype in vitro and in vivo

We chose to further investigate the role of the Hippo pathway for several reasons. First, recent evidence suggests that YAP and TAZ, effectors of the Hippo pathway, play a crucial role in the differentiation of neural crest cells (Hindley et al., 2016; J. Wang et al., 2016), which are thought to be the cell of origin of NB. Second, little is known about the role of YAP and TAZ in NB (Liu et al., 2016a; Q. Wang et al., 2014b); however, YAP signaling has been shown to promote breast cancer progression and metastases (Lamar et al., 2012; Lau, Curtis, Fillmore, Rowbotham, Mohseni, Wagner, Beede, Montoro, Sinkevicius, Walton, Barrios, Weiss, Camargo, Wong, & Kim, 2014b; F.-X. Yu et al., 2014). Third, increased YAP activity was recently found to be the only common signaling pathway to be transcriptionally upregulated specifically in relapsed NB as compared to matched diagnostic tumors (Eleveld et al., 2015; Schramm et al., 2015), suggesting that YAP activity is associated with increased aggressiveness and potentially metastasis in NB.

YAP and TAZ protein expression was elevated in B1, B5 and BR2 enhanced metastatic cells as compared to the parental cells (Figure 3.9a), confirming the results obtained with cDNA microarray (Figure 3.4). To assess the functional importance of YAP and TAZ, the expression of YAP and TAZ as well as both proteins together (YAP/TAZ) were reduced by shRNA knockdown. Consistent with a report showing compensatory up-regulation of TAZ expression upon YAP inhibition (Finch-Edmondson et al., 2015), we observed increased expression of TAZ upon YAP knockdown (Figure 3.10a). We therefore used cells in which both YAP and TAZ levels were knocked down for further studies. Knockdown of YAP/TAZ increased apoptosis, as assessed in western blots for cleaved PARP, and reduced cell growth as determined by alamarBlue assay in both enhanced metastatic and parental cells (Figure 3.9b and c). Interestingly, of the cells that survived YAP/TAZ knockdown, only the migration of the enhanced metastatic cells and not the parental cells was suppressed, suggesting that YAP and TAZ could be, in part, responsible for the increased migration phenotype seen in metastatic cells (Figure 3.9d). In addition, treatment of the enhanced metastatic cell lines with Verteporfin (VP), an inhibitor of YAP-TEAD interactions and YAP-mediated transcriptional activity (Liu-Chittenden et al., 2012) resulted in increased apoptosis (Figure 3.10b and c). To determine the effect of VP on migration and invasion, cells were pretreated with VP, the drug removed, and
viable cells assayed in Boyden chamber assays. VP pretreatment decreased the migration of the viable metastatic cells in vitro, similar to the effects observed with genetic inhibition of YAP/TAZ (Figure 3.10d). To determine whether suppressing YAP/TAZ expression could rescue the metastatic phenotype in vivo, we introduced viable B1 cells with reduced YAP/TAZ expression into mice via intra-cardiac injection. A significant inhibition of metastasis and increased disease-free survival was observed in mice injected with B1 YAP/TAZ knockdown cells as compared to control shRNA vector-expressing cells (Figure 3.9e-g). Next, we asked whether VP can also inhibit metastasis in vivo. To this end, we performed an ex vivo treatment experiment where metastatic cells were pre-treated with VP (1uM) or DMSO vehicle for 24 hours, and equal numbers of viable cells were introduced into mice by intra-cardiac injection. Although the metastatic burden of VP-treated and control cells was detected at similar levels initially as measured by bioluminescence, VP pre-treatment resulted in a significant inhibition of metastasis in vivo (Figure 3.9h). The incidence and extent of metastatic tumors in mice injected with VP pre-treated cells started to regress on day 21 post-injection, and metastases were undetectable by bioluminescence at day 42 (Figure 3.10e and f), suggesting that VP may affect both the initial colonization and the metastatic outgrowth after colonization.
Figure 3.9. The expression of the Hippo pathway effectors, YAP and TAZ, are elevated in the enhanced metastatic subpopulations and knockdown of both YAP and TAZ inhibits the metastatic phenotype in vitro and in vivo.

(a) Differential expression of YAP and TAZ in primary and metastatic subtypes from the microarray is shown (left). The increased levels of YAP and TAZ in the enhanced metastatic cells were confirmed at the protein level by immunoblot analysis with YAP or TAZ antibodies (right). (b) Knocking down both YAP and TAZ decreases cell growth and induces apoptosis in both parental and enhanced metastatic cells. Parental and B1 cells were infected with lentivirus encoding shControl alone or with shYAP and shTAZ (shYAP/shTAZ). Knockdown of YAP and TAZ and induction of cleaved (Cl)-PARP were confirmed by immunoblot analysis with Cl-PARP antibody and (c) cell growth was measured (in triplicates) by alamarBlue assay for the indicated time points. Data represent mean +/- SEM (n=4); *** represents p<0.001. (d) YAP/TAZ knockdown selectively inhibits the migration of metastatic B1 cells and not parental cells. Parental and B1 cells exhibiting YAP/TAZ knockdown were subjected to transwell migration assay. Data represent mean +/- SEM (n=4); ** represents p<0.01. (e) Immunoblot confirming the knockdown of both YAP and TAZ expression in B1 cells used for in vivo injection. (f) Knockdown of YAP/TAZ expression suppresses the metastatic phenotype in vivo. B1 cells infected with shYAP/shTAZ were introduced into mice by by intra-cardiac injection. Averages of body-wide bioluminescence from each cell line, shControl (n=9) and shYAP/shTAZ (n=8), were plotted on a log₂ scale +/- SEM (left panel); * represent p<0.05, ** represents p<0.01, *** represents p<0.001 (left panel). Representative luciferase images from day 42 post-injection are shown (right panel). (g) Kaplan-Meier curve showing the disease-free survival for mice injected with B1 cells expressing shControl or shYAP/shTAZ. The threshold for the disease was calculated as the average signal at day 7 + 3 S.D. for each condition. (h) Veterporfin (VP) pre-treatment inhibits NB metastasis in vivo. Viable B1 cells pre-treated with either DMSO or VP (1uM) for 24 hours were introduced into mice by intra-cardiac injection. Averages of body-wide bioluminescence from each condition, DMSO (n=5) and VP (n=4), were plotted on a log₂ scale +/- SEM; ** represents p<0.01, *** represents p<0.001.
Figure 3.10. Virtaporfin (VP), a pharmacological inhibitor of YAP, induces apoptosis and decreases migration of metastatic cells in vitro and inhibits the metastatic phenotype in vivo. (a) Knocking down YAP levels increases the expression of TAZ. B1 cells infected with lentivirus expressing the indicated shRNAs were lysed, and immunoblotted with YAP or TAZ antibodies. (b) VP treatment decreases the viability of the enhanced metastatic cells and induces apoptosis in vitro. IC$_{50}$ curves for VP were generated for the enhanced metastatic subpopulations. Data represent mean +/- SEM (n=3). (c) B1 cells treated with VP (2uM) for 48 hours were lysed and immunoblotted with the indicated antibodies. (d) VP pre-treatment inhibits migration and invasion in vitro. B1 cells were pre-treated with 1uM VP overnight and subjected to MIG/INV assay. Data represent mean +/- SEM (n=3); ** represent p<0.01, *** represent p<0.001. (e) Luciferase images of mice injected with VP pre-treated cells from day 21 show visible tumours (indicated with red arrows). These tumours, however, were diminished by day 42 as shown in (f).
3.3.7 Pharmacological inhibition of SPHK1 suppresses NB metastasis in vivo

To discover potential drugs that could target metastatic NB using our model, we selected genes with high expression in our metastatic signature that encoded kinases that were tractable therapeutic targets with available pharmacological inhibitors. We first focused on sphingosine kinase 1 (SPHK1), which catalyzes the phosphorylation of sphingosine to generate the lipid second messenger sphingosine-1-phosphate (S1P), as a candidate since \( SPHK1 \) is located on a region of chromosome 17q, which is frequently amplified in NB and predicts poor outcome (Schleiermacher et al., 2010). Furthermore, SPHK1 and S1P signaling has been shown to promote the growth of various cancer cells and colonization of myeloid cells at future metastatic sites (NCT01488513, NCT02490930, NCT01790269) (Deng et al., 2012). In addition, there are multiple inhibitors targeting the sphingosine kinase pathway, some of which are FDA-approved for multiple sclerosis and in early phase clinical trials for advanced solid tumors and multiple myeloma in adults (Kunkel et al., 2013). In our model, the expression of SPHK1 was significantly higher in the metastatic as compared to the primary subtype at both the RNA and protein level (Figure 3.11a). SPHK1 phosphorylates sphingosine to generate sphingosine-1-phosphate (S1P) (Figure 1.4). We detected increased S1P levels in lysates of B1, B5 and BR2 cell lines as compared to the parental cell line (Figure 3.11b). Next, we asked whether pharmacological inhibition of SPHK1 could inhibit the metastatic phenotype. Treatment with the SPHK1 inhibitors SKI II and PF-543, which are allosteric (French et al., 2003) and sphingosine-competitive (Schnute et al., 2012) inhibitors, respectively, decreased the growth of the enhanced metastatic cells without affecting their migration (Figure 3.11c and Figure 3.12a). This effect of the inhibitors was observed in other NB cell lines, as treatment of the LAN5, SHEP and BE2(C) human NB cells with SKI II and PF-543 inhibited their growth (Figure 3.12b). Given the recent evidence suggesting that SPHK1 promotes colitis-associated cancer by activating NF\( \kappa \)B-IL6-STAT3 signaling (Liang et al., 2013), we examined whether this signaling is associated with the growth or survival of the enhanced metastatic NB cell lines. Indeed, treatment of those cell lines with SKI II and PF-543 decreased phospho-STAT3 levels and induced cleaved PARP (Figure 3.11d and Figure 3.12c-e). Moreover, both SKI II and PF-543 treatments resulted in decreased SPHK1 protein expression as well as reduced S1P levels, suggesting that these inhibitors have on-target effects (Figure 3.11d and Figure 3.12f). Next, we asked whether SPHK1 inhibitors could inhibit NB metastasis in vivo independently of any effects on cell survival. To this end, we
performed an *ex vivo* pre-treatment experiment with SKI II. B1 cells were pre-treated with either DMSO or SKI II (2.5uM) for 72 hours, dead cells were removed, and equal numbers of viable cells were introduced into mice via intra-cardiac injection. SKI II pre-treatment significantly reduced both the number and size of metastatic tumors and increased disease-free survival *(Figure 3.11f and g)*. Furthermore, SKI II treatment (50mg/kg) of mice with established B1 cell line xenografts resulted in a significant delay in initial metastatic outgrowth as well as a delay in the growth of established metastases *in vivo* *(Figure 3.11h and i)*.
**D**

| DMSO | SKI II | PF-543 |

**E**

| DMSO | SKI II |

| CI-PARP | p-STAT3 | STAT3 | SPHK1 | β-actin |

**F**

- **DMSO**
- **SKI II pre-treated**

**G**

**H**

- **DMSO**
- **SKI II (50mg/kg)**

**Day 28**

% Disease-free survival

*p=0.0046*
Figure 3.11. SKI II, a pharmacological inhibitor of SPHK1, decreases the growth of enhanced metastatic cells in vitro and inhibits their metastasis in vivo.

(a) Differential expression of SPHK1 in primary and metastatic subtypes from the microarray is shown (left). The increased expression levels of SPHK1 in the enhanced metastatic cells was confirmed at the protein level by immunoblot analysis with SPHK1 antibody (right). (b) Enhanced metastatic subpopulations have increased Sphingosine-1-phosphate (S1P) levels. Lysates from parental and enhanced metastatic subpopulations grown in culture were subjected to mass spectrometry for lipid measurements. S1P measurements were normalized to total Sphingosine (S) levels for each cell line and the fold change relative to the parental cell line is shown. Data represent mean +/- SEM; ** represent p<0.01 and *** represent p<0.001. (c) SPHK1 inhibitors, SKI II and PF-543, decrease the viability of enhanced metastatic cells in vitro. The IC_{50} curves for SKI II (left) and PF-543 (right) are shown. Data represent mean +/- SEM (n=3). (d) SKI II and PF-544 induce apoptosis and decrease STAT3 signaling. B1 cells treated with SKI II (5uM) or PF-543 (50uM) for 24 hours were lysed and immunoblotted with the indicated antibodies (n=3). (e) Knockdown of SPHK1 expression in B1 cells that were used for in vivo injection was confirmed by immunoblot analysis. (f) SKI II pre-treatment inhibits NB metastasis in vivo. B1 cells were treated with DMSO or SKI II (2.5uM) for 72 hours, dead cells were washed off with PBS, and equal numbers of live cells were introduced into mice by intra-cardiac injection. Averages of body-wide bioluminescence from each cell line, DMSO (n=8) and SKI II pre-treated (n=5), were plotted on a log_2 scale +/- SEM (left panel). Representative luciferase images from day 28 post-injection are shown (right panel). (g) Kaplan-Meier curve showing the disease-free survival for mice injected with DMSO or SKI II pre-treated cells. The threshold for the disease was calculated as average signal at day 7 + 3 S.D. for each condition. (h) SKI II treatment in vivo delays outgrowth of established metastases. Control mice from (g) on day 35 were cohorted into two treatment groups, DMSO (n=4) and SKI II (50mg/kg) (n=4), based on luminescence. Averages of body-wide bioluminescence from each mouse from each treatment group were plotted on a log_2 scale +/- SEM. (i) SKI II treatment in vivo delays metastatic outgrowth. Mice injected with B1 cells were cohorted on Day 19 into two treatment groups, DMSO (n=12) and SKI II (50mg/kg) (n=12), based on luminescence. Averages of body-wide bioluminescence from each mouse from each treatment groups were plotted on a log_2 scale +/- SEM.
Figure 3.12. Pharmacologic inhibition of SPHK1 decreases the viability of NB cell lines and STAT3 and NFκB signaling in the enhanced metastatic subpopulations.

(a) SKI II pretreatment does not affect migration of the enhanced metastatic cells. B1 cells were treated with DMSO or SKI II (2.5uM) for 72 hours, dead cells were washed off with PBS, and equal numbers of live cells were subjected to migration assay. Data represent mean +/- SEM (n=3). (b) SPHK1 inhibitors, SKI II and PF-543, decrease the growth of NB cell lines \textit{in vitro}. The IC\textsubscript{50} curves for SKI II (left) and PF-543 (right) are shown. Data represent mean +/- SEM (n=2). (c) SKI II induces apoptosis in the enhanced metastatic cells. B1, B5, and BR2 cells were treated with SKI II (5uM) for 24 hours, lysed, and immunoblotted with antibodies to Cl-PARP and SPHK1. (d) SKI II treatment decreases STAT3 and NFκB signalling in metastatic subpopulations. B1, B5, and BR2 cells were treated with SKI II (5uM) for 6 hours, lysed, and immunoblotted with p-STAT3 and (e) for p-IKKα/β and p-p65 antibodies to assess STAT3 and NFκB signalling, respectively (n=3). (f) SKI II and PF-543 decreases Sphingosine-1-Phosphate (S1P) levels. Lysates from B1 cells treated with SKI II (5uM) (left panel), and PF-543 (20uM) (right panel) for 6 hours were subjected to mass spectrometry for lipid measurements. S1P measurements were normalized to total Sphingosine (S) levels for each condition and fold change relative to DMSO control is shown. Data represent mean +/- SEM (n=3); ** represent p<0.01 and *** represent p<0.001.
Gene signatures are widely used to provide clinical information about patient prognosis and in some cases can enable risk stratification as well as guide and tailor therapy (Oberthuer et al., 2006; 2010). We tested whether the 75 survival-associated genes we identified previously (Figure 3.4d) could be used as a signature to predict NB patient outcome. We initially tested the MET-75 signature in the E-MTAB-179 cohort, which include all NB patients from high risk and non-high risk groups (Oberthuer et al., 2010), that served as the training cohort to identify MET-75 genes. Elevated MET-75 signature scores were associated with a worse outcome as compared to patients with low MET-75 signature scores (HR: 11.8, p<0.0001, mean cut-point) (Figure 3.13a). As independent validation, we tested the MET-75 signature in additional NB patient cohorts. The MET-75 signature was significantly associated with survival in both the E-MTAB-16 (HR: 14, p<0.0001) & E-MTAB-38 NB (HR: 9.7, p<0.0001 cohorts) (Figure 3.13a). Overall these data suggest that the MET-75 signature is robustly associated with patient outcome. Previous studies have demonstrated the ability for randomly generated gene signatures and selected genes to predict patient outcome (Tofigh et al., 2014; Venet, Dumont, & Detours, 2011). Thus, we also compared the MET-75 with randomly generated 75-gene signatures and found that the MET-75 significantly outperformed randomly generated signatures (p=0.0273), suggesting our MET-75 comprises a unique set of genes that show a very robust relationship unlikely to be found by chance (Figure 3.14).

NB patients are stratified by risk groups where high-risk, most commonly characterized by distant metastatic disease in patients older than 18 months and/or MYCN-amplified tumors, represents the most aggressive form of disease with significantly inferior outcome as compared to low-risk patients (Maris, 2010). Among the high-risk group, however, patients experience diverse outcomes ranging from long-term survival to rapid progression and death. This suggests that NB patients currently categorized as high-risk can be further stratified by additional biomarkers to potentially identify patients with different outcomes including those with the lowest survival rates, sometimes referred to as ‘ultra’ high-risk. Since some subpopulations of our SK-N-AS cells, originally isolated from a high risk patient, had an enhanced property to metastasize, we asked whether our MET-75 signature correlates with more aggressive disease to potentially identify gene expression patterns that might be associated with ‘ultra’ high-risk
patients. By analyzing the subset of patients with distant metastatic disease (INSS stage 4), we found that high MET-75 expression correlated with poor outcome within the metastatic patient cohort (Figure 3.13b). Furthermore, MET75 expression was still associated with inferior outcome within the subset of high-risk metastatic (INSS stage 4) patients >18 months (Figure 3.13c), suggesting that our signature may identify ‘ultra’ high-risk patients and provide insight into targetable pathways in these poor prognosis patients.
Figure 3.13. Metastatic signature (MET-75) predicts survival of human NB patients. (a) Performance of the MET-75 signature in the training cohort (E-TABM-179) and two additional validation cohorts (E-MTAB-16 & E-MTAB-38). (b) Performance of MET-75 signature for overall (left panel) and event-free (right panel) survival in metastatic (INSS stage 4) patients and (c) for overall (left panel) and event-free (right panel) survival in high-risk (INSS Stage 4, >18 months) patients in E-TABM-179 cohort.
Figure 3.14. Metastatic signature (MET-75), but not random signatures, predicts survival of human NB patients.
MET-75 signature performs with superior accuracy than randomly generated 75-gene signatures. The ability of randomly generated 75-gene signatures to predict survival of NB patients was tested in E-TABM-179 cohort.
3.4 Discussion

Metastatic spread at diagnosis and recurrence are the key reasons for the lack of success in treating NB patients, however, the molecular mechanisms mediating NB metastasis are not known. Currently, *in vivo* models used to study NB metastasis do not recapitulate key features of the clinical presentation of the disease. Therefore, more faithful, quantifiable, and reproducible pre-clinical models are required to study the biology of NB metastasis and for the identification of novel therapeutic targets in metastatic NB. Here, we have developed a metastatic mouse model of NB that generates reproducible osteolytic bone metastases and CNS metastases similar to those seen in NB patients, and that allows for quantitative *in vivo* monitoring using bioluminescence. Using our model, we were able to isolate subpopulations of cells with enhanced metastatic potential. This suggests that the enhanced metastatic subpopulations have unique biological properties that facilitate increased metastatic burden. Indeed, gene expression profiling of the parental and enhanced metastatic subpopulations revealed two distinct subtypes, “parental” and “metastatic”. These two populations of cells have differentially-regulated genes and biological pathway profiles. While the enhanced metastatic subpopulations did not display specific tissue tropisms, they did exhibit increased frequency of metastasis to the sites of metastasis observed in unfavorable prognosis and relapsed NB as well as decreased latency. The gene expression profile of cells introduced into mice by intracardiac injection and that honed to the adrenal gland, the primary site of NB, clustered with the parental cell line profile, whereas the expression profile of metastatic cells isolated from bone/bone marrow and the CNS clustered together. This suggests that our method selects for cells with an increased ability to metastasize to relevant sites of NB (bone, bone marrow, brain) rather than to sites not seen commonly in NB (i.e. lung), and that those cells that hone to primary NB tumor sites do not express an enhanced metastatic gene expression profile.

We identified and characterized several genes and pathways that have not been identified to be involved in NB metastasis, including CADM1, GJA1, YAP/TAZ, and SPHK1. CADM1 is a tumor suppressor in NB and inhibits the growth of NB cells *in vitro* (Nowacki et al., 2007). We show that CADM1 re-expression in enhanced metastatic cells decreases invasion *in vitro* and inhibits metastasis *in vivo*. Strikingly, we did not observe any detectable metastases by bioluminescence in mice injected with CADM1 overexpressing cells. Since CADM1 is involved in cell adhesion, it is possible that it may play a role in the extravasation step of the metastatic
cascade, which is initiated by cell adhesion. To determine if CADM1 affects NB cell adhesion, future experiments examining the ability of CADM1 overexpressing cells for adhesion to the ECM and/or to endothelial cells \textit{in vitro} and \textit{in vivo} should be performed. For example, quantification of CADM1 over-expressing cells on the blood vessels in the metastatic sites after intra-cardiac injection can determine whether CADM1 have a functional role in adhesion to endothelial cells \textit{in vivo}.

Knockdown of GJA1, a gap junction protein that was overexpressed in the enhanced metastatic cell lines, decreased invasion \textit{in vitro} without affecting cell proliferation. These results are similar to those observed in glioma cell lines, where GJA1 knockdown attenuated glioma cell invasion (Sin et al., 2015). GJA1 knockdown, however, was not able to suppress NB metastasis by the enhanced metastatic cells \textit{in vivo}. This result suggests that while GJA1 mediates surrogate phenotypic markers of metastasis such as cell invasion, GJA1 alone is not required to maintain the metastatic phenotype. To determine whether GJA1 function in concert with other genes/pathways to promote metastasis, additional genetic manipulation of other genes/pathways can be performed in conjunction with GJA1 knockdown. For example, we can knockdown the expression of GJA1 in shYAP/shTAZ cells to determine whether knocking down GJA1 further inhibits NB metastasis \textit{in vivo} compared to shYAP/shTAZ alone.

In contrast, knockdown or pharmacological inhibition of the hippo pathway effectors YAP and TAZ inhibited the invasion of the enhanced metastatic cells and metastasis \textit{in vivo}. TAZ has been shown to regulate proliferation and tumorigenicity of NB cells (Wang et al., 2015a), however, the role of TAZ in NB metastasis had not been studied \textit{in vivo}. Previous reports have utilized a selective YAP inhibitor, Verteporfin, in preclinical studies. For example, Verteporfin treatment suppressed mutant Gq/11-induced uveal melanoma (Yu et al., 2014). Similarly, we demonstrate that \textit{ex vivo} treatment of metastatic cells with Verteporfin inhibited metastasis \textit{in vivo}, suggesting that Hippo pathway can be therapeutically targeted in metastatic NB. Targeting the Hippo pathway is further supported by recent genomic profiles comparing diagnostic and relapsed tumor samples, which demonstrate that YAP activity is specifically increased at the time of recurrence (Eleveld et al., 2015; Schramm et al., 2015).

High-risk metastatic NB is known to harbour multiple chromosomal abnormalities including segmental chromosomal losses at 1p and 11q (Attiyeh et al., 2005) and gain of 17q.
(Schleiermacher et al., 2010), however, identification of oncogenes and tumor suppressors within these regions and their roles in the progression of NB are largely unknown. Furthermore, although next generation sequencing of NB tumors has identified coding mutations in numerous oncogenes and tumor suppressors, recurrent activating driver mutations are not common with the highest prevalence detected for ALK, which harbors missense mutations in approximately 10% of tumors (Molenaar et al., 2013). Furthermore, targetable alterations specific to metastases have not been identified. We identified the kinase SPHK1 as a potential therapeutic target for metastatic NB. Previous studies have identified SPHK1 as a target in various cancers including glioblastoma, AML, prostate, hepatic, and colorectal cancers and inhibitors targeting SPHK1 showed efficacy in pre-clinical models for these cancers (Chumanevich et al., 2010; Ju, Gao, & Fang, 2016; Kapitonov et al., 2009; Leroux et al., 2007; Loveridge et al., 2010; Noack, Choi, Richter, Kopp-Schneider, & gnier-Vigouroux, 2014; Paugh et al., 2008). Mechanistically, targeting SPHK1 inhibited phosphorylation of AKT and c-Jun-NH2-kinase pathway to reduce the vascularization and the growth rate of glioblastoma tumours in vivo (Kapitonov et al., 2009).

Currently, therapies targeting SPHK1-S1P axis are in clinical trials for patients with inflammatory and autoimmune diseases and are the subject of Phase I and II oncology clinical trials for adults with solid tumors (NCT01488513, NCT02490930) and multiple sclerosis (NCT01790269) (Kunkel et al., 2013). Here, we demonstrated that two different SPHK1 inhibitors, SKI II and PF-543, inhibited the growth of metastatic NB cells in vitro. Moreover, SKI II treatment in vivo showed therapeutic efficacy as it delayed the growth of metastatic tumors. SPHK1 is located on 17q, which is frequently amplified in metastatic NB, and its expression levels were elevated in the enhanced metastatic cells as compared to the parental cell line. These data suggest that SPHK1 is a critical regulator of metastatic growth, and that patients with high levels of SPHK1 due to 17q amplification or potentially other mechanisms of upregulation may benefit from therapies targeting SPHK1.

Our functional studies of candidate genes and pathways in our metastatic mouse model together with the independent prognostic significance of these genes in multiple NB datasets suggests that we may be able to use profiles from our metastatic cells to predict patient outcome. We generated a novel metastatic signature (MET-75) that predicts outcome of NB patients. Although there are numerous signatures that predict survival of NB patients, the majority have only been used in populations of all NB patients (including high and non high-risk) but not been
shown to specifically predict outcome within metastatic subsets of patients. Notably, the majority of patients who are classified on clinical trials to have high-risk disease have distant metastatic disease or INSS stage 4/INRG stage M disease. The MET-75 signature predicted the poorest outcome patients among those with metastatic disease, and these patients may be considered to be those at the highest risk of recurrence or ‘ultra’ high-risk. It is possible that the signature correlates with a higher burden of metastases, and previous studies using quantitative scores of metastases as detected by radioisotope imaging have suggested that patients with more metastatic disease at diagnosis and following initial chemotherapy treatment have an inferior overall survival (Yanik et al., 2013). Thus, MET-75 may facilitate the identification of patients who require additional therapies and suggest pathways that may be targeted in this poor prognosis patient group. The MET-75 signature includes genes that have known roles in regulating tumorigenesis and metastasis such as APOE and MALAT1 (Gutschner et al., 2013; Pencheva et al., 2012), and a number of genes with no known function in NB. It also contains CADM1 and SPHK1 that we have functionally validated to demonstrate roles in metastasis, as well as genes that regulate or are downstream targets of the Hippo pathway. This suggests the importance of studying the functional role of novel genes within our signature that are clinically relevant, to gain insights into the biology of and possible targets for metastatic NB.

In summary, we describe a metastatic model of NB that recapitulates the clinical representation of the metastatic disease regarding tropism and burden. In addition, we identify novel regulators of NB metastasis that are potential therapeutic targets and a potentially clinically useful metastatic signature that predicts NB patient outcome specifically within the subgroup of patients with metastatic disease. Thus, our model provides a platform to study the biology of NB metastasis and to test candidate anti-metastatic drugs.

3.5 Materials and Methods

3.5.1 Generation of cell lines

The SK-N-AS and SK-N-Be2(c) cell lines were purchased from ATCC and mycoplasma and STR tested prior to the commencement of experiments. The LAN5 and SHEP cell lines were kind gifts from Dr. Patrick Reynolds. Briefly, SK-N-AS cells were infected with the retroviral TGL (thymidine kinase-GFP-luciferase) reporter (Ponomarev et al., 2004) provided by Dr.
Ronald Blasberg (Memorial Sloan Kettering Cancer Centre, New York, NY, USA). Fluorescence-activated cell sorting enriched for GFP-positive cells and luciferase activity was confirmed using the Luciferase Assay System (Promega), according to the manufacturer’s instructions. SK-N-AS cells were cultured in high glucose DMEM media (Invitrogen) containing 10% FBS, 1% non-essential amino acids (Invitrogen) and 1% pen/strep (Invitrogen).

3.5.2 Animal Studies

For xenograft experiments, male NOD/SCID mice (6-8 weeks) were injected subcutaneously with $10^6$ cells at a 1:1 ratio with growth factor-reduced matrigel; tumor volumes were monitored bi-weekly and calculated as described (Fathers et al., 2010). Intracardiac injections were performed on 4-6 week old isoflurane anaesthetized mice using $10^5$ cells as previously described (Kang et al., 2003). Mice were imaged for bioluminescence immediately after injection to ensure the proper distribution of cells through circulation. Mice were sacrificed up to 100 days post-injection or earlier depending on the metastatic burden based upon the animal’s health, weight, and appearance as per the SickKids Institutional Animal Utilization Protocol. Tumor outgrowth within bone was monitored according to (Kang et al., 2003).

3.5.3 Bioluminescence imaging and analysis

Animals were injected with D-luciferin (Caliper Life Sciences) and imaged 8-12 minutes post-injection with the Xenogen IVIS imaging system coupled to Living Image acquisition and analysis software. For bioluminescence quantification, a rectangular region was drawn over each individual animal and calculated as photon flux per second over time.

3.5.4 Isolation of tumour cells from metastatic sites

Metastatic lesions from the bone were isolated according to the procedures previously published (Kang et al., 2003). CNS and adrenal gland lesions were minced, tritutrated, and strained through a 70-µm filter before plating. After 1-3 weeks in culture (approximately 5-6 passages), isolated cell populations were GFP-sorted by FACS to obtain human NB cell line subpopulations.
3.5.5 MicroCT and MRI analysis

At endpoint (60 days or when deceased), mice were anaesthetized with isoflurane and hindlimbs were analyzed using the Siemens Inveon microCT. Briefly, the acquisition was done using the following parameters: total rotation (200 degrees), rotation steps (400), exposure time per projection (1300 ms), settle time between projections (500 ms), voltage (80kV), current (500 μA), system magnification (low-med), effective pixel size (43.29 μm). Images were reconstructed (bilinear interpolation, Shepp-Logan reconstruction filter, HU calibrated, image scale of 2.049 and image offset of -1000) then analyzed in the sagittal view for bone density using the Inveon Research Workplace software, as described by the manufacturer. Briefly, regions of interest were highlighted across 30 sections and the threshold tool was used to define the maximum and minimum voxel intensity limits for the bone and bone marrow. The mean voxel intensity for each sample was measured. Non-tumor bearing mice of similar strain and age were used as a control. The degree of bone destruction was determined as a percentage difference between the average voxel intensity of the experimental mice relative to the control animals. Mice with CNS lesions were anaesthetized with isoflurane and brains were imaged using the Biospec 70/30 USR Ultrashielded 7T MRI.

3.5.6 Histological analysis

Sections were deparaffinized and stained in either hematoxylin and eosin or freshly prepared tartrate-resistant acid phosphatase (TRAP) staining solution then scanned using a Hamamatsu slide scanner and images collected using the NanoZoomer Digital Pathology Virtual Slide Viewer.

3.5.7 RNA isolation, labeling, microarray hybridization, and databases

RNA was isolated using the Ribopure kit (Invitrogen) according to the manufacturer’s instructions. RNA quality was confirmed using Bioanalyzer software (Agilent) and hybridized onto a genechip (Affymetrix Genechip Human Genome U133 Plus 2.0 Array) as we have
described (Fathers et al., 2010). Publically available data was downloaded from ArrayExpress (https://www.ebi.ac.uk/arrayexpress/, E-TABM-38, E-MTAB-179, E-MTAB-16). The E-MTAB-179 dataset comprises 477 neuroblastoma samples profiled on the Agilent Custom Human Neuroblastoma Chip 251496110 (Oberthuer et al., 2010). E-TABM-38 comprises 199 samples profiled with DKFZ_H_sapiens_Neuroblastoma_Chip_10k_v1 microarrays (Oberthuer et al., 2006). E-MTAB-16 comprises 327 samples profiled using either DKFZ_H_sapiens_Neuroblastoma_Chip_10k_v1 or -DKFZ_H_sapiens_Neuroblastoma_Chip_10k_v3 microarrays (Oberthuer et al., 2008). For custom arrays, data was pre-processed as described by the contributor or raw data was pre-processed using RMA. Gene level expression data was obtained by collapsing probe set IDs by Gene ID, based on maximum average expression across the entire dataset. Versteeg and Seeger datasets were accessed through R2 database (http://r2.amc.nl/). Versteeg dataset comprises 88 patients and Seeger dataset comprises 102 non-MYCN amplified patients (Asgharzadeh et al., 2006).

3.5.8 Bioinformatics analysis

Gene expression analyses were completed using R (v3.2.0). Survival analysis was completed using Cox-regression implemented using the CoxPH package. GSEA was carried out as previously described (Subramanian et al., PNAS 2005), and GSEA-based enrichment maps (Enrichment map plugin) and network analysis of metastasis genes (Reactome FI plugin) were completed using Cytoscape (v2.8.2). False discovery rate (FDR) was calculated using Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995).

3.5.9 Antibodies and Immunoblot analyses

Cells were lysed in RIPA (50mM Tris, pH 8.0, 150mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) buffer supplemented with complete protease inhibitors (Roche), and protein concentration determined by the Bradford method (Bio-Rad). Immunoblot analysis was performed as described in (Seong et al., 2014). The antibodies used included anti-GJA1/Connexin 43 (#3512), YAP/TAZ (#8418), Cl-PARP (#9541S), STAT3 (#9139S), p-STAT3 (#9145S), IKKα (#2682P), p-IKKα/β (#2697S), p65 (#3033S), p-p65 (#6956S) from
Cell Signaling, CADM1 (ABT66, Millipore), SPHK1 (ab16491, Abcam), β-actin (A5316, Sigma), V5 (R960-25, Invitrogen), and Vinculin (05-386, Upstate).

3.5.10 Cellular Assays and Transductions

For migration and invasion assays, cells were plated at 2x10^5 cells per transwell and carried out and analyzed as described in (Seong et al., 2014). Briefly, cells in low serum (0.1% FBS) media (100uL) were seeded into the upper chamber and the lower chamber contained 10% FBS growth media. For invasion, 6% matrigel (BD Biosciences) in serum-free media was added to the upper chamber. Following 16 – 24 hours incubation (37°C, 5% CO₂), migrated/invaded cells were stained with crystal violet and imaged for analysis. Migration and Invasion index represent the % area of the bottom chamber covered by migrated and invaded cells, respectively. BrdU (Cell Signaling) and alamarBlue (Invitrogen) assays were performed according to the manufacturers’ directions and IC₅₀ studies were carried out as previously described (Grinshtein et al., 2011). Lentiviral-expressed GJA1 (Santa Cruz) shRNA and control shRNA lentiviral particles (Thermo Scientific) were used for viral transductions according to the manufacturer’s instructions. Lentiviral plasmids encoding shRNA to YAP and TAZ were gifts from Dr. Xiaolong Yang (Queen’s University, Kingston, ON, Canada). CCSB-Broad lentiviral vector of human CADM1 cDNA (Dharmacon) was used for overexpression studies. Sphingosine and sphingosine-1-phosphate were extracted using a modified Bligh-Dyer method and measured by The Analytical Facility for Bioactive Molecules (The Hospital for Sick Children, Toronto, ON, Canada).

3.5.11 Drug treatments

All drug treatments in vitro were conducted in low-serum (1% FBS) media unless otherwise indicated. Drugs/inhibitors used included Verteporfin (Tocris, #5305), SKI II (Tocris, #2097), and PF-543 (Selleckchem, #S7177). For SKI II treatments in vivo, mice were given IP injection of either DMSO (control) or SKI II (50mg/kg) every other day during the indicated treatment period.
3.5.12 Statistical Analysis

A two-sample variance student’s t test was used to assess for statistical significance unless indicated. A two-way ANOVA analysis was performed on the subcutaneous outgrowth and bioluminescence data. A log rank (Mantel-Cox) test was performed on the Kaplan-Meier survival curves.
Chapter 4
Conclusion and Future Directions

4 Conclusion and Future Directions

4.1 The Role of SATB2 in OS Invasion

4.1.1 Major Conclusion
Despite recent advances in multimodal treatments, the survival OS patients with metastases is <20%. Thus, better understanding the fundamental biology of OS metastasis is needed for the development of novel therapies targeting OS metastases. In chapter 2 of my thesis, we demonstrate that SATB2 is highly expressed specifically in OS cells as compared to osteoblasts, suggesting that SATB2 play an oncogenic role in OS. Moreover, we demonstrate that SATB2 OS invasion by regulating the expression of genes involved in cytoskeleton organization including EPLIN. Mechanistically, SATB2 down-regulates EPLIN expression, which leads to increased adhesion and invasion of OS cells.

4.1.2 Studying the Role of SATB2 in OS Metastasis In Vivo
We have demonstrated SATB2 mediates OS cell invasion through modulating expression of genes involved in cytoskeleton remodeling. Furthermore, we showed that knocking down the expression of SATB2 rescues invasive phenotype of OS cells in vitro, however, its pro-metastatic abilities were not examined in in vivo settings. As mentioned in Chapter 1, metastasis is a complex process that involves not only the cancer cells themselves but other cells and biological processes and structures that cannot be fully recapitulated in in vitro models. For example, the tumour microenvironment surrounding both the primary tumour and metastases comprises various cell types including cancer-associated fibroblasts (CAFs), endothelial cells, and immune cells that act dynamically to both promote and suppress tumour growth. Furthermore, the plasticity of cancer cells to coordinate cellular pathways adapt to different steps of metastatic cascade is important and cannot be tested in vitro. Thus, our findings that demonstrate SATB2 as a pro-invasive protein is OS should be further examined in vivo.
To achieve this goal, there are several in vivo models that can be employed. First, tail-vein injection model, which are widely used to study OS lung metastases (Koshkina et al., 2007; Mendoza et al., 2010; Tome et al., 2013) can be used. For this, shSATB2 (or SATB2 CRISPR KO) and control OS cells can be injected via tail-vein and metastatic nodules in the lung can be assessed. This will determine whether SATB2 is required for OS cells to colonize the lung and for metastatic outgrowth. In addition to tail-vein model, a GEM model of OS can be used. For example, we can take OS mouse models that give rise to metastases such as Osx-Cre+ p53fl/fl and Osx-Cre+ p53fl/fl-Rbfl/fl mice (see section 1.2.2.) and cross them with SATB2fl/fl mouse. This will result in mouse OS tumours that lack SATB2 expression. In these mice, we can examine both the growth of primary tumours as well as the development of metastases. Together, in vivo validations will further strengthen the critical role of SATB2 in OS metastasis.

4.1.3 Potential Role for SATB2 in Chemoresistance in OS

The survival of OS patients is greatly reduced by the resistance to therapy. Previously, our lab has demonstrated that SATB2 promotes chemoresistance by augmenting ΔNp63α-mediated transrepression of p53-family responsive genes (Chung et al., 2010). Similarly, it is possible that high expression of SATB2 in OS also promote resistance to therapies for several reasons. First, recent studies on the resistance mechanisms in OS have highlighted the importance PI3K/AKT signaling (Han et al., 2015; Z. Huang, Huang, He, & Ni, 2015; Sevelda et al., 2015; X.-J. Shao et al., 2015), which is one of the pathways that were significantly enriched in shSATB2 OS cells (Figure 2.5e). Thus, it is possible that in addition to genes that regulate cytoskeleton remodeling, SATB2 may modulate chemoresistance by regulating genes involved in PI3K/AKT signaling pathway. In addition, the expression of miR-34c was recently shown to be downregulated in OS tumours with a poor chemoresponse and inhibition of miR-34c in OS cells promotes chemoresistance in vitro (Xu, Jin, Xu, Bi, & Wang, 2014). This is interesting due to the fact that SATB2 is a well-studied target of miR-34 (Wei et al., 2012). Thus, it is possible that inhibition of miR-34c OS cells result in upregulated expression of SATB2, which mediates chemoresistance.
4.1.4 SATB2 and Chromatin Remodeling in OS Invasion

In our study, we demonstrate that SATB2 regulates the expression of EPLIN to promote OS cell invasion. Moreover, we speculate that SATB2 may interact with ΔNp73 to regulate the expression of EPLIN and possibly other genes involved in OS invasion. In addition this mechanism, it is possible that SATB2 may regulate genes more globally by remodeling chromatin structure. SATB2 binds to AT-rich regions in the DNA as well as nuclear matrix and together with other chromatin remodeling proteins, cause structural changes in chromatin structures (Dobreva et al., 2003; Gyorgy, Szemes, de Juan Romero, Tarabykin, & Agoston, 2008). This change in chromatin structure can, in turn, lead to changes in gene expression by regulating the accessibility of transcription factors to gene loci. Indeed, various chromatin remodeling proteins and epigenetic regulators, which also promote chromatin remodeling, have been implicated in metastasis in many different cancers (Gupta et al., 2011; Song et al., 2013; Tiwari et al., 2013; L. Wang et al., 2014a). Thus, it is possible that SATB2 also regulate OS invasion by modulating gene expression by regulating global chromatin remodeling and not only in a transcription factor-dependent manner.

To study this hypothesis, we can perform ATAC-sequencing and Hi-C experiments shSATB2 and control cells to assess changes in chromatin accessibility and 3-D genomic conformation, respectively. ATAC-sequencing utilizes a transposase Tn5 that preferentially incorporate into genomic regions absent of nucleosomes, which indicates accessible DNA regions (Buenrostro, Giresi, Zaba, Chang, & Greenleaf, 2013). Deep-sequencing of adapter-ligated DNA fragments can reveal accessible genomic regions. We can then examine whether genes that were differentially regulated in shSATB2 cells are located within accessible or inaccessible chromatin regions. Hi-C is based on a chromosome conformation capture technique, in which genomic DNA is cross-linked, digested, and re-ligated that only allows ligation of DNA fragments that are covalently-linked (Belton et al., 2012). Deep-sequencing of ligated products from Hi-C experiment provide insights into the interaction between both proximal and distal chromatin. The changes in these interactions can lead to differential regulation of gene transcription. Hi-C analysis on shSATB2 and control cells can potentially provide insights into the mechanism underlying differential expression of genes in shSATB2 cells.
4.1.5 Potential Novel Therapies for OS

We have shown that OS have high expression levels of SATB2 and demonstrated that SATB2 promotes OS invasion. Thus, therapeutically targeting SATB2 may lead to inhibition of OS metastasis, however, similar to transcription factors, therapeutic targeting SATB2 is invariably challenging due to its nuclear localization. Instead, we can target genes and pathways downstream of SATB2 and also upstream regulators of SATB2 that promote its function. We demonstrated that SATB2 regulates expression of genes involved in cytoskeleton remodeling, which can be potential therapeutic targets. In fact, chemotherapeutic agents that are already in clinical use including Paclitaxel and Vinblastine target microtubule dynamics to prevent the growth of cancer cells. Although actin-targeting drugs have been associated with significant toxicities in pre-clinical models due to the lack of discrimination between actin expressed in tumor cells and normal cells, recent studies suggest that specific pharmacologic targeting of actin regulatory proteins that are differentially expressed in tumor (vs. normal) cells may be a more feasible approach that will have less toxicity (Hall, 2009; Stehn et al., 2013). Thus, understanding how SATB2 regulates transcription of genes and signaling pathways, including EPLIN, which govern actin cytoskeleton effects on motility and invasion may lead to the discovery of proteins that can be targeted in metastatic OS and other cancers with high expression of SATB2.

4.1.6 Additional SATB2-regulated Genes and Upstream Regulators of SATB2

Although we have focused on the role of EPLIN in SATB2-mediated OS invasion, gene expression profiling has identified many other genes including GTPases, adhesion molecules and growth factor receptors that may be regulated by SATB2 (Figure 4.1). Some of these genes have already been implicated in promoting invasion and metastasis in other cancers including EGFR, LIFR, ITGB3, SDC2, and MMP16 (Chen et al., 2012; M. Huang et al., 2011; H. Jiang et al., 2015; Z. Liu et al., 2016b; Sun et al., 2013; Tatti et al., 2015). Thus, it is important to study additional mechanism by which genes other than EPLIN promote OS invasion downstream of SATB2. Furthermore, the mechanism by which SATB2 is over-expressed in OS is not well understood. One possible mechanism may involve the regulation by the microRNAs (miRs) that
target SATB2. For example, multiple studies have shown that the expression of miR-34, which targets SATB2 in osteoblasts, is low in OS and that over-expressing miR-34 inhibits proliferation and metastasis of OS cells (Wang et al., 2015b; Yan et al., 2012). Thus, it is possible that the effects of miR-34 in OS are, in part, mediated by SATB2 function. In addition to miRNA regulation, TGF-β and BMP signaling pathways have also been implicated in regulating the expression of SATB2 (Zhao et al., 2014). This suggests that studies to identify exact mechanism that regulate SATB2 in osteoblast and osteosarcoma cells will better our knowledge in both the development and pathogenesis of OS.

SATB2 can bind to other transcription factors and act as a co-activator or a co-repressor (Dobreva et al., 2006). Previous work from our lab have demonstrated that SATB2 binds to p53 family proteins p63 and p73 and that SATB2 augments the activity of the N-terminally truncated p63 isoform ΔNp63α to promote chemoresistance in HNSCC (Chung et al., 2010). In addition, different isoforms of p63 and p73 (mainly TA vs truncated N-terminal isoforms) have been demonstrated to regulate metastasis (Bergholz et al., 2012; Rodriguez Calleja et al., 2016; Steder et al., 2013; Su et al., 2010; J. Wu et al., 2014). For example, ΔNp73 promotes melanoma metastasis by inhibiting the expression of LIMA resulting in EMT phenotype and increased AKT/STAT3 signaling (Steder et al., 2013). Moreover, ΔNp63 represses the expression of miR-527 and miR-665 to promote metastasis by enhancing TGFβ-induced metastasis (Rodriguez Calleja et al., 2016). Thus, it is possible that SATB2 enhances metastatic phenotype by modulating the activity of p53 family protein members.

4.1.7 The Role for SATB2 as a Possible Tumour Suppressor

Our lab has identified oncogenic roles for SATB2 in HNSCC and OS. There are, however, multiple studies that suggest a tumour suppressor role of SATB2 in colorectal cancer. These studies showed that down-regulated expression of SATB2 is associated with metastasis and poor prognosis and that SATB2 over-expression suppresses progression of colorectal cancers in vitro and in vivo (Eberhard et al., 2012; Mansour et al., 2015; S. Wang et al., 2009). The ability for SATB2 to have both oncogenic and tumour suppressive effects indicate that the role of SATB2 is cell type and context-dependent. This is likely due to the ability for SATB2 to interact with diverse transcription factors. Thus, better understanding of the transcription factors
that interact with SATB2 in specific cell type and context will help determine the exact of SATB2.

4.1.8 The Role of SATB2 in NB Metastasis

Interestingly, the expression of SATB2 was found to be up-regulated in the metastatic subpopulations in our NB metastasis model. Given that SATB2 promotes OS cell invasion, it is possible that SATB2 also plays a pro-invasive role in NB to promote metastasis. In order to test this hypothesis, I will knockdown the expression SATB2 in metastatic cells lines using shRNA and/or CRISPR and characterize the effects on proliferation and invasion in vitro. I have already generated NB cells with SATB2 knockdown via shRNA and CRISPR. These can be used for intra-cardiac injection to examine whether knocking down SATB2 expression can rescue metastatic phenotype in vivo. If SATB2 knockdown rescues metastatic phenotype in vivo, I will perform further experiments to characterize the transcription partner of SATB2 as well as genes regulated by SATB2 to elucidate the mechanism by which SATB2 promotes NB metastasis. Furthermore, to gain insights into potential downstream regulators of SATB2 in NB metastasis, we can compare the microarray data from OS shSATB2 cells and NB metastasis model. For example, by overlaying genes that were up-regulated in NB model with genes that were down-regulated in shSATB2 OS cells may provide potential SATB2 targets that regulate NB metastasis.
Figure 4.1. Working model of SATB2-mediated invasion and metastasis. SATB2 promotes OS invasion by regulating the expression of genes involved in various biological processes including cell-cell adhesion, actin polymerization/de-polymerization, Rho GTPases, cell-extracellular matrix (ECM) and growth factor receptor signaling. Differentially regulated genes in shSATB2 cells identified by microarray are indicated in red boxes.
4.2 Modeling Metastatic NB and Identification of Genes and Pathways to Target NB Metastasis

4.2.1 Major Conclusion

Recent sequencing studies have identified novel genetic determinants in NB tumours including mutations in ALK and ATRX (Pugh et al., 2013). However, the genes and pathways that regulate NB metastasis, which is the leading cause of death in children with NB, are less well understood. In my thesis, we demonstrate the development of a novel mouse model of NB metastasis that recapitulates human metastatic NB in terms of tissue tropism and burden of disease. Moreover, we isolated metastatic subpopulations that display enhanced metastatic burden and identified novel regulators of NB metastasis including CADM1, GJA1, SPHK1, and the Hippo pathway. Although our model has identified novel functional regulators of NB metastasis, there are important limitations and interesting questions that require further investigations. In this section, I discuss some of the outstanding questions as well as provide insights into the additional studies that may be needed.

4.2.2 NB Metastasis Model and the Microenvironment

In chapter 3, we demonstrate novel genes and pathways that regulate NB metastasis. However, we have only characterized the genes and pathways that affect NB cell behavior. There are many cell extrinsic factors such as the extracellular matrix as well as other cell types including fibroblasts and immune cells that regulate metastatic cascade. In fact, recent studies have demonstrated the importance of the microenvironment in supporting metastasis (see section 1.1).

This suggests the possibility that the enhanced metastatic cells in our model have increased capability to transform the microenvironment in the metastatic niche to support the growth of metastases. Indeed, metastatic subpopulation showed increased expression of genes that both compose and remodel the extracellular matrix including COL4A1, COL4A2, FBLN2, LAMC2, LOXL3, and HAS2. In fact, LAMC2 has already been implicated invasion and metastasis in many cancers (Moon et al., 2015; Smith et al., 2010; Yamamoto, Itoh, Iku, Hosokawa, & Imai, 2001; Yamamoto et al., 2009). For example, the expression of LAMC2 is upregulated in lung adenocarcinoma metastatic cells and promotes metastasis by increasing the traction force
and invasion, which was accompanied by epithelial-to-mesenchymal transition (EMT) (Moon et al., 2015). Thus, analyzing the microenvironment of the NB metastases as well as ECM genes that were differentially regulated in metastatic cells in more detail could help us understand how cell non-autonomous factors can contribute to NB metastasis.

4.2.3 Characterization of the Molecular Mechanisms of Novel Regulators of NB Metastasis

The development of NB metastasis model led to the identification of novel functional regulators of NB metastasis, some of which rescued metastatic phenotype in vivo. However, there are several important remaining questions to be answered in order further elucidate the mechanism by which these novel regulators modulate NB metastasis.

CADM1 was down-regulated in metastatic cells and over-expression of CADM1 into metastatic cells rescued metastatic phenotype both in vitro and in vivo. Strikingly, we did not observe detectable metastases in mice injected with CADM1 over-expressing cells as measured by bioluminescence (Figure 3.7e). There are many possible explanations for this observation. First, CADM1 over-expressing cells may not have survived the anchorage-independent condition in the circulation. Second, CADM1 over-expressing cells may have successfully extravasated into the secondary site but failed to colonize the tissue for outgrowth. Our finding that CADM1 does not affect growth of metastatic cells (Figure 3.7c) and anchorage-independent growth (Figure 4.2), suggest that alternative mechanism is involved. During metastasis, cancer cells require appropriate adhesion to endothelial to initiate proper extravasation into the secondary tissue (Cao et al., 2013; Jouve et al., 2014; S.-A. Kang et al., 2015; F. T. Wu et al., 2015). Thus, it is possible that CADM1 regulates adhesion dynamics between the metastatic NB cells and the endothelial cells in the blood vessel. For example, CADM1 over-expression may inhibit the adhesion metastatic NB cells and the endothelial cells or may promote excessive adhesion to limit extravasation into metastatic sites.
Figure 4.2. CADM1 over-expression does not affect anchorage-independent growth of metastatic cells.

B1 cells over-expressing CADM1 and LacZ control cells were plated on ultra-low attachment plates and cell viability was measured by alamarBlue assays for indicated time points. Data represent mean +/- SEM (n=2).

The pathway analyses of the gene expression data revealed an enrichment of genes belonging to the Hippo pathway in the metastatic subpopulations (Figure 3.4b and c). Importantly, the expression of the Hippo pathway effectors YAP and TAZ were up-regulated in metastatic cells and knocking down the expressions of these genes suppressed NB metastasis in vivo. YAP and TAZ are transcriptional co-factors that lack the ability to bind to DNA. To induce downstream transcriptional program, they bind to other transcriptional factors including TEADs, SMADs, p73, β-catenin, and Runx transcription factors (Piccolo, Dupont, & Cordenonsi, 2014). In breast cancer and melanoma, Lamar and colleagues showed that the ability for YAP to promote metastasis is dependent on the interaction with TEAD transcription factors (Lamar et al., 2012). It is possible that TEADs are also critical for NB metastasis, which is partially supported by the fact that high expression of TEAD4 predicts poor survival in NB patients (data not shown). However, it is also possible that other transcription factors mediate pro-metastatic effects of YAP and TAZ. For example, multiple studies have shown that Wnt signaling transcriptional effector β-catenin promotes NB tumorigenesis (Cantilena et al., 2012; Flahaut et al., 2009; X. Liu et al., 2007; MIddlebeek et al., 2015; Tringali et al., 2012). In addition, Wnt signaling has already been implicated in metastasis in various cancers including breast, lung,
pancreatic, and melanoma (Damsky et al., 2011; W. Liu et al., 2012a; Malladi et al., 2016; Nguyen, Bos, & Massagué, 2009; F.-X. Yu et al., 2012). Thus, it is possible that pro-metastatic function of YAP is dependent on the interactions and crosstalk with Wnt signaling pathway. To study whether pro-metastatic function of YAP/TAZ is dependent on β-catenin/Wnt signaling, I will first determine whether over-expressing β-catenin and/or activating Wnt signaling rescues decreased metastatic phenotype in metastatic NB cells with YAP/TAZ knockdown. Similarly, I will examine whether β-catenin knockdown in metastatic cells can phenocopy YAP/TAZ knockdown. Furthermore, I will perform ChIP-seq experiments to examine whether there are target genes that overlap between YAP/TAZ and β-catenin.

Furthermore, we have identified SPHK1 as a therapeutic target in metastatic NB cells and showed that a SPHK1 inhibitor, SKI II, suppressed NB metastasis in our in vivo preclinical model. To move forward SKI II into human clinical trials, however, there are additional preclinical studies that need to be completed. For example, we need to test the efficacy of SKI II on NB cell lines and tumours from various genetic backgrounds. As mentioned previously, our model was generated with non MYCN-amplified NB cell line. Thus, it would be interesting to test whether SKI II show efficacy towards NB cell lines with different genetic background such as MYCN-amplified and ALK mutated NB cell lines, as well as in PDX models of NB. Increasingly, precision medicine treatment strategies should be tailored to and account for the genetic and biological traits of individual’s tumours. Since SPHK1 is located on chromosome 17q, which is frequently gained in NB, treatment strategy to target SPHK1 may benefit subset of NB patients with 17q amplification.

Lastly, SKI II treatment in vivo resulted in a delay in growth of metastatic tumours and did not cause tumour regression (Figure 3.11g and i). This may be due to multiple pro-survival mechanisms that become activated due to SKI II treatment, which may eventually lead to resistance. One mechanism by which this might occurs is through reactivation of the pathway inhibited by SKI II. For example, we demonstrated that SKI II inhibits STAT3 signaling (Figure 3.12d), which may become reactivated. Indeed, a study by Lee et al. showed that various oncogene-addicted cancers cells become resistant to drugs by reactivating STAT3 signaling through positive feedback loop (H.-J. Lee et al., 2014). Thus, therapeutic strategy to use SKI II in combination with STAT3 inhibitors may result in a more effective inhibition of metastatic NB tumours.
4.2.4 Proteomics Approach to Identify Genes and Pathways Regulating NB Metastasis

We have characterized the metastatic subpopulations in NB cells using gene expression profiling, which led to the identification of genes and pathways that regulate NB metastasis. The transcriptome, however, have limitations in accurately reflecting the proteome, which are the functional workhorse of a cell. For example, certain proteins are activated by phosphorylation, which are not measured by transcriptomic studies. This means that increased RNA expression of those genes does not necessarily indicate increased activity of those proteins. Thus, proteomics/phospho-proteomics analysis on metastatic and primary subpopulations can provide additional novel pathways that regulate NB metastasis. Indeed, proteomics studies have identified key regulators of metastasis in various cancers including lung, gastric, and breast cancers (Bouchal et al., 2015; Cho et al., 2012; Costa-Silva et al., 2015; Dun et al., 2015; Lin et al., 2013). Notably, one group compared proteomics data with the matched transcriptomic data, they found that only 43% of the changes in protein expression corresponded with the mRNA expression (Dun et al., 2015). Similarly to proteomics approach, other ‘omics’ including miRNA array and methylation profiling can be performed to further our knowledge about the NB metastatic subpopulations.

4.2.5 Identification of Therapeutic Targets Using the Connectivity Map

One of the major goal following the development of the metastatic NB model is to identify therapies that can potentially be used clinically to target NB metastases. Although we have identified SPHK1 as a potential therapeutic target, there are many other potential genes from our expression profiling data that needs further investigation. To more systemically examine potential therapeutic vulnerabilities in the metastatic subpopulations using our gene expression data, we exploited the Connectivity Map, which is a collection of transcriptional expression data from human cells treated with genetic and pharmacological agents. This enables researchers to make functional connections between drugs, genes, and diseases. Using this approach, we have identified potential bioactive molecules that are associated with the conversion of metastatic to
primary gene signature. These compounds should be further investigated in cell-based experiments to study their efficacy and to elucidate the mechanism of action.

4.2.6 In Vivo CRISPR Screening to Identify Regulators of NB Metastasis

Gene expression profiling of \textit{in vivo} selected NB cell lines in our model revealed many genes that were significantly differentially regulated between the metastatic and parental cells. To select genes for functional validation, we prioritized our genes based on several factors including 1) fold change in expression 2) ability to predict outcome in NB patients and 3) known roles in invasion and metastasis and 4) novelty in NB. However, it is inefficient to study genes individually for their effects in NB metastasis \textit{in vivo}. To overcome this, we can employ multiplex \textit{in vivo} screening strategy to more efficiently identify functional regulators of NB metastasis. To do this, I will design and make a customized CRISPR knockout library that comprise of sgRNA targeting genes that were significantly up-regulated in the metastatic subpopulation. I will then infect metastatic cells with the customized CRISPR knockout library, select for infected cells, and inject them into mice via intra-cardiac injection. Next-generation sequencing will then be performed to metastatic tumours arising in mice as well as CRISPR library infected cells in culture as a reference point. This will identify sgRNA that have been lost in the metastatic tumours, which will allow us to identify genes critical for NB metastasis. Similar approach can be applied for down-regulated genes in metastatic subpopulation by infecting metastatic cells with over-expression library that comprises down-regulated genes.

4.2.7 Identification of Genes and Pathways Regulating Chemoresistance in Metastatic NB

In addition to enhanced metastatic capability, we have also observed that metastatic subpopulations have increased chemoresistance compared to the parental (\textbf{Figure 3.3f}). Chemoresistance invariably develops following therapy in many different cancers and is one of the major characteristics of metastatic tumours (Acharyya et al., 2012; Ferretti, Bhutkar, McNamara, & Lees, 2016; Fischer et al., 2015; X. Zheng et al., 2015). Thus, understanding the mechanism by which metastatic cells acquire chemoresistance will lead to better therapeutic
strategies to target NB metastases. To study chemoresistance in our NB metastasis model, I will perform a genome-wide CRISPR knockout. Similar to CRISPR screen proposed above to identify regulators of NB metastasis, I will infect both metastatic and parental cells line with genome-wide CRISPR knockout library, select for infected cells, and treat them with a chemotherapeutic of interest. After the treatment, chemotherapy treated and control cells will be harvested and sequenced to detect sgRNAs. Specific set of sgRNAs that are selectively lost in chemotherapy-treated metastatic cells but not in the control or chemotherapy-treated parental cells will identify genes that mediate chemoresistance in the metastatic subpopulations. Furthermore, similar experiments can be performed with a drug library instead of the CRISPR library to identify potential drugs that selectively target metastatic NB.

4.2.8 Improving NB Metastasis Models

Finally, one of the limitations of our model is the fact that it is based on one cell line, SK-N-AS, which lacks MYCN amplification. In MYCN-amplified NB, MYCN regulates vast majority of the transcriptome, and thus, likely drive the biology of NB cells. Thus, our model may only represent non-MYCN amplified tumours and not MYCN amplified tumours, which generally have more aggressive phenotype. To improve and extend our metastatic model, a MYCN amplified NB cell line can be exploited to select subpopulations of cells with enhanced metastatic capability. Furthermore, cells from patient-derived xenograft (PDX) NB models can also be used to increase the genetic diversity of NB metastasis models. This will help us to understand and identify molecular determinants of NB metastasis that are shared by NB cells from different genetic backgrounds as well as those that are more specific to NB tumours with MYCN amplification or other genetic aberrations. This will aid in the development of therapeutic strategies that will more precisely target NB with different genetic backgrounds.
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