Roles of a Putative Tumor Suppressor Gene, *Chc1L*, in Tumorigenesis

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Abstract

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Human chromosome 13q14 has been identified as one of the hotspots of deletion in prostate cancer, multiple myeloma, and chronic lymphocytic leukemia. Chromosome Condensation 1-like (CHC1L) is an uncharacterized gene in this region. CHC1L is found within the smallest common region of loss of heterozygosity in prostate cancer, and its decreased expression is linked to pathogenesis and progression of both prostate cancer and multiple myeloma. In the present study, we have generated Chc1L gene knockout mice and demonstrated that loss of this gene increases tumorigenesis in two year old mice. Knockout and heterozygous mice are predisposed to development of Histiocytic Sarcoma and Histiocyte-Associated Lymphoma. Bone marrow and splenic cells from 8-12 week old knockout mice have elevated viability ex vivo. These data provide the first direct evidence that CHC1L is a tumor suppressor gene involved in suppression of histiocyte-rich neoplasms.
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List of abbreviations

aa: amino acid
AKT: protein kinase B
ARLTS1: ADP-ribosylation factor-like 11
Arf: Alternate Reading Frame tumor suppressor, aka p16
ATP: adenosine triphosphate
BCL: B cell lymphoma
BCL2: B cell lymphoma 2
BOB.1: POU domain, class 2, associating factor 1
Blimp1: B lymphocyte-induced maturation protein 1
BRCA1: Breast Cancer 1, early onset
BRCA2: Breast Cancer 2, early onset
BTB/POZ: Broad Complex, Tram-Trac, Bric-a-Brac/Pox virus and Zinc finger
CCND1: cyclin D1
CD: Cluster of Differentiation
CDR: Commonly deleted region
CEBPβ: CCAAT-enhancer-binding protein β
CHC1L: Chromosome Condensation 1-Like
CLL: Chronic Lymphocytic Leukemia
CML: Chronic Myelogenous Leukemia
COP9: constitutive photomorphogenic homolog 9
COPS4: COP9 signalosome complex subunit 4
CRM1: Chromosome region maintenance protein-1
CSN: COP9 signalosome
CUL: Cullin
DICE1: deleted in cancer cells 1
DLEU1: deleted in lymphocytic leukemia 1
DLEU2: deleted in lymphocytic leukemia 2
DLEU7: deleted in lymphocytic leukemia 7
Dmp1: dentin matrix acidic phosphoprotein 1
DU145: human prostate cancer cell line
E2A: ITF1 Immunoglobulin enhancer binding, aka Transcription factor 3
EBF1: Transcription factor COE1
ECH: ECHIDNA protein
ENU: N-ethyl-N-nitrosourea
ERK: mitogen activated protein kinase
Ex4-F, Ex5-R: Primers for detection of Chc1L transcript
F4/80: EGF-like module-containing mucin-like hormone receptor-like 1
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>FISH</td>
<td>Fluorescent In Situ Hybridization</td>
</tr>
<tr>
<td>FL</td>
<td>Follicular Lymphoma</td>
</tr>
<tr>
<td>Ink4a</td>
<td>Cyclin dependent kinase inhibitor 2A, aka p16</td>
</tr>
<tr>
<td>FOXOA1</td>
<td>Forkhead in human rhabdomyosarcoma A1</td>
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<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
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<tr>
<td>GAP</td>
<td>GTPase activation protein</td>
</tr>
<tr>
<td>GATA1</td>
<td>GATA binding factor 1</td>
</tr>
<tr>
<td>GATA2</td>
<td>GATA binding factor 2</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>H+E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HA tag</td>
<td>human Influenza hemagglutinin</td>
</tr>
<tr>
<td>HAL</td>
<td>Histiocyte-Associated Lymphoma</td>
</tr>
<tr>
<td>HE</td>
<td>Heterozygote</td>
</tr>
<tr>
<td>HECT</td>
<td>Homologous to the E6-AP Carboxyl Terminus</td>
</tr>
<tr>
<td>HeLa</td>
<td>cervical cancer cell line</td>
</tr>
<tr>
<td>HepG2</td>
<td>liver cancer cell line</td>
</tr>
<tr>
<td>HERC</td>
<td>Homologous to the E6-AP Carboxyl Terminus and Regulator of Chromosome Condensation-1 protein</td>
</tr>
<tr>
<td>HS</td>
<td>Histiocytic Sarcoma</td>
</tr>
<tr>
<td>HURP</td>
<td>hepatoma up-regulated protein</td>
</tr>
<tr>
<td>IgK</td>
<td>immunoglobulin κ</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>Int4-F, Int4-R</td>
<td>primers for detection of WT locus</td>
</tr>
<tr>
<td>JH</td>
<td>immunoglobulin heavy chain joining region</td>
</tr>
<tr>
<td>KCNRG</td>
<td>Potassium channel regulator</td>
</tr>
<tr>
<td>Keap-1</td>
<td>Kelch-like ECH-associated protein 1</td>
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<tr>
<td>Ki67</td>
<td>antigen identified by monoclonal antibody Ki-67</td>
</tr>
<tr>
<td>KMS11</td>
<td>myeloma cell line</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LNCaP</td>
<td>prostate cancer cell line</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>Mac-2</td>
<td>Cyclophilin C-associated protein</td>
</tr>
<tr>
<td>MDM2</td>
<td>murine double minute 2</td>
</tr>
<tr>
<td>MDR</td>
<td>Minimally Deleted Region</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>MGUS</td>
<td>monoclonal gammopathy of undetermined significance</td>
</tr>
</tbody>
</table>
miR15/16: microRNA cluster 15a/16-1
MM: Multiple Myeloma
MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MY5: myeloma cell line
Nedd8: Neural precursor cell expressed, developmentally down-regulated 8
Neh2 domain: Nrf2-ECH homology domain
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NLS: nuclear localization signal
NOD-SCID: nonobese diabetic/severe combined immunodeficiency
Nrf2: Nuclear factor (erythroid-derived 2)-like 2
NZB: new Zealand black
Oct2: POU domain, class 2, transcription factor 2
p27: Cyclin-dependent kinase inhibitor 1B
p53: protein 53
Pax5: Paired box protein 5
PC: Prostate Cancer
PC3: prostate cancer cell line
PCLI: plasma cell labeling index
PHF11: Plant homeodomain finger protein 11
PI3K/Akt pathway: phosphoinositide 3-kinase/Protein Kinase B pathway
RanBP1: Ran binding protein 1
RanBP2: Ran binding protein 2
RanGDP: Ran in GDP-bound form
RanGTP: Ran in GTP-bound form
RanGAP: RanGTPase Activating Protein
RanGEF: RanGTP Exchange Factor
RanQ69L: constitutively active Ran mutant
RanT24N: constitutively inactive Ran mutant
RB1: Retinoblastoma-1
RCB1: Regulator of chromosome condensation and BTB domain-containing protein 1
RCC1: Regulator of Chromosome Condensation 1
RFP2: Ret finger protein 2
RING: Really Interesting New Gene
RLD: RCC1-like domain
RNASEH2B: ribonuclease H2, subunit B
RT-PCR: reverse transcription-polymerase chain reaction
SAF: spindle assembly factor
SCDR: Smallest Commonly Deleted Region
SETDB2: Su(var) 3-9, enhancer-of-zeste, trothorax domain-containing protein possessing potential histone H3K9 methyltransferase activity
SMM: smoldering multiple myeloma
TC-F, TC-R: primers for detection of knockout allele

TNF: Tumor Necrosis Factor

TSG: tumor suppressor gene

TPX2: Targeting protein for Xenopus plus end-directed kinesin-like protein

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

U266: myeloma cell line

WHO: World Health Organization

WNT3A: wingless type mouse mammary tumor virus integration site family, member 3A

WT: Wild-type

ZHX-2: Zinc fingers and homeoboxes protein 2
Chapter 1: Introduction

1.1 The burden of cancer

Every year, approximately 12 million people are diagnosed with cancer worldwide (Jemal et al. 2011). Eventually, these 12 million newly diagnosed patients will contribute to the almost 8 million annual cancer-caused deaths, making the disease the second most prevalent cause of death in developing countries, and the leading cause in the developed world (Jemal et al. 2011).

Hematopoietic neoplasms account for approximately 8% of new cancer diagnoses within the developed world (Jemal et al. 2011). They can be divided into three main types: lymphoma, which presents as a solid tumor of lymphocytes typically affecting the lymph nodes; leukemia, a cancer of circulating malignant hematopoietic cells, affecting the blood and bone marrow; Multiple Myeloma, a cancer of the plasma cell, which often aggregate within bone causing deleterious effects on bone marrow hematopoiesis, amongst other symptoms. As with other cancers, hematopoietic neoplasms are treated with various combinations of chemotherapy, radiotherapy, and immunotherapy. Additionally, they are often treated by bone marrow stem cell transplantation, whereby the patient’s malignant cell population as well as his/her body’s normal bone marrow stem cells are destroyed and replaced with the patient’s own hematopoietic stem cells (autologous), or those of an appropriate donor (allogeneic).
Although much progress has been made in understanding cancer and its pathogenesis, many forms remain incurable. This is largely due to heterogeneity between cancers. While virtually any cell in the human body may become neoplastic, even cancers of the same cell type retain a remarkable heterogeneity, and thus effective treatments remain elusive for many cancer subtypes. Part of the reason for this is the variety of genetic lesions that may promote cancer development. Molecular profiling of cancer subtypes is a quickly emerging field dedicated to grouping cancers of a particular tissue type into subgroups that bear similar expression profiles. This has recently been performed in considerable detail for breast cancer (Curtis et al. 2012). The goals of this endeavour are the identification of the affected pathways common to various subgroups that may be therapeutically targetable, and with an improved understanding of the cancer’s molecular biology will also come improved screening approaches, and more accurate prognostic evaluation.

The subgrouping of cancer types relies on the identification of frequently occurring mutations and changes in gene expression of particular genes, which implicates the pathways that these genes act upon in neoplastic development. Once recognized, novel molecular pathways may be targeted therapeutically. For example, the characterization of the Philadelphia Chromosome in 1960 (Nowell and Hungerford 1961) and the fusion protein bcr-abl lead to the discovery of drugs like Gleevec that were able to block the ATP binding site (Takimoto CH 2008) of the constitutively active tyrosine kinase, greatly improving the odds of survival for patients suffering from Chronic Myelogenous Leukemia (CML).

The laboratory mouse is a powerful model organism for the validation of gene function as it relates to cancer pathogenesis. Originally, mice were used to study cancer through examination
of tumor xenograft and carcinogenesis models. However, the establishment of the first transgenic mouse lines reiterating activating mutations in proto-oncogenes (Brinster et al. 1984; Stewart et al. 1984), which were eventually complemented by gene targeting technology to knock out tumor suppressor genes (Donehower et al. 1992; Jacks et al. 1992), allowed a reversal in how genes involved in cancer were studied: Mutations frequently observed in human cancer through the conventional forward genetics approach could be mirrored in an animal model, validating the proneoplastic effects of the mutation if the genetically engineered strain developed cancer. Changes in expression levels of other genes can be measured using microarray chips to identify downstream effector pathways leading to neoplastic progression. Additionally, if the cancer pathogenesis in a genetically engineered model closely recapitulates the human form of the disease, the mouse model may be used for detailed analysis of tumor progression, as well as screening of therapeutic compounds.

1.2 Chromosome 13q14 harbors putative tumor suppressor genes

1.2.1 13q14 deletions in CLL, MM and PC

Chronic Lymphocytic Leukemia:

Chronic lymphocytic leukemia (CLL) is a highly heterogenous B cell neoplasm comprising many genomic profiles and clinical courses (Chiorazzi et al. 2005). It is the most common adult leukemia is the Western world (Dohner et al. 2000).
The most common genomic lesions are deletions within chromosome arms 13q, 11q, 17p and 7q, as well as trisomy of chromosome 12, which collectively occur in approximately 80% of patients (Dohner et al. 2000). Deletions affecting the 13q arm are highly heterogeneous, but the most common is deletion of region 13q14, affecting a single allele in approximately 70% of cases, while biallelic deletions occur in 19% of CLL patients (Dewald et al. 2003). Patients with a large percentage of 13q-deleted nuclei and/or large deletions that include the \( RB1 \) locus have a shorter time-to-treatment, and often require an aggressive clinical course (Dal Bo et al. 2011).

Despite these associations, as the sole abnormality, monoallelic 13q14 deletion as determined by fluorescent in situ hybridization (FISH) remains associated with a good prognosis compared to CLL with no cytogenetic abnormalities (Chena et al. 2008).

**Multiple Myeloma:**

Multiple myeloma (MM) is presently an incurable hematopoietic neoplasm of plasma cells. It is characterized by several common genetic changes, including deletion of 13q14 (40-50% of patients) (Fonseca et al. 2004), amplification of 1q21 (~40% of patients) (Hanamura et al. 2006), deletion of 17p13 (~20% of patients) (Fonseca et al. 2003), and IgH translocations (Fonseca et al. 2009).

Del(13q14) is strongly associated with an elevated plasma cell labelling index (PCLI) (Hose et al. 2011; Li et al. 2011), which is itself an important independent prognostic factor in newly diagnosed MM. Additionally, the proportion of plasma cells carrying deletion within 13q is observed to increase as the disease progresses from Monoclonal Gammopathy of Undetermined Significance to Smoldering Multiple Myeloma, and finally to MM (Lopez-Corral
Del(13q14) remains a negative predictor of remission (Schilling et al. 2008). 13q14 loss has also been correlated with increased bone marrow microcirculation, and this should be taken into account when considering therapies with antiangiogenic activity (Schreiber et al. 2000; Hillengass et al. 2008). However, the prognostic implications of 13q deletions remain to be fully elucidated in MM. Originally thought to imply a worsened prognosis (Zojer et al. 2000), 13q14 deletions remain associated with shortened overall and event-free survival by univariate analysis, which considers a single variable in relation to prognosis (Avet-Loiseau et al. 2007). However, as 13q14 deletion often co-occur with other mutations, when multiple genetic lesions are considered and related to prognosis (multivariate analysis), only del(17p) and t(4;14) are independent genetic prognostic indicators (Avet-Loiseau et al. 2007).

Prostate Cancer:

Prostate cancer (PC) is the most commonly diagnosed men’s cancer in the developed world, accounting for an estimated 29% of newly diagnosed cases and 11% of cancer-related deaths in 2011 (Siegel et al. 2011). The molecular etiology of this disease is unknown, however several genomic changes are well recorded: common losses occur with chromosome arms 6q, 8p, 10q, 13q, 16q, and 18q and gains frequently occur at 1q, 2p, 7, 8q, 18q, and Xq (Nupponen and Visakorpi 2000).

Deletions in the q arm of chromosome 13 have long been known to occur in human PC. 13q deletions in PC are associated with advanced stages (Afonso et al. 1999; Yin et al. 1999; Lu et al. 2006). Approximately one third of cases demonstrate loss of heterozygosity (LOH) at 13q14 (Cooney et al. 1996). Loss of this region is particularly responsible for the clinical
significance of advanced prostate cancers compared to other regions of 13q (Dong et al. 2001; Misumi et al. 2010), and for early biochemical relapse (Brookman-Amishah et al. 2007).

1.2.2 Minimally deleted regions at 13q14

The high frequency of 13q14 deletions in these cancers suggests the existence of one or more tumor suppressor genes within this region. As the affected region is highly heterogeneous between patients, identification of the commonly affected chromosomal location would point to genes that are being preferentially targeted for deletion due to their cancer inhibiting function.

Chronic Lymphocytic Leukemia:

Ouillette et al. have defined two general types of 13q14 deletion (Ouillette et al. 2008) (Figure 1). This grouping of deletion-types is supported by other data indicating two molecularly distinct groups of deletion (Mosca et al. 2010). Type 1 deletions comprise 60% of all 13q14 deletions. Type 1a deletions encompass a minimally deleted region (MDR) that overlaps with the miR15a/16-1 cluster within the DLEU2 gene, plus DLEU1. Type 1b deletions encompass the same region as Type 1a, but the centromeric breakpoint may occur anywhere up to, but excluding, RB1. Type 2 deletions, making up the remaining 40% of cases, are the largest of 13q14 deletions, and do involve loss of RB1. They possess the same telomeric breakpoints as Type 1, occurring at 51.2-51.5 Mbp physical position, however breaks extending closer to the telomere are documented (Parker et al. 2011).
Multiple Myeloma:

Mapping of 13q deletions in MM found that D13S272 and D13S31, microsatellite markers within the 13q14 region, are the most commonly deleted markers of the 13q arm, deleted in 70% and 64% of cases, respectively (Shaughnessy et al. 2000). This indicated the presence of a putative tumor suppressor gene within the vicinity of 13q14.2-.3. Further, these deletions are homozygous in 12% of patients (Shaughnessy et al. 2000), while LOH has been reported in 26% of heterozygous patients (Ahmed et al. 2003).

The region affected by this deletion has not been mapped in as great detail as in CLL. As with CLL, there is great heterogeneity in long arm chromosome 13 deletions in MM, but it was initially very difficult to study because cytogenetic analysis originally required the analysis of metaphase spreads, which are less commonly found in MM (Chang et al. 1999). However, with the development of FISH, which allows for analysis of both metaphase and interphase nuclei (Haines 1995), the MDR has been mapped to a 350kb sequence overlapping with DLEU1, DLEU2, and RFP2, among other genes not shown (Elnenaei et al. 2003) (Figure 1).

Prostate Cancer:

A large-scale genetic linkage analysis of over 1,200 families suggested one or more susceptibility loci for Familial Prostate Cancer at 13q14 (Xu et al. 2005). 13q14 deletion is the region of 13q most commonly affected in PC (Afonso et al. 1999; Latil et al. 1999; Ueda et al. 1999; Yin et al. 1999), within which the most commonly deleted region occurs between markers D13S153 and D13S273 (Ueda et al. 1999). As D13S153 is located within the RB1 locus, this heavily implicated its activity as a tumor suppressor gene in PC, which was not surprising due to
its well-known involvement in cell cycle regulation. However, these allelic imbalances did not correlate with low levels of $RB1$ expression, indicating the existence of another tumor suppressor gene in the region (Cooney et al. 1996; Li et al. 1998; Latil et al. 1999). Further mapping indicated that the candidate tumor suppressor was located telomeric to $RB1$ (Afonso et al. 1999; Yin et al. 1999). High resolution mapping of 13q14 LOH events in PC eventually revealed a MDR at 13q14.2, overlapping with $CHCIL$ (Latil et al. 2002) (Figure 1).
In PC, the MDR of LOH overlaps with the CHC1L locus (Latil et al. 2002). The two main types of 13q14 deletion in CLL are shown (Ouillette et al. 2008). In MM, a 350 kb MDR extends from RFP2 to within the DLEU2 locus (Elnenaei et al. 2003).
1.2.3 Putative tumor suppressors at 13q14

miR15a/16-1:

Existing within introns of DLEU2, a gene that does not demonstrate tumor suppressor activity (Bullrich et al. 2001; Migliazza et al. 2001; Calin et al. 2002; Mertens et al. 2002), the miR15a/16-1 (hereafter referred to as miR15/16) cluster is expressed at high levels in normal B cells, suggesting the importance of these miRNAs in B cell homeostasis (Calin et al. 2002). A study by Ouillete et al. identified only 2 of 171 CLL cases with 13q14 deletions shorter than Type 1a, implying a MDR spanning miR15/16 and DLEU2, supportive of findings from several other projects (Bouyge-Moreau et al. 1997; Kalachikov et al. 1997; Kapanadze et al. 2000; Kitamura et al. 2000; Migliazza et al. 2001; Wolf et al. 2001; Calin et al. 2002; Hammarsund et al. 2004; Pfeifer et al. 2007). Further investigation has shown that the miRNAs are downregulated or deleted in most CLL cases (Calin et al. 2002; Calin and Croce 2006; Nicoloso et al. 2007) as well as in other cancers (Bottoni et al. 2005; Bonci et al. 2008). Indeed, in CLL, several other genes in the area of miR15/16 were unaffected by 13q14 deletion (Bullrich et al. 2001; Migliazza et al. 2001; Pekarsky et al. 2005). Interestingly, these miRNAs are underexpressed in a transgenic murine model of CLL (Pekarsky et al. 2005). NZB mice, which are predisposed to development of CLL, possess a point mutation in the miR15/16 locus, and these miRNAs are expressed at low levels in NZB lymphoid tissues (Raveche et al. 2007). Perhaps the strongest demonstration of the tumor suppressing effects of miR15/16 is that conditional deletion of miR15/16 in mouse B cells is sufficient to cause development of CLL (Klein et al. 2010).
In MM, miR15/16 have been shown to functionally regulate proliferation of MM cells both in vitro and in vivo through inhibition of members of the Akt and MAPK pathways (Roccaro et al. 2009). MiR15/16 levels can be affected by the bone marrow microenvironment, where bone marrow stromal cells cause decreased expression of this microRNA cluster through IL-6 signalling (Hao et al. 2011). Suppression of miR15/16 in MM cells results in increased drug resistance, while ectopic expression causes G1/S checkpoint arrest (Hao et al. 2011).

In advanced prostate tumors, miR15/16 are significantly underexpressed, whereas the levels of their targets are abnormally high (Bonci et al. 2008). Delivery of antagonomirs targeting miR15/16 to normal mouse prostates resulted in hyperplasia, and in vitro knockdown increased survival, proliferation and invasiveness of healthy prostate cells, which became neoplastic in NOD-SCID mice (Bonci et al. 2008). Ectopic expression of miR15/16 resulted in arrest of growth, induction of apoptosis, and regression in a prostate cancer xenograft model (Bonci et al. 2008).

The mechanism of CLL pathogenesis beginning with 13q14 deletion had remained elusive until the miR15/16 cluster was identified as a likely contributor in the tumor suppressing effects of this region. Analysis into potential targets revealed a well-known oncoprotein, BCL2, as a target (Cimmino et al. 2005). BCL2 is an oncogene frequently overexpressed in CLL (Kitada et al. 1998). In a small fraction (<5%) of CLL cases, this overexpression is achieved by a translocation placing BCL2 under the IgH enhancer (Adachi et al. 1990), but, as miR15 and 16 have both been shown to target BCL2, miR15/16 deletion may also contribute to BCL2 overexpression.
Fabbri et al. recently found evidence of a feedback loop, whereby TP53 binds upstream of miR15/16 and activates their expression, decreasing BCL2 levels (Fabbri et al. 2011). They also found that miR15/16 represses TP53, and so miR15/16 and TP53 co-regulate through mutual repression. TP53 binds upstream of another miRNA cluster, miR34b/34c, activating its expression (Corney et al. 2007). This cluster is found at chromosomal location 11q, another region deleted in CLL. MiR34b/34c targets ZAP70, a protein overexpressed in aggressive CLL.

Fabbri et al. proposed a mechanism whereby, in cancer-free conditions without loss of 13q14, miR15/16 are expressed normally, and BCL2 is kept at normal levels, so apoptosis will occur as expected, while miR15/16 and TP53 co-regulate. TP53 transactivates miR34b/34c, which downregulate their target ZAP70, keeping it at normal levels and preventing development of aggressive CLL.

However, in indolent CLL where 13q14 is deleted and miR15/16 are lost, levels of BCL2 elevate, allowing evasion of apoptosis. Loss of miR15/16 also releases TP53 from repression. This overactivates the miR34 pathway, causing decreased expression of ZAP70, and reduction of its effector pathways. By keeping levels of ZAP70 and apoptosis low, the CLL remains indolent yet has a capacity to proliferate if there are subsequent mutations, such as deletion of 11q (containing miR34b/34c) or 17p (containing TP53). Once either of these deletions occur, ZAP70 will be overexpressed. Indeed, del(11q) and del(17p) are found in 18% and 7% of cases, respectively, and are associated with aggressive CLL (Dohner et al. 2000; Dewald et al. 2003).
KCNRG and RFP2:

Despite the wealth of information suggesting the role of the miR15a/16-1 cluster as the elusive tumor suppressors existing within 13q14, there is evidence for other important genes lost in this common deletion, the roles of which remain to be uncovered. *KCNRG* and *RFP2* are both found within the MDR of MM and are affected in Type 1b and Type 2 deletions in CLL. *In vitro* models have shown the capacity of *KCNRG* to suppress division and to promote apoptosis (Birerdinc et al. 2010). *RFP2* has recently demonstrated the ability to enhance apoptosis via ubiquitin ligase activity, which causes degradation of MDM2 and AKT, resulting in enhanced p53 signalling (Joo et al. 2011).

DICE1, FOXOA1, and ARLTS1:

Several genes located at 13q14, but outside of the MDR, may be PC tumor suppressors. Low expression of *DICE1* is found in PC cell lines DU145 and LNCaP as a result of promoter hypermethylation, and the same hypermethylation has been found in patient tumors (Ropke et al. 2005). *FOXOA1* is deleted in approximately one third of prostate cancers (Dong et al. 2006). Ectopic expression inhibited cell survival and proliferation, inhibiting androgen- and androgen receptor-mediated gene regulation (Dong et al. 2006). As a PI3K/Akt signalling pathway inhibitor (Biggs et al. 1999), the activity of *FOXOA1* as a tumor suppressor is consistent with its known functions. An *ARLTS1* variant (Cys148Arg) has been associated with prostate cancer and breast cancer (Siltanen et al. 2008). In a larger study, it was confirmed that the variant had a higher incidence in PC patients, and was associated with an increased risk of diagnosis and of cancer aggressiveness (Siltanen et al. 2011). The variant was accompanied by lowered
expression of the gene, and significantly lowered expression of ARLTS1 was found in clinical samples as well as in previously published microarray data (Siltanen et al. 2011).

**Other genes with tumor suppressive activity:**

The heterogeneity in the size of 13q14 deletions due to the existence of many breakpoints clustering in this region implicates many different genes as putative tumor suppressors. Parker et al. have found that 15 genes are typically deleted in Type 1b CLL deletions, 14 of which are located within a 1Mb region (49.2-50.2Mb physical position) between miR15/16 and RB1 (Parker et al. 2011), which includes SETDB2, PHF11 and RCBTB1. Interestingly, RCBTB1 is a paralogue of CHC1L, and the two have been shown to heterodimerize (Plafker et al. 2009).

There is evidence that genes telomeric to miR15/16 may play roles in CLL as well. A mouse model with B cell-specific DLEU2-miR15/16 deletion has been used for validation of the causative role of the MDR, showing that it is sufficient to induce CLL pathogenesis (Klein et al. 2010). More recently, the same group has generated a strain to reiterate larger 13q14 deletions that extend telomerically, named the commonly deleted region (CDR) (Lia et al. 2012). Mice with heterozygous deletion of the CDR have a similar penetrance of lymphoproliferations compared to +/- MDR mice, however the impact of the larger deletion was toward a more aggressive disease course (Lia et al. 2012). Three genes of interest reside in this region: DLEU1, DLEU7, and RNASEH2B.

Expression of DLEU7 (51.285Mb position) is frequently lost or reduced in CLL as a result of promoter methylation (Hammarsund et al. 2004; Palamarchuk et al. 2010) or deletion (Ouillette et al. 2008). Overaction of the NF-κB pathway as a cause of CLL has been described
using transgenic mice (Planelles et al. 2004). *DLEU7* has been shown to inhibit the NF-κB pathway by inhibiting Tumor Necrosis Factor (TNF) receptors (Palamarchuk et al. 2010). The authors of this pathway propose a mechanism for CLL development whereby minimal deletion of 13q14, abrogating miR15/16 and *DLEU7* expression, leads to overactivation of BCL2 and the NF-κB pathway, which is consistent with a double transgenic mouse model which overexpresses *Bcl2* and a TNF receptor-associated factor, causing development of B cell lymphoma which leads to leukemia (Zapata et al. 2004).

RNASEH2B is a subunit of RNase H, which coordinates hydrolysis of RNA in DNA:RNA hybrids formed during normal cellular processes, as well as removing misincorporated ribonucleotides during DNA synthesis, thus maintaining genomic integrity (Reijns et al. 2011). *DLEU1* expresses a noncoding RNA with over 20 different splice variants (Wolf et al. 2001), however no function has been proposed. No role in preventing tumorigenesis has been demonstrated for either, although it is conceivable for RNASEH2B to play a tumor suppressive role based on its function.

**Chromosome Condensation 1-Like:**

Mapping of LOH events in PC puts *CHC1L* inside the MDR. *CHC1L* expression was decreased at least 2-fold in 58% of all tumors studied, as well as in the three prostate cancer cell lines LNCaP, DU145, and PC3, compared to normal prostate epithelial cell lines (Latil et al. 2002). When considering only tumors with LOH at 13q14, *CHC1L* is significantly down-regulated in 78% of tumors (Latil et al. 2002). Only *CHC1L* and three other genes appear to be targets of 13q14 deletion in PC based on altered expression patterns (Latil et al. 2003).
Importantly, although $RB1$ is also located adjacent to the MDR, its expression levels do not correlate with LOH at 13q14, suggesting it is not a critical component of 13q14 deletion for prostate tumorigenesis (Cooney et al. 1996; Li et al. 1998; Latil et al. 1999; Latil et al. 2002).

Although $CHC1L$ lies outside of the MDR in MM, low expression levels of $CHC1L$ are frequently observed in MM patients (Legartova et al. 2010). Through comparative gene expression profiling of CD138-purified cells from newly diagnosed myeloma patients vs healthy controls, expression levels of three genes were simultaneously significant in determining prognosis (Harousseau et al. 2004). Patients with high $RAN$ expression had increased risk of event, while patients with high $ZHX-2$ or high $CHC1L$ had lowered risk. Since expression of each gene has independent prognostic significance, the collective expression of the three genes was shown to be a strong predictor of event-free survival. The coefficient of determination ($R^2$) using the three gene model was 66%, compared to the 30% $R^2$ of clinical models used at the time the study was published (Harousseau et al. 2004).

Collectively, this data indicates that multiple tumor suppressor genes reside within 13q14, with evidence suggesting membership of $CHC1L$ in this group.

1.3 Chromosome Condensation 1-like: Structure and putative function

1.3.1 CHC1L: Gene structure, mRNA isoforms, and protein product

Chromosome Condensation 1-Like, $CHC1L$, is located within 13q14 (Devildier et al. 1998), and is a candidate tumor suppressor gene in B cell chronic lymphocytic leukemia
(Ouillette et al. 2008), multiple myeloma (Schreiber et al. 2000), and prostate cancer (Misumi et al. 2010). The human gene is 30 kb in length, and contains 14 exons, ubiquitously expressing 4 alternatively spliced mRNA isoforms of approximately 3 kb (Figure 2). Isoforms A and B possess exon 3, which contains an ATG start codon at position 229, suggesting a protein 551 amino acids in length. mRNAs C and D, lacking exon 3, have a first in frame start codon at nucleotide position 301, within exon 4 (Devilder et al. 1998). This is consistent with a protein product of 526 amino acids for both C and D isoforms.

Figure 2 Human CHC1L transcript isoforms

CHC1L encodes four mRNA isoforms, producing protein isoforms of 551 and 526 amino acids. mRNA isoform A contains all exons, and initiates translation of the 551aa protein from a start codon in exon 3. Isoform B also initiates translation from exon 3, but exon 2 is spliced out. mRNA isoforms C and D both lack exon 3, and produce the 526aa protein from a start codon in exon 4. Isoform C possesses exon 2, whereas this exon is spliced out of isoform D.
Recently, tissue-specific expression of two classes of mRNA isoforms was studied in detail in the mouse (Wang et al. 2012). Expression of the long isoform was found in the testes, while the short isoform was expressed in all other tissues examined (heart, brain, spleen, lung, liver, kidney, ovary and seminal vesicle), with the exception of smooth muscle. Protein expression was found in the heart and testes only, revealing a protein of 61kDa, with cross-reaction at 25kDa in the brain, and 50kDa in the liver. GFP-tagged CHC1L localizes to vesicles at the surface of the nuclear envelope, indicating that it may be in close association with Ran, which traverses the nuclear envelope regularly.

The identifying characteristics of the human CHC1L protein are the presence of 6 RCC1-like (Regulator of Chromosome Condensation 1-like) repeats on the N terminus, and two BTB/POZ (Broad Complex TramTrac Bric-a-Brac/Pox virus and Zinc finger) domains toward the C terminal end (Devilder et al. 1998; Solomou et al. 2003) (Figure 3).

**Figure 3 Human CHC1L protein structure**
The 551aa isoform of CHC1L possesses 6 RCC1-like repeats on its N-terminal, and may be involved in interaction with Ran. There are two BTB/POZ domains toward the C-terminal end. BTB domains are often involved in protein-protein interactions.
1.3.2 Function of CHC1L RCC1-like domain

**Putative Guanine Nucleotide Exchange Factor activity**

The RCC1-like domains (RLDs) are 52-53 amino acid domains, and show significant homology to the domains of the RCC1 protein, which are responsible for interaction with Ran (Renault et al. 1998). RCC1 is a well-known regulator of RanGTPase. Ran is responsible for nuclear protein import as well as spindle assembly, nuclear envelope dynamics, and control of cell cycle transitions (Figures 4 and 5) (Clarke and Zhang 2008). RCC1 is the main Ran Guanine Nucleotide Exchange Factor (RanGEF), catalyzing the exchange of GDP for GTP (Clarke and Zhang 2008). RanGTPase Activating Protein (RanGAP) is responsible for activating Ran’s intrinsic GTPase activity, thus hydrolyzing GTP, rendering Ran inactive. RanGAP is located cytoplasmically, whereas chromatin-bound RCC1 is within the nucleus, maintaining concentrations of Ran bound to GTP (RanGTP) within the nucleus and bound to GDP (RanGDP) within the cytosol (Solomou et al. 2003). This sequestering of Ran in its two states is an integral aspect of its ability to import and export proteins with nuclear localization and nuclear export signals, respectively. This is achieved through interaction of RanGTP with importins carrying proteins destined for the nucleus, causing dissociation of the cargo. Interaction with chromosome-region maintenance protein-1 (CRM1) causes RanGTP to form export complexes carrying proteins with nuclear export sequences, which dissociate upon hydrolysis of GTP in the presence of cytoplasmic RanGAP (Clarke and Zhang 2008). During mitosis, the nuclear membrane dissolves, and the discrete sequestering of RanGTP from RanGDP is lost (Clarke and Zhang 2008). A concentration gradient is formed, with the GTP-bound form at highest
concentration proximal to the chromosomes due to RCC1’s association with chromatin. This causes release of spindle assembly factors, proteins with nuclear localization signals, from their carrier importin proteins, allowing their association and proper formation of the spindles close to the chromosomes.
The Ran cycle and its role in nucleocytoplasmic transport

a. The RanGTP-GDP cycle. In the presence of RCC1, GDP is exchanged for GTP, and Ran is able to interact with Karyopherin, a transport factor of the importin-β superfamily. Ran’s intrinsic GTPase activity is activated by RanGAP1 and RanBP1 or 2, hydrolyzing GTP to GDP.

b. Ran’s function in nucleocytoplasmic transport. In the presence of RCC1, which is located in the nucleus, Ran is bound to GTP. This causes Ran to associate with importin-β, causing release of proteins containing a nuclear localization signal (NLS). Similarly, Ran bound to GTP interacts with chromosome region maintenance protein-1 (CRM1), promoting the assembly of export complexes that carry proteins with nuclear export signals into the cytosol. Within the cytosol, associated with the nucleopore, RanGTP is hydrolyzed by RanGAP1 in the presence of RanBP1 or 2, resulting in hydrolysis of GTP and dissociation of the export complex. RanGDP re-enters the nucleus and the cycle continues.
Figure 5 Role of RanGTPase during mitosis

During mitosis, the nuclear envelope dissolves, and the discrete partitioning of RanGTP from RanGDP is lost. Since RCC1 is chromatin-bound, a gradient of RanGTP is created. Since RanGTP concentration is highest near the chromosomes, spindle assembly factors (SAFs) may be released from importin shuttling proteins, resulting in spindle formation near the chromosomes.
There are 5 groups of proteins containing RLDs, and only in the case of RCC1 has RanGEF activity been shown (Hadjebi et al. 2008). The amino acid residues necessary for forming the seven-bladed propeller structure of an RCC1-repeat are highly conserved across RCC1-like family members, but there is room for specific variation within each individual RLD. For example, RCC1 itself contains an extra β wedge between the third and fourth β strands of propeller blade three (Hadjebi et al. 2008). This wedge is critical for destabilizing the interaction between Ran and GDP (Renault et al. 1998), allowing GTP to be incorporated, and conferring GEF activity to RCC1, and is not found in other RLDs (Hadjebi et al. 2008). Family member-specific structural motifs, analogous to RCC1’s β wedge, likely confer specific functions to each, and so a RanGEF activity may not be present in all proteins containing RLDs since they do not possess this β wedge.

The other subgroups of the RCC1 family have varying functions. The RLDs of CHC1L share significant homology with those of HERC1+3 (Devilder et al. 1998), members of the HERC subgroup of the RCC1-like protein family. The HERC subgroup has the common function of acting as ubiquitin ligases (Hadjebi et al. 2008), targeting various proteins for degradation. This function is achieved through the HECT domains on the C terminal of these proteins, which catalyze formation of a thioester bond with ubiquitin before transferring it to a target substrate (Hochrainer et al. 2005).

**Putative E3 ligase substrate adaptor activity**

Through proteome-scale studies of the human protein-protein interactome using high throughput yeast two-hybrid technology, it was shown that CHC1L can interact with COPS4.
COPS4 is the fourth subunit of the COP9 signalosome (CSN). The CSN is a highly conserved regulator of cullin E3 ubiquitin ligases, the largest family of E3 proteins (Lyapina et al. 2001). E3 ligases are the final enzymes in a coordinated cascade required for polyubiquitylating a target molecule. Ubiquitin is first linked to the E1 enzyme, which then transfers the ubiquitin moiety to E2, which interacts with E3, bringing it into proximity of a target molecule, and subsequently transferring multiple ubiquitin moieties onto the target through action of E3 ligase. This process results in degradation of the target by the 26S proteasome, and is key for regulating cell cycle progression (Pines 2006), as well as DNA repair and gene expression (Wolf et al. 2003). The CSN possesses Nedd8 isopeptidase activity (Cope et al. 2002), which removes Nedd8 from the cullin subunit of the cullin-RING family of E3 ubiquitin ligases (Schwechheimer et al. 2001). The nature of CSN regulation of E3 is somewhat paradoxical: neddylation increases the recruitment of E2 ligases and promotes ubiquitin transfer to the target molecule (Saha and Deshaies 2008). However, this increased activity can also lead to auto-ubiquitination, leading to self-destruction (Enchev et al. 2010). Therefore, while deneddylation by CSN decreases ubiquitin-transfer to substrates destined for proteasomal degradation, it also serves to protect the CSN complex from self-destruction, thereby serving as a positive regulator of E3 ligases in vivo (Schwechheimer et al. 2001; Wolf et al. 2003).

CHC1L possesses two BTB/POZ domains close to its C terminus. Cullins (CUL), the catalytic cores of E3 ligases, are able to act on target molecules through intermediate substrate adaptor proteins (Willems et al. 2004). The common characteristic of CUL3, one of 8 types of cullins found in humans, is that its substrate adaptors possess a BTB domain (Xu et al. 2003). The BTB domain interacts with CUL3, and the other end of the protein often contains a protein-protein interaction motif, specifying target molecules (Plafker et al. 2009).
RCBTB1, the parologue of CHC1L whose locus is also found at 13q14.3, associates with CUL3 \textit{in vitro} through interaction of its BTB domain with the substrate adaptor binding domain of CUL3 (Plafker \textit{et al.} 2009), providing evidence for its activity as an E3 ligase substrate adaptor. Through yeast two-hybrid analysis, protein pulldown, and co-localization studies, this group has also shown that RCBTB1 and CHC1L can homo- and heterodimerize (Plafker \textit{et al.} 2009), a characteristic common to CUL3 substrate adaptors (McMahon \textit{et al.} 2006). This dimerization may act to specify targets for degradation, however none have yet been identified.

Based on its homology to and capacity to interact with RCBTB1, as well its association with the CSN, there is good evidence to suggest that CHC1L acts as a substrate adaptor protein for E3 ubiquitin ligases, specifying other proteins for degradation, thereby regulating molecular processes within the cell. However, if it is acting as an E3 adaptor, its overall function depends on which proteins it is silencing.

Keap-1 is a BTB domain-containing CUL3 substrate adaptor, responsible for regulating Nrf2, a transcription factor that promotes survival following oxidative stress (Cullinan \textit{et al.} 2004). Through its N terminal BTB domain, Keap-1 has been shown to homodimerize and interact with CUL3 (Zipper and Mulcahy 2002). Once dimerized, the C terminal Kelch domain binds to the Neh2 domain of Nrf2 (Li \textit{et al.} 2004), causing it to become tethered to the E3 complex, both sequestering it in the cytosol away from the nucleus, and leading to its degradation (Li \textit{et al.} 2004). Keap-1’s target specificity is thus determined by its other protein interaction domain. Therefore, it is reasonable to propose that the substrate specifying capability of BTB domain-containing proteins rests in the other structural motifs of the adaptor. Since the β wedge of the RCC1 repeats found on the original RCC1 protein is necessary for exchanging
GDP for GTP (Renault et al. 1998), and it is not found on other RCC1-like domains (Hadjebi et al. 2008), it is possible that the RLD repeats on the N terminal of CHC1L are only able to interact with Ran without causing guanine nucleotide exchange, and therefore CHC1L may regulate Ran activity not by acting as a GEF, but by targeting it for degradation.

Whether CHC1L acts as a GEF for RanGTPase or specifies it for ubiquitin-mediated degradation, if it does indeed interact with Ran, has important implications. As a GEF, loss of CHC1L would decrease levels of activated Ran. As a ubiquitin ligase adaptor protein targeting Ran, it would increase total levels of Ran. Since RanGDP is functionally inert, the functional effect of excess Ran would arguably be an increase in RanGTP.

1.3.3 RanGTPase and cancer

Ran is widely overexpressed in human cancer (Xia et al. 2008), and its overexpression is associated with poor prognosis in ovarian cancer (Ouellet et al. 2005; Ouellet et al. 2006), breast cancer (Papaconstantinou et al. 2006), and multiple myeloma (Legartova et al. 2010). Data suggests that RanGTP-triggered pathways are exploited by cancer cells. Several of its downstream effectors are differentially expressed in cancer: the kinase Aurora A (Giet et al. 2005), and a microtubule-associated protein HURP (Koffa et al. 2006), for example. Its silencing in cultured tumor cells results in dramatic defects in mitotic spindle assembly and apoptosis (Xia et al. 2008). One of its effectors, survivin, a negative regulator of apoptosis (Altieri 2006), is depleted following Ran knockdown. This downregulation of survivin is required for Ran knockdown-induced apoptosis (Xia et al. 2008). In comparison, Ran knockdown is well-tolerated in normal cells (Xia et al. 2008). This may indicate a cancer cell dependence on Ran-
directed cell division. The concept of oncogene addiction, in which cancer cells, but not normal
cells, have a major reliance on a specific growth-promoting pathway (Weinstein and Joe 2006)
has also been seen for other proteins, particularly cell division kinases (Landis et al. 2006; Liu et al. 2006; Xia et al. 2008). It is not presently known why overactivation of and dependence on the
Ran pathway would promote cellular transformation, but it may be related to its role in
chromosome segregation during mitosis, in which deregulation may promote chromosomal
instability (Xia et al. 2008). Indeed, the Ran targets TPX2 and Aurora A have been identified in
a chromosomal instability gene signature associated with poor prognosis in multiple cancer types
(Carter et al. 2006). Additionally, it has been suggested that altered localization of tumor
suppressors and oncoproteins due to faulty nucleocytoplasmic transport may promote
tumorigenesis (Kau et al. 2004). Commonly overactive cell signaling pathways such as the
PI3K/Akt and Ras/MEK/ERK pathways exert their effects by altering subcellular localization of
transcription factors (Kau and Silver 2003; Grant 2008). Ran activity has been shown to be
activated by the PI3K/Akt and growth factor signalling pathways (Ly et al. 2010). Tumor cells
with a highly overactive PI3K/Akt signalling pathway are particularly susceptible to apoptosis
through Ran silencing (Yuen et al. 2012), suggesting Ran-targeted therapy as a potentially
effective course of treatment.
1.4 Histiocytic Sarcoma

As our mouse gene knockout study of Chc1L demonstrated that knockout mice have a higher incidence of Histiocytic Sarcoma (HS), I would like to give a detailed background introduction to HS.

The term histiocyte has undergone many transformations, and is currently used to describe cells of the monocyte/macrophage lineage and well as those of the Langerhans cell/dendritic cell series (Cline 1994). HS is a rare and poorly understood hematopoietic neoplasm, representing <1% of all non-Hodgkin’s lymphomas (Jaffe ES 2001). It affects adult men and women equally, with average age at diagnosis between 46 and 55 years (Pileri et al. 2002; Hornick et al. 2004), and more rarely affecting children (Buonocore et al. 2005; Kumar et al. 2011; Mainardi et al. 2011). It may present as a localized disease in the lymph nodes, skin and intestinal tract, or may be disseminated to multiple organs (Weiss LM 2001; Pileri et al. 2002). Historically, it has been a difficult cancer to recognize due to inconsistencies in terminology and diagnostic criteria (Vos et al. 2005).

As knowledge of cellular differentiation markers improved, many tumors originally diagnosed as HS turned out to be a spectrum of neoplasms including B- and T-cell lymphomas (Morris and Davey 1975; Isaacson et al. 1985; Stein et al. 1985; van der Valk et al. 1990; Arai et al. 1993; Egeler et al. 1995), but particularly diffuse large B cell lymphomas (Jaffe ES 2001). After this elucidation, in 2001, the World Health Organization (WHO) declared the requirements for diagnosis of HS: the presence of immunophenotypic characteristics of the histiocyte lineage and the absence of markers found on cells of other large cell malignancies such as lymphomas (Jaffe ES 2001). However, the distinction between HS and lymphoma broke down as increasing
evidence of lymphocyte plasticity became apparent. In one of the first studies on the topic, a study of 8 patients with both HS and Follicular Lymphoma (FL), Feldman et al. (Feldman et al. 2008) provided evidence for trans- or de-differentiation from FL into HS. Using Fluorescent In Situ Hybridization (FISH), the group identified the presence of t(14:18), the genetic hallmark of FL, within the histiocytic tumors of 6 of the 8 patients (FISH was not possible in the other two patients). The two patients in which FISH could not be successfully performed had other characteristics of FL present in both the HS and FL tumors; BCL2/JH and immunoglobulin rearrangements with identical breakpoints in the paired tumors. In addition to this, other rare cases of HS containing immunoglobulin gene rearrangements, specific to B cell maturation (Weiss et al. 1985; Hanson et al. 1989; Feldman et al. 2008; Chen et al. 2009), including cases of HS where past and concurrent diagnoses of lymphoma were excluded (Chen et al. 2009), have been found.

Expression of B cell markers in HS has also been explored. Oct2 is a transcription factor involved in B cell development. Oct2 is expressed at high levels in B cells, and at lower levels in T cells, cells of the central nervous system, and in kidney and testis (Stoykova et al. 1992; Pfisterer et al. 1994; Matthias 1998; Luchina et al. 2003). Oct2 expression is responsible for activity of many B cell-specific genes including the Ig locus (Thevenin et al. 1993; Corcoran and Karvelas 1994). Expression in B cells ranges from low levels in pro- and pre-B cells to high levels in mature B cells (Staudt et al. 1988; Miller et al. 1991). In a study by Chen et al. (Chen et al. 2009), 4 out of 7 HS patients without concurrent or previous diagnosis of lymphoma had tumor cells expressing Oct2, with none expressing B cell markers Pax5, CD20, or BOB.1. 6 of the 7 possessed IgH rearrangement, and 4 had IgK rearrangements. Oct2 expression has been
detected in other cases as well (Chen et al. 2009; Wang et al. 2010). The significance of Oct2 expression is unknown, but may be indicative of the HS cells’ B cell origin.

This evidence indicates a complex relationship between the neoplasms and may represent the lymphocytic origins of some cases of HS. Following these discoveries, in 2008 the WHO removed the requirement of B cell-specific trait exclusion in diagnosis of HS. Since then, similar data has emerged supporting lineage infidelity in development of HS (Zhang et al. 2009; Wang et al. 2010; Wang et al. 2011; Zeng et al. 2011), including evidence of T cell transdifferentiation (Castro et al. 2010).

1.4.1 Murine HS

A good animal model of HS is required to better understand the etiology of this disease. Incidence of HS in the mouse varies greatly according to sex, age and strain, being most common in C57/BL6J mice with an incidence of 22.2% in 24 month-old males and 10.4% in females (Frith 1990; Lacroix-Triki et al. 2003). In mice, liver and uterus are typically affected, but most other organs may also be involved (Frith CH 2001). More recent experimental models have found the spleen to be the primarily affected organ, with the lymph nodes as the first site of dissemination, and the liver being a commonly affected non-lymphoid organ (Hao et al. 2010). The bone marrow may also present features of malignant histiocytes (Mashima et al. 2010). Extramedullary hematopoiesis in the spleen (Frith 1990) and liver (Lacroix-Triki et al. 2003) is another common feature of murine HS. In a study of 41 cases of spontaneous mouse HS (Hao et al. 2010), expression of histiocyte markers (Mac-2, lysozyme, F4/80) and germline configuration of B cell immune receptor loci were characteristic of HS. Increased expression of F4/80 is
known to correlate with maturation of the monocyte/macrophage lineage (Lee et al. 1985; McKnight et al. 1996; Schaller et al. 2002). F4/80 expression was observed at higher levels in HS cells of round morphology, compared to those of spindle morphology, and cases displaying transition from round cell-type to spindle shape indicate that the two forms are developmentally related (Hao et al. 2010). In general, murine histiocytes are negative for B and T cell markers (Hao et al. 2010). Strictly defined, true HS in the mouse is also negative for Ig and T cell receptor translocations (Morse et al. 2001; Hao et al. 2010). However, in a study by Hao et al. (Hao et al. 2010), 2 cases of spindle cell HS contained histiocytes positive for Pax5, suggestive of lineage infidelity, similar to that seen in some human cases. Although this confounds the suggestive importance of Pax5 for maintaining B cell commitment seen in humans, this may represent a difference between the two species, or an alternate pathway of trans-/de-differentiation in the mouse.

1.4.2 Pathway involvement in HS pathogenesis

Several models of murine HS have been generated to study genes involved in HS development. Infection of mice with malignant histiocytosis sarcoma virus (MHSV) resulted in the accumulation of mononuclear phagocytes, originally characterized as malignant macrophages (Franz et al. 1985; Lohler et al. 1987). However, consistent with the trend of misdiagnosis in humans, recent re-evaluation of the model has shown that not only macrophages, but dendritic cells and precursor cells of the bone marrow were affected by the Ras-expressing virus, and this results in a malignancy that is more heterogenous than originally believed (Leenen et al. 2010). The affected cells demonstrate a dendritic cell or macrophage-like phenotype, and this phenotype seems to depend on the microenvironment characteristic of the tissue to which they have homed.
The heterogeneity in this mouse model is reminiscent of the plasticity and heterogeneity seen in human cases (Pileri et al. 2002), with fewer cases than initially suspected representing true HS.

Functional knockout of Cdkn2a, which encodes the tumor suppressors p16Ink4a and p14Arf, results in elevated frequency of lymphomas and fibrosarcomas early in life through altered regulation of the Rb1 and p53 pathways (Serrano et al. 1996). Infection of Cdkn2a<sup>−/−</sup> mice with Moloney murine leukemia virus (MoMuLV) was performed to identify loci whose disruption synergizes with knockout of Cdkn2a in tumor development (Lund et al. 2002). In addition to an increased frequency of lymphoma, there was a 55% incidence of HS affecting the spleen and liver. 40% of cases were a mixture of lymphoma and HS, and 15% were exclusively diagnosed as HS. 6 loci were identified as common insertion sites specifically for HS, representing either activated proto-oncogenes or inactivated tumor suppressor genes: Hcph, ZNF220 mouse orthologue, Dgke, Kif13a, as well as 2 expressed sequence tags located on mouse chromosomes 13 and 17. 17 loci were identified as being involved in both lymphoma and HS. These sequences may represent genes involved in HS pathogenesis, and their involvement in the PI3K pathway indicates the potential importance of this pathway in preventing HS.

In a smaller study, primary hematopoietic stem/progenitor cells were transduced with a retrovirus encoding the large tumor antigen of simian virus 40, which, like Cdkn2a, also inactivates Rb1 and p53 (Li et al. 2007). These cells were found to differentiate into malignant histiocytes or other neoplastic cells of the myeloid lineage. This study also implicated several other genes in development of HS.

Studies of Cdkn2a and Pten double knockout mice (Carrasco et al. 2006) indicate the importance of these genes in suppressing HS and display an altered pattern of expression during
pathogenesis of the disease. *Cdkn2a*−/− mice develop B and T cell marker-expressing biphenotypic lymphomas, with a low frequency of HS. However, *Cdkn2a*−/− *Pten*+/− mice had expanded populations of biphenotypic B220+, CD117+ myelolymphoid cells which preceded development of HS at an elevated frequency compared to controls, while frequency of B and T cell lymphomas compared to *Cdkn2a*−/− mice remained unaltered. The lack of histiocytic hyperplasia in *Pten*+/− mice and low frequency in *Cdkn2a*−/− mice compared to *Cdkn2a*−/− *Pten*+/− mice indicates a potentially important cooperative effect. Furthermore, almost all cases of HS had subsequent LOH of *Pten* and were associated with aberrant activation of the PI3K/Akt and Ras/MAPK pathways. In a translational study, the group similarly found *Cdkn2a* and *Pten* to be genetically or epigenetically inactivated in human HS.

Dok-1, Dok-2, and Dok-3 proteins are substrates that inhibit protein tyrosine kinase pathways (Lemay et al. 2000; Yamanashi et al. 2000; Songyang et al. 2001; Mashima et al. 2009), such as the Bcr-Abl pathway found in acute myelogenous leukemia. Dok-1,2,3 associate with the p120 ras GTPase activator protein (Carpino et al. 1997; Yamanashi and Baltimore 1997). Machima et al. (Mashima et al. 2010) knocked out all three genes in mice and found a severe neoplastic phenotype, whereas Dok-1−/− and Dok-2−/−Dok-3−/− mice did not develop aggressive tumors. Triple knockout mice initially develop abnormal macrophage accumulation in the lung and eventually succumb to HS spreading to multiple organs without elevated incidence of other tumors. The tumors are transplantable into lethally irradiated mice. *In vitro*, triple knockout macrophages show a higher than normal proliferative response to M-CSF and GM-CSF, while Dok-1−/− and Dok-2−/−Dok-3−/− mice also have a high response, though significantly lower than the triple knockouts. As these proteins interact with Ras, which has many downstream mediators including MAPK, several pathways may be involved in the genesis of HS. Further
evidence for the importance of these genes in inhibiting HS stems from studies of the Lyn protein. Lyn is required for phosphorylation and activation of Dok-1 and 3. Lyn knockout in vitro gives macrophages an enhanced growth potential in response to M-CFS and GM-CSF, and deficiency in a murine model caused development of macrophage tumors that may bear similarities to HS, supporting the role of this pathway in HS development (Harder et al. 2001).

Although it is also a rare disease in dogs, some breeds have a predisposition to development of HS, indicating the importance of the genetic component. Several deleted genomic regions in HS that may host tumor suppressor genes have been identified, and support the involvement of the pathways implicated in murine HS. The Arf and Ink4a/b locus is affected in 62.8% of HS found in Burnese Mountain Dogs and Flat-Coated Retrievers (Hedan et al. 2011). Deletions of the regions containing Rb1 and Pten were found in 55.8% and 40.7% of cases, respectively.
Chapter 2: Research aims and hypotheses

The aim of this project is to better understand the mechanism by which 13q14 deletion contributes to tumorigenesis. Specifically, we will elucidate the role of CHC1L deletion in promoting cancer pathogenesis. I hypothesize that CHC1L is a tumor suppressor gene, and that deletion of its locus promotes tumorigenesis through a pathway involving altered regulation of RanGTPase and/or disrupted degradation of other proteins as a potential ubiquitin ligase adaptor protein.

The evidence for its tumor suppressive activity stems from clinical observations. CHC1L is located within chromosome 13q14, which is a common site of deletion in CLL, MM, and PC. Within 13q14, it is located at a LOH hotspot in PC, appearing to be the main target of deletion. Its expression is reduced significantly in poor prognosis cases of PC and MM suggesting that loss of CHC1L activity promotes neoplasticity.

In order to examine the putative tumor suppressor role of CHC1L, a murine knockout model has been created in our lab. Since our model possesses deletion of the Chc1L locus, we may infer its function by the phenotype that arises in its absence.

We wish to confirm that loss of Chc1L confers susceptibility to tumorigenesis. By determining the incidence of tumorigenesis, we will better understand the significance of this deletion in developing cancer. Further, by studying the spectrum of disease, we will see if its loss is associated with a specific cancer phenotype or with tumorigenesis in general.
Chapter 3: Materials and Methods

3.1 Mice and gene knockout strategies

Mice were backcrossed and maintained on a C57/BL6J background, and housed at the Animal Resource Facility of the Princess Margaret Hospital.

The mouse \textit{Chc1L} gene is located on chromosome 14. It spans a genomic region of 40 kb with 16 exons. The start codon ATG is located on exon 4. The complete cDNA of \textit{Chc1L} is 3 kb. Since a codon of methionine (ATG) is identified in exon 5, this ATG was prevented from potentially initiating translation by designing targeting strategies to delete both exons 4 and 5. Our targeting strategy (Figure 6) is based on targeting vector pSC22 using a 7.6 kb 5’-arm and a 4 kb 3’-arm with homology to murine \textit{Chc1L}. Because of the limit of cloning size, exons 4 and 5 were cloned separately and ligated together in deleting more than 6 kb of intron 4. Two \textit{loxP} sites were inserted before and after exon 4 and 5 in the same direction. This vector system employs neo as a positive selection marker. Next, the targeting vector was electroporated into ES cells and ~600 neo-resistant clones were screened by Southern Blotting. The targeting event can be screened by a 5’-probe with EcoRI digestion. It detects a 20 kb band in the wild-type locus, but a 14 kb band in the targeted locus. From this screen, 5 targeted ES cell clones were identified and microinjected into embryos that were then implanted into pseudopregnant females. The resulting chimeras were bred for germline transmission of the targeting vector. Mice possessing germline insertion were bred to mice expressing Cre recombinase in the germ line, under the action of the \textit{Blimp1} promoter, to generate mice with non-conditional deletion of \textit{Chc1L}. 

Knockout of exons 4 and 5 was confirmed by RT-PCR using two separate primer pairs. The resulting amplicons were sequenced to confirm specificity for exons 4 and 5.

3.2 Genotyping

Murine ear tissue was taken and submerged in 100uL protease K solution (2µg Protease K in PCR buffer) and incubated at 55°C overnight. 2µL of dissolved tissue solution containing DNA was diluted into 8µL autoclaved ddH2O in a 200µL PCR tube. The PCR tube was placed in a thermocycler (Eppendorf Thermocycler PCR MasterCycler 5332) and kept at 95°C for 10 minutes to denature protease K. 20µL PCR master mix was added to the tube, mixed, and briefly spun to bottom of tube. The PCR reaction program is indicated in Table 1. PCR buffer and master mix recipes are found in Table 2.

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<th>Annealing Temperature</th>
<th>PCR reaction conditions</th>
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<td>GTGTTACTTTTGCCCGTGGT GTGACAGGGCAAACCAAGTT</td>
<td>58°C</td>
<td>1. 93°C for 03:00 2. 93°C for 00:30 3. Annealing temp. for 00:45</td>
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<td>4. 70°C for 01:00 5. Repeat steps 2-4 40 times</td>
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<tr>
<td>Int4-R2</td>
<td>AGGGACTGCACAGGACTGAT CAAGCCTGACGAATCAGTC</td>
<td>60°C</td>
<td>6. 70°C for 10:00 7. Hold 4°C</td>
</tr>
<tr>
<td>TC-F</td>
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<td>58°C</td>
<td></td>
</tr>
<tr>
<td>TC-R</td>
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<td>58°C</td>
<td></td>
</tr>
<tr>
<td>Ex4F2</td>
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<td></td>
</tr>
<tr>
<td>Ex5R2</td>
<td>GGCCACTTTCCCCACATCTAA</td>
<td>60°C</td>
<td></td>
</tr>
</tbody>
</table>
3.3 Gel purification

DNA fragments were purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen, cat. no. 28704). Briefly, DNA bands were excised from the gel with a scalpel. Each gel slice was massed and three volumes of dissolution buffer were added. One volume of isopropanol was added, and the tube was briefly centrifuged through a DNA-binding column at 13,000rpm. Washing buffer was then added, and column was briefly centrifuged. DNA was eluted by adding water to column, and collected into a microcentrifuge tube via centrifugation.

3.4 RNA extraction and cDNA synthesis

Tissues were collected in RNase-free conditions and snap frozen using liquid N$_2$. RNA was collected using QIAGEN RNeasy Plus (Qiagen TM, Cat. No. 74134) mini kit according to
manufacturer’s protocol. Briefly, 30mg of tissue was added to a lysis buffer (Buffer RLT) and disrupted by sonication. The lysate was transferred to a gDNA Eliminator column and centrifuged a 8,000g for 30 seconds. An equal volume of ethanol was then added to the flow-through, and mixed. Each sample was then transferred to RNeasy spin column and centrifuged. The flow-through was discarded, and buffer RW1 was added to the spin column and spun, again discarding the flow-through. Two more washes of the spin column were performed with buffer RPE. The RNA was then collected into a 1.5mL collection tube by elution with RNase-free water. RNA integrity was verified on an Agilent 2100 Bioanalyzer.

RNA was reverse transcribed using Applied Biosystems’ High Capacity cDNA Reverse Transcription Kit (Applied Biosystem™, 4368814) according to manufacturer’s instructions. 1µL cDNA was diluted 1:10 in RNase-free ddH2O, and master mix was added (recipe in Table 2), using primers Ex4F2 and Ex5R2 or Ex4F3 and Ex5R3. The PCR program is displayed in Table 1.

3.5 Histological preparation

All tissues were collected into 10% neutral-buffered formalin, and fixed overnight. Tissues were subsequently embedded in paraaffin, sectioned, and stained by hematoxylin and eosin. For immunohistochemistry (IHC) using antibodies for Mac-2 and B220, standard tissue sections were deparaffinized and rehydrated, then post-fixed in 10% neutral-buffered formalin for 1 hour and washed 5 minutes in PBS. Sections were soaked in 10mM citrate buffer (pH6.0) at 85°C for 3 hours (for Mac-2 only). Slides were air-dried for 10 minutes, and washed in PBST for 5 minutes. Slides were soaked in 3% hydrogen peroxide in methanol for 30 minutes, and
washed 3 times in PBST for 5 minutes each wash. Each slide was blocked using Dako protein block serum free (Dako: catalogue number X0909) for 30 minutes. 1:100 diluted rat anti-Mac-2 (Cedarlane: catalogue number CL8942AP) or rat anti-B220 (BD Bioscience: catalogue number 550286) was pipetted onto slide surface and incubated overnight at 4°C. The slides were washed 3 times in PBST, and 1:100 anti-rat IgG-biotin (Vector Labs: catalog number BA-4001) was pipetted onto surface, and incubated at room temperature for 60 minutes. Slides were washed 3 times in PBST. A and B from kit ABC (Vector Labs: catalogue number PK-6100) were pipetted onto slide surfaces, and incubated 30 minutes room temperature, then washed 3 times in PBST for 5 minutes each. Slides were immersed in DAB colour development solution, then washed in distilled water for 5 minutes. Slides were then counterstained with hematoxylin for 30 second. Slides were dipped in 1% acid alcohol, then immersed in Scott’s tap water for 30 seconds. Finally, slides were dehydrated and cleared, then coverslipped with Permount.

3.6 Histological analysis

All histological analyses were performed by service from the Toronto Center for Phenogenomics. The pathologist was blinded to genotype during histological analysis. Diagnosis of HS and HAL or HS co-occurring with BCL was performed based on previously outlined morphological features of these tumor cells (Hao et al. 2010), those being cells varying from round to spindle-shaped in morphology, containing large amounts of lightly staining cytoplasm and pleiomorphic nuclei, while also taking into account the distribution of affected organs and other findings associated with tumorigenesis such as extramedullary hematopoiesis and the presence of multinucleated giant cells. HAL was diagnosed in cases consistent with HS and
possessing large number of lymphocytes. To confirm the accuracy of this diagnostic approach, IHC for histiocyte-specific and B cell-specific markers (Mac-2 and B220, respectively) was performed on representative tumors, whereby HS is diagnosed as Mac-2+/B220-, HAL is Mac-2+/B220+, an HS and BCL composite is Mac-2+/B220+, and BCL is Mac-2-/B220+, as outlined by Hao, Fredrickson et al. (2010).

3.7 Flow cytometry

Blood was collected from the lateral tail vein. Each mouse was heated by a lamp to increase blood flow to the tail if necessary. Mice were restrained in a box with a hole for the tail. Using a 22½G needle, the lateral vein was punctured. As the blood droplet formed, it was quickly pipetted into a tube containing PBS+2mM EDTA. Approximately 20µL was collected into 180µL PBS/EDTA.

Approximate cell density was measured using flow cytometry. Cell solutions was centrifuged at 300g for 10 minutes, and supernatant was then aspirated. The pellet was resuspended to $10^7$ nucleated cells/100µL flow buffer. 10µL anti-CD45-Vioblue (Miltenyi Biotech, catalogue number 130-092-910) per 100µL cell suspension was added to stain mononuclear cells, then mixed and incubated for 10 minutes at 4°C. Alternatively, Ack lysis buffer (0.15M NH₄Cl, 10mM KHCO₃, 0.1mM EDTA) was used to remove RBCs. Cells were washed by adding 1mL FACS buffer, then spun down (300g for 10min), and supernatant was aspirated. The solution was resuspended into 100µL FACS buffer. 3µL of antibody of interest (Table 3) diluted 1:10 in FACS buffer was added. Solutions were mixed and incubated on ice for
20 minutes. They were then washed with 1mL FACS buffer, and spun down (300g, 10 minutes). Solutions were finally resuspended into 1mL FACS buffer prior to proceeding to flow cytometry.

Flow cytometry was performed on a MACSQuant Analyzer or BD FACSCalibur. Approximately 20,000 cells were counted per stain.

<table>
<thead>
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<th>Antibody</th>
<th>Supplier</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
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<td>BD Pharmingen</td>
<td>558597</td>
</tr>
<tr>
<td>CD3-FITC</td>
<td>Abcam</td>
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<td>CD11b-APC</td>
<td>BD Pharmingen</td>
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</table>

Table 3 Antibodies used for flow cytometry

3.8 Bone marrow and spleen primary culture

Bone marrow was collected from both femurs and tibia. After each mouse is dissected, both hindlegs were removed and placed into a tube containing PBS. Leg bones were transported on ice to the lab. Muscle tissue was removed, and the bone marrow aspirated from the femur and tibia using a 25G needle into culture media (DMEM/10%FBS/1%Antibiotic).
To obtain a splenocyte suspension, the spleen was removed and placed into culture media for transport. The spleen was placed between two microscope slides, which were pressed together. The slides were rubbed together until only the lining of the spleen remained. Splenocytes were continually transferred into culture media as the spleen was ground to pulp.

The cell suspension was pipetted up and down using the same needle and passed through a 70µm cell strainer to remove clumps of cells. The resulting suspension was spun down (500rpm for 2min), and the pellet resuspended into 2mL Ack lysis buffer and incubated for 2min at room temperature. The cell suspension was washed and resuspended into 3mL culture media. Cell concentration was calculated using a hemocytometer. The cell suspension was plated into a 6 well plate at 5x 10^6 cells/well and cultured in DMEM/10%FBS/1%Antibiotic +5µg/mL LPS for splenocytes, or 100ng/mL LPS for bone marrow. Cells were cultured for 2 days.

**3.9 MTT assay**

Bone marrow cells were cultured in triplicate at 2x10^5 cells per well in a 96-well plate with 200µL DMEM/10%FBS/1%Antibiotic + LPS for 48 hours. 10µL MTT solution (5mg/mL MTT (Invitrogen™, catalogue number M-6494) in PBS) was added to each well, and cultured for 4 hours. 100µL solubilization solution (10% SDS in ddH₂O+100µL of 37% HCl per 100 mL) was added to each well, and cultured for 4 hours. OD was read at 570nm.
3.10 PI staining

Cell suspension was pipetted into centrifuge tube, and centrifuged for 5 minutes at 500xg. Supernatant was then aspirated. Cells were resuspended with 0.5mL of PBS. A labeled 15mL Falcon Tube was prepared with 4.5mL of ice cold 70% ethanol for each sample. The resuspended cells were added in a dropwise fashion to the 15mL Falcon Tube while simultaneously mixing the tube to ensure that cells don’t clump.

Cells were fixed on ice at 4°C for 2 hours on a shaker in the cold room. Cells were pelleted (centrifuge for 5 minutes at 500xg) at RT. Cells were then washed twice with 5mL of PBS (centrifuge for 5 minutes at 500xg and discard supernatant). Cells were resuspended in 1mL of 0.1% Triton X-100 in PBS. 10 µL of RNAse was added to each tube. Tubes were then incubated at 37°C for 15 minutes. 20 µL of 2mg/mL PI was added and the suspension was incubated for 20 minutes prior to proceeding to flow cytometry.

3.11 Protein lysate preparation

One half tablet Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Cat. No. 11 836 170 001) was added to 1ml Lysis Buffer (0.05M TrisHCl, 1mM EDTA, 1%NP40, 0.15M NaCl, adjusted to pH 7.4) to make 10x protease inhibitor in lysis buffer, which can be stored in the -20°C freezer. 10x protease inhibitor in lysis buffer was diluted 1:10 in lysis buffer, and the serine protease inhibitor PMSF was added to a final concentration of 0.1ng/mL. The resulting solution is the protease-complete lysis buffer.

Approximately 100mg of fresh tissue, stored at -80°C, was placed into 2mL ice-cold protease-complete lysis buffer. Tissue was homogenized using an electric homogenizer. Tissue
homogenate was placed on ice for 30 minutes. For isolation of protein from cultured cells, cells were washed in cold PBS, and 1mL (per 100mm culture dish at 70-95% confluency) ice-cold protease-complete lysis buffer was washed over monolayer. Using a cell scaper, cells were physically disrupted. The resulting solution was collected and incubated on ice for 30 minutes. Lysates were then spun down at high speed for 20 minutes, and supernatant was collected.

Protein concentration was determined using the BCA protein assay. Reagent S was diluted into reagent A at a ratio of 1:50, to form reagent A’. 5µL of standards (ranging from 0 to 2mg/mL) and 5µL of samples (in triplicate) were pipetted into 96-well plate. 25µL A’ and 200µL reagent B were added to each well. Plate was agitated and incubated at room temperature for 15 minutes. Absorbance was read at 750nm.

3.12 Polyacrylamide gel electrophoresis

An 8% polyacrylamide resolving gel was made according to the following recipe: 4.2mL ddH$_2$O, 1.6mL 40% acrylamide, 2mL 1.5M Tris pH 8.8, 80µL 10% SDS, 80µL 10% APS, 8µL TEMED. A 4% polyacrylamide stacking gel was made with the following recipe: 3.1mL ddH$_2$O, 0.5mL 40% acrylamide, 1.25mL 0.5M Tris pH6.8, 50µL 10% SDS, 50µL 10% APS, 5µL TEMED.

20µL of 5x SDS sample buffer (10% SDS, 10mM DTT, 20% glycerol, 0.2M TrisHCl pH 6.8, 0.05% Bromophenol blue) was mixed with 80µL tissue lysate. The resulting mixture was boiled for 5 minutes. The polyacrylamide gel was immersed in Running Buffer (25mM Tris, 200mM glycine, 0.1% SDS in ddH$_2$O). 50ng of total protein was used per well, and the gel was run at 100-150V for 1.5 hours.
Prior to transferring to a nitrocellulose membrane, the membrane, gel, filter paper and sponges were all soaked in Transfer Buffer (25mM Tris, 200mM glycine in 20% methanol) for 20 minutes. The gel and membrane were then sandwiched together and placed in the transfer apparatus, which was run overnight at 22V within a 4°C fridge.

3.13 Western Blotting

The transferred nitrocellulose membrane was washed 3 times in TBST (20mM Tris, 0.138M NaCl, 0.05% Tween20, buffered to pH 7.6) for 5 minutes each to remove residual transfer buffer. The membrane was then blocked using 5% milk in TBST for 2 hours at room temperature. The membrane was briefly washed to remove excess blocking solution. Anti-Chc1L antibody is diluted 1:500 in blocking buffer (Table 4). Membrane was submerged in antibody solution and incubated at 4°C for 8 hours or overnight. The membrane was washed 3 times in TBST for 5 minutes each. Goat-anti-rabbit-HRP was diluted 1:5000 in blocking buffer. The membrane was incubated in secondary antibody (Table 4) for 1 hour at room temperature. Following secondary antibody incubation, the membrane was washed 4 times in TBST, then one time in TBS, for five minutes each. The membrane was then exposed for approximately 5 minutes onto x-ray film (CL-XPosure Film, 8 x 10in., Thermo Scientific™, catalogue number 34091).
3.14 Transfection protocol

HepG2 cells were plated at 1.8 x 10^5 cells/well in a 24-well plate containing DMEM+10% FBS+antibiotics, and grown for 24 hours. Cells were transfected at ~70% confluency using Lipofectamine 2000 (Invitrogen, Cat. No. 11668-019). Final concentrations were optimized to: 0.12µM siRNA, 8ng/µL lipofectamine.

60 pmol siRNA was added to 50µL Extreme MEM. 4µg Lipofectamine 2000 was added to 50µL Extreme MEM and incubated for 5 minutes at room temperature. The 50µL siRNA and Lipofectamine 2000 solutions were combined, and incubated for 20 minutes to allow siRNA/Lipofectamine complex formation. Media was removed from cells, and the monolayer was washed twice in PBS. 400µL of Extreme MEM was added to each well, followed by slow addition of the 100µL siRNA/Lipofectamine complex solution. Cells were incubated at 37°C for

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**Table 4 Western Blotting antibodies**

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47
24 hours, at which point the transfection media was removed and replaced with DMEM containing FBS and antibiotics.

### 3.15 Statistical methods

P values for determining significance of tumor incidence in each genotype compared to one another were calculated using the Chi-squared test.

The two-tailed homoscedastic student’s T test was used to calculate significance of peripheral blood population count changes and MTT cell survival differences.
4.1 Generation of Chc1L gene knockout mice

To examine whether Chc1L suppresses tumor formation in vivo, knockout mice were generated using homologous recombination (Figure 6). In deleting more than 6kb of intron 4 of the murine orthologue, a targeting vector was created that incorporated two unidirectional loxP sites flanking exons 4 and 5, both of which contain an ATG methionine codon. By removing the two potential start codons, initiation of translation will be deleted. The targeting vector was electroporated into murine ES cells, using neomycin resistance as a selection marker. Successful targeting was confirmed with Southern Blotting to detect the 6kb length difference of the targeted and non-targeted alleles, following EcoRI digestion. Gene-targeted ES cells were microinjected into mouse embryos, which were transplanted into pseudo-pregnant females, generating chimeric mice. Mice were then bred to homozygosity, detecting knockin again using Southern Blot.

Gene-targeted knockin mice (Chc1L<sup>loxP/+</sup>) were then crossed to a strain expressing Cre recombinase under the Blimp1 promoter. Blimp1 was originally identified for its capacity as a master regulator of plasma cell differentiation (Shaffer <i>et al.</i> 2002). Additionally, it is responsible for germ cell determination in the mouse embryo, and is thus active in primordial germ cells (Ohinata <i>et al.</i> 2005; Vincent <i>et al.</i> 2005). By crossing exon 4 and 5-floxed knockin mice to Blimp1-Cre transgenic mice, progeny possessing germ-line deletion of both exons were
generated. Interbreeding of germ-line deletion-carrying mice gave offspring with nonconditional deletion. These mice were interbred to generate WT, HET and KO mice, with no need for Cre expression in any tissues.

A genotyping method was developed based on the presence of intron 4 in the WT allele. Primers that target sequences within intron 4 were used to detect the WT allele, whereas primers that target introns 3 and 5, which are brought into proximity upon deletion, detected the KO locus (Figure 7A). Successful knockout of gene product was detected by RT-PCR. Two sets of forward and reverse primers were designed to complement sequences in exons 4 and 5, respectively (Figure 7B) (Primer IDs: Ex4F2-Ex5R2 and Ex4F3-Ex5R3, see Table 1 for primer sequences). A PCR amplified product was detectable using cDNA generated from wild-type tissues (Figure 7C). No amplification was detectable in the knockout lanes. The amplified products from both PCR primer pairs were gel-purified and sequenced, confirming their specificity for exons 4 and 5 of Chc1L.
Figure 6 Chc1L gene targeting strategy and generation of knockin mice

A. Cloning and knockout strategy

- **A.** A targeting vector containing loxP-flanked exons 4 and 5 was used for gene targeting of *Chc1L*.
  - (a) A targeting vector was constructed by purifying and subcloning exons 4 and 5 into a vector containing loxP sites, thereby flanking the exons with unidirectional loxP sequences. The “floxed” exons were then ligated into a targeting vector containing 5’ and 3’ *Chc1L* homology arms, allowing homologous recombination to occur.
  - (b) The resulting knockin locus contains exons 4 and 5 flanked by loxP sites, and the neomycin resistance gene.
  - (c) In the presence of Cre recombinase, active in germ cells, the ATG-containing exons 4 and 5 are deleted.

B. Successfully targeted ES cells were selected for neomycin resistance, and the knockin locus was detected as a 14 kb sequence by Southern blot.

C. Chimeric mice were generated by microinjecting successfully targeted ES cells into embryos, which were transplanted into pseudopregnant mothers.

D. Chimeric mice were bred and germline transmission was detected and confirmed by Southern Blot.
Figure 7 PCR and RT-PCR strategies

A. The WT allele was detected using primers that recognize intron 4 (Int4-F and Int4-R). The KO allele is detected using primers specific for sequences flanking the recombined loxP locus (TC-F and TC-R). In the WT allele, these sequences are too far apart to amplify by conventional PCR. B. The RT-PCR strategy uses primers designed to target exons 4 and 5, which are not present in the KO transcript. C. Knockout of Chc1L expression was detected by RT-PCR in various tissues, showing expression of the WT transcript in WT lanes only. The bands were purified and sequenced to confirm successful deletion of exons 4 and 5.
4.2 HET and KO mice succumb to Histiocytic Sarcoma and Histiocyte-Associate Lymphoma

Wild-type (WT), heterozygote (HET) and knockout (KO) mice were bred from a C57/BL6J background and born at expected Mendelian frequency. HET and KO mice have normal embryonic development and do not develop prostate cancer (Figure 8). Two cases had features of plasma cell lymphoma, indicating with may suggest it is a component of the disease spectrum of these mice.

Figure 8 Knockout of Chc1L in mice does not result in PC
The H+E staining of Chc1L knockout prostates demonstrated normal histological structure.
In order to track cancer development, we performed preliminary analyses on young mice. 5 knockout mice, age 8-12 weeks, were necropsied. No visible pathology was observed. Histopathological analysis was performed on spleen, lymph nodes, and liver, however no evidence of early neoplastic events was present.

To allow more time for neoplastic progression, mice were sacrificed between the ages of 20-26 months, and detailed necropsies were performed. Tissues with gross abnormalities were documented and fixed in formalin whenever possible. Table 5 summarizes all tumors identified based on gross pathological analyses.

Prevalence for gross pathology was evident in HET (55.6%, n=18) and KO (80.0%, n=25) mice, compared to wild-type controls (22.2%, n=9) (Figure 9). The difference between tumor incidence in WT and KO mice was significant using the Chi-square test (p=0.02). These pathologies were enlargements of the spleen, mesenteric lymph nodes and liver, as well as masses appended to an organ, all classified as tumors. Tumors were concentrated in the spleen, lymph nodes of the mesentery, liver and intestines/colon (Figure 10). Interestingly, although tumor incidence in HE’s was lower than that of KO mice, HET mice tended to have more organs affected (Figure 11). These may represent metastases from a primary tumor which may arise in the spleen or lymph node, based on the frequent observation of tumors in these tissues. However, without demonstrating common clonality of the tumor cells, it is not possible to confidently state that tumors found in multiple locations within the same individual do not represent independent proliferations.
**Table 5 Tumor distribution**

Each row represents a mouse in which at least one tumor was found. Commonly affected organs in HET and KO mice were the spleen, lymph node and liver.

<table>
<thead>
<tr>
<th></th>
<th>Intestine</th>
<th>Lung</th>
<th>Liver</th>
<th>Colon</th>
<th>Spleen</th>
<th>LN</th>
<th>Kidney</th>
<th>Skin</th>
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*Note: The table entries indicate the presence of tumors in various organs across different genotypes (WT, HET, and KO).*
Figure 9 Tumor incidence observed by gross pathology
Tumor incidence by genotype. Incidence of observable tumors was highest in HET and KO mice (WT: 22%, HET: 56%, KO: 80%). WT vs KO: p=0.02; WT vs HET: p=0.10; HET vs KO: p=0.09.
Tumors were found most often in the spleen, mesenteric lymph nodes and liver.
Figure 11 Incidence of multiple organ tumors
HET mice typically had multiple tumor-bearing organs.
To gain insight into the nature of these tumors, conventional histopathological studies, with a focus on commonly affected organs, were performed by an experienced veterinary pathologist.

The spleen and mesenteric lymph nodes were frequently enlarged due to proliferations of transformed cells, which expanded the tissues and destroyed their normal architecture (Spleen: Figure 13; lymph node: Figure 14). The liver was often involved, and many cases demonstrated proliferations of neoplastic cells emanating from blood vessels (Figure 15). Tumors found on the lower gastrointestinal tract often caused destruction of the mucosal surface (Figure 16). This appears to be due to abnormal proliferations affecting the Peyer’s patches, lymphoid nodules found within the ileum.

The morphology of the tumor cells in question varies from round to spindle-shaped, with abundant eosinophilic cytoplasm and pleiomorphic (round, oval and folded) nuclei (Round: Figure 12a; Spindle: Figure 12b). Tumor cells of a single morphology (round or spindle shaped) were found existing either discretely within a proliferation, or a mixture of cells of each morphology were found admixed in the same tumor (Figure 12c). In the mixed cell-type tumors, the round and spindle-shaped cells are often intermingled, and these groups of cells appear to transition from one morphology to the other. These features are consistent with the morphology of malignant histiocytes.

Often, large proliferations of lymphocytes were found co-occurring in the histiocyte-rich neoplasms (Figure 12d). Occasionally, Mott cells, plasma cells defective in immunoglobulin secretion, were found associated with the lymphocytes (Figure 12d). Sometimes admixed in populations of tumor cells were large numbers of multinucleated giant cells (Figure 12e). Mitotic
figures and apoptotic bodies were also abnormally prevalent. Extramedullary hematopoiesis was recurrently observed in the liver as well as the spleen (Figure 12f).

Tumors within the commonly affected tissues (spleen, lymph node, liver) were collected from HET and KO mice (HET: n=8; KO: n=11), and compared to the same tissues of age- and sex-matched WT mice (WT: n=4). However, not every organ was analyzed for histopathology.

Most of these findings, namely cell morphology, affected organs, presence of giant cells, and extramedullary hematopoiesis, are indicative of HS. Additionally, the large numbers of lymphocytes in some cases may indicate the co-existence of B cell lymphoma (BCL) a common neoplasm in C57/BL6 strains, along with HS. An alternative possibility to the concurrent pathogenesis of these two cancers is that these mice are succumbing to Histiocyte-Associated Lymphoma (HAL).

Table 6 summarizes the histological findings in tissues collected for analysis. Of the eight HET mice examined, two cases were diagnosed as HS, and five cases were differentially diagnosed as either HAL or a composite of HS and BCL. Of tumors extracted from eleven KO mice, six were diagnosed as HS and three as HAL or HS+BCL. Other diagnoses for HET and KO mice were cystadenoma and follicular BCL of the lymph node. One KO case had age-related inflammation resulting in lymph node enlargement, and was classified as “no significant findings.” Four WT mice were chosen as histological controls. Of the four WT mice studied, one case of HAL or HS+BCL was found in the intestine. However, the enlarged spleen and lymph node from one WT was used for other applications, and not studied for its histopathology.
Figure 12 Tumor cell morphology and pathological findings

(a) HS, round cell morphology. Pale eosinophilic cytoplasm is abundant and ranges from smooth to coarse. Nuclei are mainly large with open chromatin pattern and prominent eccentric nucleoli. Cells have a low nucleus: cytoplasm ratio. (b) HS, spindle cell morphology. Nuclei are elongated and convoluted. Cells are elongated and appear in sheets. (c) HS, round (thin arrow) and spindle cell composite (bold arrow). (d) Mott cell admixed with histiocyte-resembling cells and lymphocytes. Note displaced crescent nucleus, bright pink cytosol. (e) Multinucleated giant cells are admixed throughout HS proliferation. Mitotic figures are abundant. (f) Extramedullary hematopoiesis in the liver, consisting of proliferations of precursor cells and neutrophils.
Figure 13 Spleen H&E

Comparison of WT and representative KO spleen at 4x, 20x, 100x. HET and KO spleens were often enlarged, with normal structure obliterated by proliferations of tumor cells with abundant, eosinophilic cytoplasm, and irregular nuclei with open chromatin and prominent nucleoli.
Figure 14 Lymph node H+E

Comparison of WT and representative mesenteric lymph node at 4x, 20x, 100x. HET and KO lymph nodes were frequently enlarged, with normal structure displaced by tumor cells with morphology as described in the spleen.
Figure 15 Liver H+E

Comparison of WT and representative liver at 4x, 20x, 100x. Frequently, multifocal areas of tumor cell infiltration that destroy the hepatic parenchyma were observed in HET and KO mice.

Table 6 Summary of histopathological analyses

Representative tumors were harvested and analyzed for histopathology, and compared to WT organs.
Figure 16 Small Intestine H+E
WT compared to representative intestine at 4x, 20x, 60x. Peyer’s patch is severely enlarged by tumor cells which have destroyed the mucosa.
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**Table 6: Summary of Histopathological analyses**

HEF and KO mice were frequently diagnosed with HS or HAL+H5+BC1
While normally necessary to definitively diagnose HS and HAL or concurrent HS+BCL, IHC for histiocyte marker Mac-2 and B cell marker B220 was performed on representative tumors from two HET and one KO mouse rather than on all tumors collected as a method of confirming our histopathological findings. IHC was also performed on one WT control mouse.

Figure 17, panels c and d show a lymph node tumor with cells stained positive for Mac-2 admixed with B220-positive cells. The case may be either HAL/HS+BCL. Panels e and f display a lymph node tumor containing Mac-2-positive tumor cells only, and is definitively diagnosed as HS. Figure 18 shows a tumor occurring within a spleen, in which tumor cells abnormally express Mac-2, which has a different expression profile in the normal spleen, due to macrophages that are normally present in this organ. In the liver (Figure 19), Mac-2-positive histiocytes were observed in proximity to a blood vessel and were distributed throughout the tissue, in comparison to control liver, which has less frequent Mac-2-expressing Kupffer cells. Figure 20 illustrates the remaining IHC findings.

Although IHC analysis was not performed on every HS and HAL/HS+BCL case observed, these results indicate that our diagnoses based on the histopathological appearances of tumors stained by H+E are accurate.
**Figure 17 Lymph node IHC**

**a+b:** Wild-type mesenteric lymph node. Non-neoplastic, Mac-2 positive macrophages are present between follicles. B220 positive cells are present in cortical region.

**c+d:** Enlarged mesenteric lymph node from heterozygous mouse. The normal cortical structure is blurred by heterogenous population of mixed round and spindloid cells. Nearly 30-40 % of the cortical cells and numerous cells within the medullary sinuses are Mac-2 positive. Nearly 60-70 % of the cortical cells are B220 positive. Differential diagnoses: Histiocyte-Associated Lymphoma or composite of HS and B cell lymphoma.

**e+f:** Enlarged mesenteric lymph node from a separate heterozygote. Cortical structure is obliterated: lymphatic nodules have been replaced by spindle shaped cells positive for Mac-2, which are invading the cortical region. Erythroid myeloid cells are abundant. Neoplastic cells are negative for B220. Diagnosis: HS and erythroid leukemia.
Figure 18 Spleen IHC

a+b: Wild-type spleen. Healthy Mac-2 positive macrophages are evenly spread throughout cortical red pulp. Occasional cells stain positive for B220.

b+c: Enlarged heterozygote spleen. The splenic white pulp is replaced by large numbers of heterogenous spindle-shaped cells. The red pulp is expanded by erythroid myeloid cells. Megakaryocytic cells are also markedly increased in number. Nearly 90% of the cells within the white pulp proliferation are positive for Mac-2. Approximately 40-50% of the cells in the red pulp have strong cytoplasmic Mac-2 expression. Scattered cells within the proliferation are positive for B220. Diagnosis: Histiocytic Sarcoma and erythroid leukemia.
Figure 19 Liver IHC
a+b: Wild-type liver. Organ appears histologically normal. Occasional Kupffer cells and other macrophages stain positive for Mac-2, with slight background positivity. All cells are negative for B220.

c+d: Heterozygote liver. Mac-2 positive cells are evenly spread throughout organ. Heavily activated cells are strongly positive for Mac-2, and located adjacent to blood vessels. Diagnosis: Metastatic Histiocytic Sarcoma.
Figure 20 Other IHC findings

a+b: HS tumor of the testis.
c+d: HAL or HS+BCL in the kidney.
e+f: HS cell accumulation in the lung.
4.3 **Peripheral blood cell populations are unaffected by loss of Chc1L**

In order to detect circulating tumor cells, peripheral blood cell populations were measured by flow cytometry on mice aged two years. Age- and sex-matched WT and KO mice were compared. Antibodies for the following markers were used: CD3+CD8, cytotoxic T cells; CD3+CD4, T helper cells; IgM/IgD/B220, B cells; CD138, plasma cells; cKit, hematopoietic stem cells; CD11b, monocytes. This data is summarized in Table 7.

**Table 7 Summary of peripheral blood cell populations measured by flow cytometry**

No significant difference was detected. *Of the 3 pairs of mice analyzed, two KO mice had a distinctly increased CD138-positive population (see Figure 22).*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Wild type (%)</th>
<th>Knockout (%)</th>
<th>P value</th>
</tr>
</thead>
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<td>CD3+8</td>
<td>0.22 ±0.17</td>
<td>0.72 ±0.27</td>
<td>0.24</td>
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<td>CD3+4</td>
<td>0.80 ±0.27</td>
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<td>IgM</td>
<td>24.23 ±20.81</td>
<td>25.49 ±20.31</td>
<td>0.97</td>
</tr>
<tr>
<td>IgD</td>
<td>11.34 ±7.28</td>
<td>11.19 ±4.48</td>
<td>0.93</td>
</tr>
<tr>
<td>B220</td>
<td>4.55 ±2.34</td>
<td>9.56 ±6.16</td>
<td>0.44</td>
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<td>CD138*</td>
<td>1.72 ±1.06</td>
<td>4.46 ±1.96</td>
<td>0.29</td>
</tr>
<tr>
<td>cKit</td>
<td>0.60 ±0.17</td>
<td>0.36 ±0.12</td>
<td>0.33</td>
</tr>
<tr>
<td>CD11b</td>
<td>6.16 ±0.08</td>
<td>8.05 ±0.96</td>
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No significant differences between WT and KO mice were detected. However, a noticeable increase in CD138-positive cells was found in the peripheral blood of two KO mice.
compared to age-, sex-matched WT controls. The third pair had a less severe increase in circulating plasma cells. Data from these three pairs is shown in Figure 21.
Figure 21 Circulating plasma cells
In two pairs of mice (A and C), a distinct increase in CD138-positive cells was detected. The other pair (B) had a slight increase.

Note: Data for C was collected with MACSQuant Analyzer, while data for other dotplots was collected using BD FACSCalibur.
4.4 Splenocytes and bone marrow cells from young mice have increased viability

*ex vivo*

At 8-12 weeks, no visible pathology was observed in KO mice. However, in order to determine whether a difference in cell cycle progression or viability exists in KO cells at this age, PI staining of bone marrow cells and MTT assay-based analysis of bone marrow and spleen cells were performed on age- and sex-matched pairs.

Splenocytes and bone marrow cells from WT and KO mice were plated at equal densities and cultured for two days in the presence of LPS to promote B cell proliferation in the spleen and maturation of dendritic cells in the bone marrow.

PI staining of primary bone marrow cultures did not demonstrate any differences between WT and KO cell cycle progression (Figure 22A; n=3).

However, using the MTT assay, a consistent increase in KO cell survival was seen for splenic and bone marrow KO cell suspensions, compared to WT (Splenocyte fold-survival (KO/WT)=1.45±0.29, p<0.05; Bone marrow fold-survival (KO/WT)=1.26±0.11, p<0.01; n=3 for both assays) (Figure 22B).
A. PI staining of bone marrow cells did not show a significant difference in cell cycle progression. A representative cell cycle assay is shown (n=3). B. KO splenocytes and bone marrow cells have increased viability as determined by MTT assay (n=3).

Figure 22 Cell cycle analysis and viability
A. PI staining of bone marrow cells did not show a significant difference in cell cycle progression. A representative cell cycle assay is shown (n=3). B. KO splenocytes and bone marrow cells have increased viability as determined by MTT assay (n=3).
Chapter 5: Conclusions

In conclusion, we have successfully generated Chc1L gene knockout mice using an embryonic stem cell based gene targeting strategy. In this study, we have characterized the null mutation. Chc1L gene knockout does not affect embryogenesis as wild-type, heterozygote and knockout mice are born at expected frequency. We have not observed development of prostate cancer or multiple myeloma in knockout mice at two years of age. However, we have observed increased peripheral blood CD138 plasma cell populations in some knockout mice, as well as some cases of plasma cell lymphoma, which may indicate a myeloma-like phenotype as part of the disease spectrum.

At 8-12 weeks of age, Chc1L knockout mice do not develop cancer. However, knockout splenocytes and bone marrow cells cultured ex vivo have increased viability compared to wild-type. Importantly, we observed an increased incidence in development of Histiocytic Sarcoma and Histiocyte-Associated Lymphoma in knockout mice. These findings provide the first direct evidence that CHC1L may be a tumor suppressor gene, suggesting that deletion of CHC1L at 13q14 as seen in human cancer may initiate and promote cancer development. These findings also suggest that mutation of CHC1L alone is not sufficient to induce myeloma and prostate cancer, and that the combination of other genetic changes might be necessary for development of these cancer types.

However, our results are mainly based on analysis of two year old mice. By analyzing mice at an earlier age in future studies, we will provide stronger evidence for development of Histiocytic Sarcoma. Analyzing all tumors observed upon necropsy will help confirm the
increased incidence of this cancer. Furthermore, it is important that we also perform a detailed analysis of the incidence of other cancers observed in this model. Several cases of B cell lymphoma and plasma cell lymphoma were observed in combination with Histiocytic Sarcoma or discretely, but a detailed investigation of their incidence remains to be completed.

Our studies support that CHC1L is a tumor suppressor gene. Through further characterization the mouse gene knockout mutation and associated molecular mechanisms, we may identify and validate pathways involving CHC1L as therapeutic targets for the treatment of Histiocytic Sarcoma.
Chapter 6: Discussion

6.1 Knockout strategy

This project studied the function of Chc1L through a reverse genetics approach. Using gene targeting, Chc1L was nonconditionally deleted in a C57/BL6J mouse strain. By analyzing Chc1L knockout mice, we are able to infer its role in tumorigenesis.

Based on our analyses, knockout of Chc1L appears to be successful. Deletion did not affect embryonic viability, indicating that it is not necessary for early development. Removal of exons 4 and 5 was verified by DNA-based methods, using PCR primers specific for the deletion allele in combination with primers specific for the wild-type allele. These primers were then used for genotyping.

I was unable to confidently confirm loss of CHC1L protein production by Western Blot. Several different primary antibodies were used to probe for CHC1L (see Table 4). A discrete 60-62kDa band (the predicted size of CHC1L) was not detectable in WT protein lysates collected from various tissues. Frequently, nonspecific bands were present, which may have overlapped with and obscured CHC1L. The commercially available antibodies are targeted against the human orthologue of CHC1L, and this may have reduced its specificity. Two of the antibodies used were raised in mouse, resulting in an immunoglobulin smear at 55kDa, which was often so large that it could have masked a band at 60kDa. Very recently, a group studying CHC1L
localization during spermatogenesis performed Western Blot on an array of adult mouse tissues (Wang et al. 2012). However, they produced primary antibodies by cloning the N terminal and C terminal portions of the mouse homologue themselves, producing His-tagged recombinant proteins in vitro, which were shipped to a commercial organization for generation of rabbit antisera. Since these antibodies are generated against the mouse orthologue of CHC1L, they are likely more specific than the antibodies used in this project, which are specific for the human orthologue. In the future, we may consider using these antibodies to confirm successful knockout.

Since these antibodies were not available at the time of this study, knockout was verified using an RT-PCR based approach. To confirm that exons 4 and 5 are deleted from the Chc1L transcript, we designed two separate primer pairs that amplify a sequence across the two exons. We found that Chc1L mRNA is expressed in all WT and HET tissues examined, but not in KO tissue (Figure 7C). The amplified products were sequenced to confirm their identity.

These results demonstrate at the DNA and RNA levels that Chc1L was successfully targeted in our strain. The murine Chc1L transcript does not possess an in-frame ATG sequence in its 5’UTR, nor are there any within the coding sequence of the remaining exons following recombination. It should not, therefore, be possible for translation to initiate through recruitment of a different ATG as a start codon. If this were to occur from another ATG sequence, it would result in a frameshift and a non-functional protein. However, in the future, to definitively demonstrate successful knockout at the protein level, showing knockout at the protein level is necessary.
6.2 Early phenotype

To detect a phenotype in early development, five knockout mice were necropsied at 8-12 weeks of age. No visible pathologies were observed by gross pathological analysis. Tissues were collected and analyzed for histopathology, and no lesions were detected.

As we frequently observed splenomegaly, and the bone marrow is the source of monocytes, spleen cells and bone marrow was aspirated from three pairs of young mice and cultured in cell media containing LPS. LPS, a component of the outer membrane of gram-negative bacteria, acts as a mitogen for inducing proliferation of B cells (Smithwick et al. 2003). LPS also induces maturation of dendritic cells in primary bone marrow cultures (Granucci et al. 1999).

After two days of culture, cell survival was measured using the MTT assay. A significant increase in cell survival was demonstrated for cells isolated from both tissues. This suggests that B cells of the spleen, and dendritic cells from the bone marrow of KO mice are displaying an exaggerated proliferative response. Based on this information, even though mice at 8-12 weeks have not developed cancer, splenic B cells and possibly immature B cells resident to the bone marrow are capable of hyperproliferating in response to mitogens. Additionally, dendritic cells that have undergone maturation from bone marrow progenitor cells in the presence of LPS are also hyperproliferative.

To better understand the increase in survivability, cell cycle analysis of bone marrow cells was performed by flow cytometric analysis of DNA content. However, relative proportions of cells in each phase did not change significantly. It was expected that if an excess of KO cells
were leaving a quiescent state to enter cell division, there would be a marked increase in G2/M and S phase cells. Also, if fewer cells were undergoing apoptosis or senescence, a difference in cells with subdiploid DNA content would have been observed. It is possible that the increase in survival is due to faster cell division due to unchecked cell cycle transitions, which would result in a larger number of cells, but not necessarily change the proportion of cells in each phase.

The MTT assay relies on the reduction of MTT by mitochondrial reductase, which is present in living cells. Therefore, another interpretation of these results is that mitochondrial metabolism is increased in bone marrow and spleen cells of young KO mice. However, it has long been understood that cancer cells rely on glycolytic energy production, and mitochondrial metabolism is typically reduced, a phenomenon known as the Warburg effect (Warburg 1930). Since the cells have not progressed to a malignant stage, the Warburg effect may not have initiated. Also, it is possible that an increase in mitochondrial replication resulting in a larger number of active mitochondria could have caused this effect. Indeed, some cancer cells have been shown to have heightened mitochondrial replication (Shapovalov et al. 2011).

Since the MTT assay is a colourimetric representation of the number of living cells, to definitively demonstrate increased proliferation of KO splenocytes and bone marrow cells, the Ki67 assay should be used, which specifically measures proliferation.

In the future, a more defined cell population would also be beneficial in understanding the effects of Chc1L knockout on B cells and histiocytes. Here, the use of total bone marrow and spleen populations may have diluted the effects on the cell types of interest. In the case of B cells, purification from total spleen homogenate will improve the meaningfulness of our results. Also, as we only studied the changes of dendritic cell growth, complementing this study by
measuring macrophage proliferation in the presence of macrophage growth factors M-CSF and GM-CSF will help us define the changes in histiocyte growth following Chc1L knockout.

6.3 HS and HAL pathogenesis

While no tumors were found in 8-12 week old mice, aged KO mice sacrificed at approximately 2 years of age had a significantly greater incidence of tumorigenesis compared to WT. Tumors from the most commonly affected organs, the spleen, mesenteric lymph nodes, and liver, as well as several tumors found in other tissues, were collected for histopathological analysis. Diagnosis of HS was made based on the morphological appearance of the tumor cells, as outlined previously (Hao et al. 2010): cells round to spindle-shaped in morphology, containing large amounts of lightly staining cytoplasm and pleiomorphic nuclei. HAL or tumors that may have been composites of HS and BCL were diagnosed based on these same morphological characteristics of histiocytes, plus the appearance of large populations of lymphocytes within tumor cell foci. The cellular morphology, frequent co-occurrence of multinucleated giant cells within tumors, extramedullary hematopoiesis occasionally occurring within the liver and spleen of affected mice, as well as the distribution of affected organs were all symptoms highly suggestive of HS. Several histiocyte-rich neoplasms also contained abnormal proliferations of B lymphocytes. These may represent cases of BCL co-occurring with HS, or may be instances of HAL. Currently, there is no way to differentiate between HS+BCL vs HAL as no marker for malignant histiocytes has been discovered.
As described in the legend of Table 6, not every organ was analyzed for histopathology, and, therefore, instances of tumorigenesis not observable upon gross pathology may have been missed. However, the 22% tumor incidence observed by gross pathological analysis parallels literature incidence of HS for C57/BL6 mice, which ranges from 10.4% in females to 22.2% in males at two years of age (Frith 1990; Lacroix-Triki et al. 2003). Also, the number of HS cases occurring in KO mice represents an incidence already greater than that predicted by literature.

Based on current diagnostic criteria to definitively diagnose HS (Hao et al. 2010), IHC for histiocyte markers must be performed. The current WHO standards for HS diagnosis no longer require the absence of B cell markers due to evidence of transdifferentiation from B cell to histiocytes. While two cases of Pax5-expressing HS cells have been described in the mouse (Hao et al. 2010), these observations are preliminary, and in the vast majority of murine cases, HS cells do not express B cell markers in the mouse. Therefore, to confirm our diagnostic method, IHC for a histiocyte marker (Mac-2) and a B cell marker (B220) was performed on several representative tumors to validate our H+E-based diagnostic approach.

Three representative cases diagnosed as HS or HAL/HS+BCL were chosen to confirm the accuracy of the diagnoses made based on analysis of H+E tumor sections. The IHC results were in accordance with the initial diagnoses, and revealed instances of HS and HAL/HS+BCL occurring in the spleen, lymph nodes, liver, testis, kidney and lung.

As mentioned previously, to conclusively differentiate HS from B cell neoplasms, IHC analysis must be performed on each tumor. Not every tumor diagnosed by conventional H+E histopathology was stained for cellular markers. However, the IHC analysis performed on representative tumors has thus far confirmed our diagnosis of HS and HAL/HS+BCL. In the
future, IHC will be performed on all tumors to confidently diagnose each. Additionally, we will use a larger panel of B cell and histiocyte markers to validate our findings. In order to track development of the malignant processes, detailed evaluation of circulating histiocytes and B cells needs to be performed at earlier time points.

Based on this data, loss of Chc1L in mice predisposes to HS development. The frequent occurrence of HAL/HS+BCL may be explained by the common finding that mouse models of HS often develop B cell lymphomas at a similar frequency (Martin-Caballero et al. 2001; Carrasco et al. 2006; Mashima et al. 2010), and the two may be found occurring together. Perhaps this is suggestive of a common mechanism of pathogenesis that parallels evidence of a B cell precursor to HS cells found in humans (Weiss et al. 1985; Hanson et al. 1989; Feldman et al. 2008; Chen et al. 2009).

6.4 Prostate Cancer and Multiple Myeloma

Since CHC1L falls within the MDR of 13q14 in human PC, and low levels of expression have been correlated with prostate tumorigenesis, special attention was also paid toward prostate cancer pathology. However, in the 21 male HET and KO mice (8 HEs, 13 KOs) in which tumors were observed, no cases of prostate tumorigenesis were detected via gross pathology or histopathology (Figure 8). Therefore, Chc1L deletion does not influence prostate cancer pathogenesis, contrary to literature findings in human disease.

Based on literature findings, particular attention was paid to the presence of myeloma-like features. Two mice possessed malignant cells resembling plasma cells, which made up the
majority of the tumor composition, suggestive of plasma cell lymphoma or late stage, germinal center-derived B cell lymphoma. Histiocytes were a minor component within these tumors. Another case had Mott cells occasionally distributed through a tumor engulfing the intestine. Mott cells are plasma cells defective in their secretory pathway, staining brightly eosinophilic due to immunoglobulin buildup within the cytosol, which causes displacement of the nucleus to the cell’s periphery. In addition to being associated with MM, Mott cells may be found in BCL, and this finding could be attributed to B cell proliferations as were frequently observed. However, the possibility of myeloma-like features in Chc1L KO mice parallels clinical data where low CHC1L expression correlates with poor prognosis (Harousseau et al. 2004).

Indeed, flow cytometry of peripheral blood detected an increase in circulating plasma cells in some mice. In two of the three pairs examined, a distinct CD138-positive population increase was found, while the third pair had a slight increase. High levels of circulating plasma cells may be due to MM-related processes as seen in the cases discussed about, or, alternatively, they may have formed from the B lymphocyte component of HAL/HS+BCL tumors. Therefore, there is some evidence to suggest that MM or a myeloma-like process may be a component of the disease spectrum, however at a much lower incidence than HS and HAL/HS+BCL.

The lack of PC development and relatively low incidence of MM features compared to HS or HAL/HS+BCL may be due to retained activity of other genes found at 13q14, loss of which may preferentially promote these cancers. Alternatively, in MM and PC patients where CHC1L expression was decreased or the gene itself was deleted, unrelated mutations may have also predisposed to these particular phenotypes. In futures studies, this area may be explored by
crossing \textit{Chc1L} KO mice with strains possessing other mutations seen in MM and PC, and detecting the synergy of these genetic changes in promoting development of these cancers.

\section*{6.5 Penetrance and haploinsufficiency}

While only 22\% of wild-type mice had visible signs of pathology, the incidence of tumors in HET and KO mice was remarkably high, suggesting a powerful role for Chc1L in inhibiting tumorigenesis. 56\% of HET mice had at least one tumor upon gross necropsy, while the incidence was, as expected, even higher in KO mice (80\%). Despite not reaching significance compared to WT incidence, the tumor incidence in HET mice is intermediate between WT and KO incidences, and therefore may be an accurate representation of tumor occurrence for mice of this genotype. This could be validated using a larger sample size of both WT and HET mice.

The severity of the HET phenotype was surprising. The incidence was dramatic for a heterozygous genotype, and most HET mice had multiple affected organs. Knudson’s two-hit hypothesis defines a tumor suppressor gene as being haplosufficient (Knudson 1985). Many well-known tumor suppressors are associated with familial cancer susceptibility syndromes. These patients are born with germ-line mutations in one allele of a tumor suppressor gene. The remaining functional copy is sufficient to regulate cell growth, but when the second allele is targeted by mutation, proliferation becomes abnormal and results in tumorigenesis. By this definition, \textit{CHC1L} does not appear to encode a tumor suppressor gene. However, since the two-hit hypothesis was originally proposed, its requirement as a tumor suppressor characteristic is no longer absolute. Tumor suppressor genes (TSGs) are thought to exist on a spectrum of ranging
from totally haplosufficient to haploinsufficient (Cook and McCaw 2000). BRCA1 and 2 are well-studied tumor suppressors that are considered totally haplosufficient. Germline heterozygosity for BRCA1 and 2 is known to confer susceptibility to breast and ovarian cancer (Lancaster et al. 1996; Lengauer et al. 1998). In vitro, MEFs heterozygous for BRCA2 show no difficulty in performing DNA repair (Lancaster et al. 1996). This functional capacity underscores the primary characteristic of a Knudson two-hit tumor suppressor gene. The inability of heterozygous BRCA1 and BRCA2 cells to undergo clonal expansion is likely what makes these mutations rare in sporadic cancers (Quon and Berns 2001), since the initial mutation of one allele is unlikely to provide a selective advantage to the cell. It is this trait that characterizes haplosufficient tumor suppressor genes.

A single hit in a haploinsufficient tumor suppressor gene will provide enough of a proliferative advantage for the affected cell to clonally expand, thus increasing the target cell population available for the multistep genetic pathway to tumorigenesis. Dmp1+/− mice are prone to an array of tumors at two years of age (Inoue et al. 2001). Indeed, the affected target genes are dysregulated similarly to Dmp1−/− (Mallakin et al. 2010). Other tumor suppressor genes are haploinsufficient only under certain circumstances, such as p27+/− mice that are challenged by gamma irradiation or chemical carcinogenesis (Fero et al. 1998). In fact, homozygous loss of p27 has not been documented in human cancers (Pietenpol et al. 1995). In these cases, the malignant cells retained the functional copy of the gene of interest, indicating that LOH is not necessary for transformation. Some have argued that haploinsufficiency is due to the requirement for abnormally high levels of gene product, and that haploinsufficiency arises under conditions of insufficient levels of a certain protein (Deutschbauer et al. 2005).
However, in both of the p27 and Dmp1 models, homozygously deleted mice had a more severe phenotype than the heterozygotes. The second allele must retain some amount of activity such that its deletion will further promote cancer development. This suggests that even Dmp1 and p27 are not completely haploinsufficient. In fact, most TSGs probably fall at an intermediate position on the continuum of haplosufficiency-haploinsufficiency. This study resembles these findings in that HET mice display an obviously increased incidence in tumorigenesis compared to WT mice, implying a degree of haploinsufficiency, yet the tumorigenic response to loss of both alleles is measurably higher, suggesting the presence of a single wild-type allele has tumor suppressive activity.

The data presented here suggests that *Chc1L* lies toward the haploinsufficient end of the continuum. As both heterozygous losses at 13q14 occur in MM, CLL and PC (Latil *et al.* 1999; Hanlon *et al.* 2009; Hanlon *et al.* 2009), there is evidence to suggest some degree of haploinsufficiency of *CHC1L* in human cancer. Studies of *CHC1L* expression in MM correlate low expression with a severe prognosis (Harousseau *et al.* 2004). This suggests a dose-dependent effect of CHC1L on disease phenotype, where lower expression levels may be promoting disease progression.

LOH analyses of *Chc1L*+/- tumors would improve our understanding of the degree to which *Chc1L* is haploinsufficient. If the remaining copy is still functional in highly malignant tumors, this would imply that although Chc1L may be acting as a tumor suppressor, it is profoundly haploinsufficient. *In vitro* studies of the effects of low, moderate and high levels of *CHC1L* will increase our understanding of the functional capacity of CHC1L protein.
6.6 Transdifferentiation

Several mice displayed a cancer phenotype consistent with both HS and B cell lymphoma. Many of these mice have likely succumbed to HAL. HAL represents a diversity of histiocyte-rich lymphomas, wherein normal histiocytes are either admixed with malignant populations of lymphocytes with clonal Ig rearrangements (B cell lineage HAL), OR admixed with cells possessing Ig and TCR rearranged loci (HAL with B and T cell lineages) (Morse et al. 2001). However, it has been proposed that some cases of HAL may in fact be composites of both HS and B or T cell lymphomas (Hao et al. 2010). While Mac-2 is a marker for both malignant and normal histiocytes and is useful for detecting these cells in tumors as well as healthy tissue, the lack of established phenotypic or genotypic features for differentiating malignant histiocytes and normal histiocytes means it is presently impossible to definitively diagnose a tumor as HAL or HS and lymphoma co-occurring. Further characterization of HS cells will prove or disprove the existence of these composites.

If some of the tumors described here are indeed HS co-occurring with BCL, it may suggest a relationship between the two cancers. Indeed, the first data suggesting transdifferentiation from malignant B lymphocytes to HS cells comes from studies of HS co-occurring with lymphoma (Weiss et al. 1985; Hanson et al. 1989; Feldman et al. 2008; Chen et al. 2009). In these studies, B cell genotypes as well as expression of B cell markers were found in the HS cells. Presently, there is very little evidence for transdifferentiation from BCL into HS in the laboratory mouse. There have been no studies of murine HS that revealed a genotype suggestive of lymphocytic lineage, however a pair of cases of HS cells expressing the B cell transcription factor Pax5 have been identified (Hao et al. 2010). Nevertheless, it is interesting
that knockout of a gene involved in B cell neoplasms would produce this phenotype. This finding may be due to Chc1L’s tumor suppressive functions in a range of cell types, but the possibility for transdifferentiation is worth further analysis.

The plasticity of hematopoietic cell lineages has been a topic of great interest in recent years (Graf 2002). Recent discoveries demonstrating the plasticity of cells of the hematopoietic system suggest three mechanisms: dedifferentiation, transdifferentiation, or a combination of the two (Xie and Orkin 2007). B cell lineage commitment is established by the expression of three genes, E2A, EBF1, and Pax5. Failure to express any one of these genes will halt B cell development at an early stage (Busslinger 2004). The effects of loss of Pax5 expression in development of macrophage and other myeloid phenotypes are well studied. B cell lymphopoiesis will proceed up until a progenitor-B cell stage in adult Pax5−/− mice (Nutt et al. 1997), and Pax5−/− pro-B cells maintain the ability to differentiate into macrophages under in vitro ectopic expression of myeloid transcription factors CEBPα (Heavey et al. 2003), β (Xie et al. 2004) and GATA-1,2 and 3 (Heavey et al. 2003), with proceeding expression of myeloid genes and repression of B cell genes (Heavey et al. 2003). Enforced expression of C/EBPα and β in mature B cells also leads to loss of Pax5 expression, downregulation of its target CD19 and development of a myeloid phenotype (Xie et al. 2004). Pax5−/− pro-B cells are unable to differentiate into mature B cells unless Pax5 expression is restored retrovirally (Nutt et al. 1999).

Further, conditional deletion of Pax5 in mature B cells causes dedifferentiation into uncommitted precursors, and subsequent T lymphopoiesis in T cell deficient mice (Cobaleda et al. 2007). This data suggests that Pax5 is required for commitment to and differentiation along the B cell lineage, and that cells of the B lineage lacking Pax5 may acquire an uncommitted potential. Recent data from patients with B cell genotypic HS show that the malignant histiocytes are
negative for Pax5 (Vos et al. 2005; Chen et al. 2009; Hayase et al. 2010; Hure et al. 2012). Based on this information, loss of Pax5 expression may be an important event in trans-/de-
differentiation into a histiocyte-like tumor cell. However, the existence of cases of murine HS
with tumor cells that express Pax5 may be due to alternative pathways of lineage plasticity or a
cross-species difference (Hao et al. 2010).

We have crossed Chc1L^{loxp/+} mice to a strain expressing Cre recombinase under a B cell-
specific promoter to generate conditional knockout in B cells. HS pathogenesis in this model
would provide strong evidence for transdifferentiation. Additionally, further IHC analyses of the
nonconditional knockout strain using primary antibodies for stage-specific B cell markers may
provide evidence for transdifferentiation.
Chapter 7: Future Directions

7.1 *Validate increased prevalence of HS*

This study has provided evidence for the tumor suppressing activity of *CHC1L*. Specifically, *Chc1L* knockout appears to increase incidence of HS in two year old mice. Our primary objective for future investigation is to validate the results presented here. We have already bred two new generations of *Chc1L* knockout mice, and plan to study tumorigenesis at 6 months and 1 year of age. Each group will consist of 30 mice split evenly amongst WT, HET and KO genotypes. Mice will be sacrificed collectively at each time point, and all organs will be collected for histopathological analysis, with special interest in spleen, mesenteric lymph node, liver and bone marrow pathology, based on their frequent involvement in HS and HAL. We will also search for macrophage accumulation in the lungs as a precursor to HS development, as seen in a previous triple knockout model of HS (Mashima *et al.* 2010). For these future studies, every organ, including WT and healthy-looking HET and KO organs, will be collected and analyzed for histopathology. Therefore, cases of early tumorigenesis that are not observable during necropsy will be accounted for. This will allow for a more accurate quantitative analysis of tumor incidence and disease spectrum. Also, by studying disease progression at an earlier age, an elevated incidence of tumorigenesis will be more meaningful because age-related neoplasias will be reduced.
Immunohistochemical staining will be applied to all tumor sections to definitively diagnose neoplasms. Antibodies for Mac-2 and CD163 will be used to detect histiocytes, and B220 antibodies for detection of B cells. To further characterize these tumors, other markers may be probed. Expression of F4/80 is inversely correlated with histiocyte maturity (McKnight et al. 1996; Schaller et al. 2002; Hao et al. 2010), and staining for this marker will provide insight into the degree of differentiation of malignant histiocytes. A panel of B cell-specific antibodies may also be used to detect lineage infidelity in histiocytic proliferations: Oct2, Pax5, CD20 and BOB.1.

Flow cytometric analyses of peripheral blood and bone marrow aspirates will be performed prior to pathological analysis. Particular attention will be paid to levels of circulating B lymphocytes, stained with B220, and histiocytes, stained with CD163. Also, cKit/Sca1 double staining will be used to specifically detect circulating stem cells. T cells will again be detected using CD3, CD4 and CD8 antibodies. At these younger ages, there will be less variation to due age-related pathology.

As a single mutation in a single subset of cells may not be sufficient to induce tumorigenesis in young mice, we may plan to use the mutagen N-ethyl-N-nitrosourea (ENU) to initiate tumorigenesis in younger mice. Mice will be divided into two groups: Group A, consisting equally of WT, HET and KO mice, will be injected with saline as controls; Group B, also made up of WT, HEs and KOs, will be injected with low doses of ENU. These animals will be necropsied and organs will be studied for histopathology. A significantly high incidence of tumorigenesis in HET mice will promote our understanding of the degree of haploinsufficiency.
7.2 Investigate B lymphocyte transdifferentiation and myeloma

We have crossed the $Chc1L^{loxp}$ knockin locus to CD19/Cre mice and generated many $Chc1L^{loxp}$-CD19/Cre mice. These mice will be used for conditional gene mutation in B cells. Since only B cells will possess deletion, the development of HS in this model will provide strong evidence of B lymphocyte transdifferentiation to a malignant HS cell.

Specific deletion of $Chc1L$ in B cells may also result in B cell tumorigenesis or MM-tumorigenesis, as MM stem cells have been shown to have features of memory B cells including CD19 expression (Matsui et al. 2008; Delude 2011). Formation of MM will support clinical evidence correlating low $Chc1L$ expression with prognosis in MM patients (Harousseau et al. 2004). B cell tumorigenesis would suggest that loss of $CHC1L$ at 13q14 in CLL promotes pathogenesis. We may also use ENU mutagenesis to initiate tumor development if loss of $Chc1L$ in a single cell-type is not sufficient to induce tumorigenesis.

7.3 Investigation of mechanistic pathway

To elucidate the mechanism of CHC1L activity, the following experiments may be performed in the future. To check for putative E3 ligase substrate adaptor functionality, interaction with CUL3 ligase must first be demonstrated. Immunoprecipitation of CHC1L-overexpressing lysates followed by Western blotting for CUL3 will be performed. Alternatively, CHC1L may be FLAG-tagged, and an antibody for the FLAG-tag can be used for co-immunoprecipitation. HA-tagged CUL3 plasmids already exist (Plafker et al. 2009), and can be co-transfected with Flag-CHC1L. Pulldown may similarly be performed to demonstrate
interaction with Ran. Antibodies that bind Ran nonspecifically in both its forms may be used for measuring changes in Ran protein levels when expression of CHC1L is altered. Shifts in electrophoretic mobility due to polyubiquitylation in the presence of CHC1L may also be detected using this method. Other potential substrates can be identified by yeast-two hybrid analysis.

To look into Ran guanine exchange factor activity, Western Blot using antibodies specific to the GDP or GTP-bound forms of Ran may demonstrate altered activity of RanGTPase in conditions of CHC1L under- and over-expression. Fluorescence Resonance Energy Transfer (FRET) can also be used to detect Ran activity using an antibody that binds to Ran and, upon excitation, emits light of a particular wavelength in each of Ran’s guanine nucleotide-bound states, thus allowing real-time dissection of CHC1L’s potential impact on Ran activity. Changes in other major cell cycle pathways will also be explored by Affimetrrix microarray, and confirmed by RT-PCR and Western Blot.

To complement in vivo analyses, functional characterization of CHC1L will be performed in vitro. I have already demonstrated using Western Blot that HepG2 cells express CHC1L, as the antibodies were more specific for the human homologue (Figure 23). siRNA knockdown will be performed, and the effects on cell cycle regulation, proliferation, and apoptosis will be studied. I have previously screened a series of cancer cell lines for expression of CHC1L, identified HepG2 as expressing high levels of CHC1L, and optimized the transfection protocol using FAM-labelled siRNAs (Figure 24). Changes in cell cycle will be measured by propidium iodide staining of synchronized, siRNA-transfected cells. The effects on proliferation and apoptosis will be assessed using the MTT cell survival assay and, if necessary for validation,
Ki67 and TUNEL staining. The same studies will be performed in the context of *CHC1L* overexpression.

**Figure 23 Human cell line analysis of CHC1L expression**
A panel of MM, PC, and liver cancer cell lines were analyzed for CHC1L expression to identify candidates for siRNA knockdown. The antibody used specific for the human orthologue, as this antibody did not work on mouse samples. HepG2 had the highest expression levels of CHC1L and was chosen for knockdown experiments.

**Figure 24 siRNA transfection optimization**
Figure shows successful transfection of FAM-labelled siRNA, which was optimum at 0.12uM siRNA, 8ng/μL Lipofectamine.
7.4 Clinical disease association studies

If Chc1L’s tumor suppressive role in murine HS is confirmed, it will be important to extend our findings to translational studies. Primary human HS samples will be acquired. Direct sequencing will enable detection of CHC1L mutations. A tissue microarray will be constructed from patient samples and IHC may be used for quantification of CHC1L expression. Mutation frequency and expression levels will be compared to patient prognosis to evaluate the effects of CHC1L on HS disease progression.
References


Mashima, R., K. Honda, et al. (2010). "Mice lacking Dok-1, Dok-2, and Dok-3 succumb to aggressive histiocytic sarcoma." Lab Invest 90(9): 1357-64.


