Chromatin-Modifying Factors in Zebrafish Models of Rhabdomyosarcoma and Hematopoiesis

Dissertation Advisor: Leonard I. Zon Colleen E. Albacker

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Abstract

Epigenetics, or the reversible and heritable marks of gene regulation not including DNA sequence, encompasses modifications on both the DNA and histones and is as important as the DNA sequence itself. Gene transcription, DNA repair, DNA replication, and the cell cycle are each impacted by the chromatin structure. A variety of enzymes modulate these modifications, and a suite of factors interacts with them to aid in promoting or inhibiting cellular functions. Many of these chromatin-modifying factors are deregulated in cancer, making them novel therapeutic targets. This dissertation describes the identification of an H3K9 histone methyltransferase, SUV39H1, as a suppressor of rhabdomyosarcoma formation in zebrafish. This suppressor is dependent on the methyltransferase domain of the enzyme, ruling out any scaffold effects since this enzyme is a part of a multiprotein complex. SUV39H1-overexpressing and control tumors share many of the same characteristics, including proliferation rate, muscle differentiation state, and tumor growth rate. The tumor suppressive phenotype cannot be rescued by alterations in the downstream muscle program alone. However, SUV39H1-overexpressing fish initiate fewer tumors, which results in the observed suppressive

phenotype. This initiation defect occurs between 5 and 7 days of life in the zebrafish, likely by impacting cyclin B1 expression. This dissertation also describes the development of a novel F1 transgenic screening strategy in the zebrafish. This approach was utilized to screen a variety of chromatin-modifying factors for their effects on hematopoietic development. The developed strategy will have future applications as a zebrafish screening tool. Our data suggest that chromatin-modifying factors play an important role in rhabdomyosarcoma and illustrate the use of the zebrafish in discovering genes involved in tumorigenesis and hematopoiesis.

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and my husband, Lee Albacker,

with the sentiment that only the best can convey:

Baby, baby, baby

when all your love is gone

who will save me

from all I'm up against out in this world

-mbt

Chapter 1

Introduction

Chromatin

Every living creature carries DNA in its cells as its blueprint for life. From bacteriophages that contain their eleven genes in 5000 base pairs (bp) to humans that have their twenty thousand genes encoded across 3.3 billion bp, each cell of the organism contains the entire genome. This DNA must be packaged efficiently yet remain accessible for potential gene transcription. The chromatin structure of a nucleus, in which the DNA and proteins are packaged together, ensures this balance. In eukaryotic organisms, DNA is packaged into nucleosomes, where 147 bp are wrapped twice around an octamer of histone proteins; the octamer contains two copies each of histones H2A, H2B, H3, and H4 [1-5]. The linker histone H1 sits at the DNA entry and exit points of the nucleosome beads, and the neighboring beads are connected by up to 80 bp of DNA [5-8]. This somewhat open structure is known as euchromatin. The nucleosome structure, resembling beads on a string, can be compacted further along the H1 proteins to create a 30 nm fiber (heterochromatin), and the addition of other scaffolding proteins can compact the structure even further, eventually resulting in the most densely packed metaphase chromosome [9-12].

To control the thousands of eukaryotic genes buried among billions of compacted base pairs requires another level of regulation known as epigenetics. Epigenetics is the reversible and heritable marks of gene regulation, which have proven to be just as important in the field of genetics as the DNA sequence itself. Epigenetic marks seem to be among the first developmental instructions that an

embryonic cell receives and continue through the life of an organism to mark changing states of a cell without ever affecting the nucleotide sequence [13, 14].

One method of epigenetic regulation is to mark the DNA itself. This is done through methylation of cytosine bases with the family of DNA methyltransferase (DNMT) enzymes. This mark silences the genes and non-coding regions in the methylated sequence and tends to occur in repetitive regions [15, 16]. The covalent methyl mark can be maintained through cell divisions when it occurs at the dinucleotide pair of a cytosine next to a guanine (CpG) [17, 18]. Of the three main DNMTs, DNMT1 is responsible for maintaining the methylations after cell division whereas DNMT3A and DNMT3B are *de novo* enzymes that initiate new methylations [19-22]. By methylating CpG islands in promoter regions, DNMTs can directly shut off gene expression by making the promoter inaccessible to transcription factors. Additionally, methyl-CpG-binding domain (MBD) proteins can bind the DNA methylations; MBD proteins can recruit other chromatin factors to silence the gene or promote further methylation of the DNA [23, 24].

A second level of epigenetic regulation occurs on the tails of the histone proteins. Each of the histones in the nucleosome has a flexible charged N terminal tail coming out of the nucleosome bead containing twenty to thirty amino acids that can be modified by methylation, acetylation, ubiquitination, phosphorylation, and sumoylation [25-27]. There are over sixty residues associated with these modifications, and new marks and residues are constantly being discovered [27, 28]. These marks are generally associated with activation or repression of transcription. This can depend on the marks physically altering the chromatin structure into an

open or closed conformation. Additionally, other proteins can recognize and bind the different marks, such as chromodomain proteins binding acetyl marks and bromodomain proteins binding methyl marks, which then recruit other factors important for cellular and transcriptional function [29, 30]. A combination of marks at different sites is known as the histone code, with the precise combinations resulting in particular biological functions. The collections of enzymes that add and remove these modifications as well as the proteins that interact with them are known as chromatin-modifying factors.

Histone methylation is associated with both transcriptional activation and repression, depending on the specific mark. Lysine residues on the histone tails are subject to mono-, di-, and tri-methylation [27]. Two classes of HMTs (histone methyltransferases) catalyze the reaction on the lysines, those with a SET domain and those without [31, 32]. Arginine residues can accept mono- or di-methyl groups [27]. PRMTs (protein arginine methyltransferases) catalyze the reaction on the arginines [33]. Methylation was thought to be a permanent histone mark until the more recent discovery of histone demethylases, completing the understanding of how the mark is reversible [34]. Methylation on histone H3 lysine 4 (H3K4) is a mark of transcriptional activation and is associated with the enzymes MLL and Set7 [35, 36]. Also linked to transcriptional activation are H3K36, which is methylated by Set2, and H3K79, which is methylated by DOT1 [32, 37]. In contrast, H3K9 is associated with repression and heterochromatin formation; this is mediated by enzymes such as SUV39H1, G9a, and SETDB1 [31, 38, 39]. Additionally, methylation at H3K27 is a mark of transcriptional repression as well, mediated by G9a and EZH2

[38, 40]. The arginine methylations also have mixed outcomes since PRMT5 methylates H3R8 to repress transcription, whereas CARM1 methylates H3R17 to activate transcription [41, 42].

Acetylation is most often seen as an epigenetic transcriptional activator, with hyperacetylated histones a hallmark of active chromatin. Both histone acetyltransferases (HATs) and histone deacetylases (HDACs) have been found in vertebrates, using acetyl-CoA to place and remove the acetyl mark, respectively [43]. A commonly acetylated mark associated with transcriptional activation is H3K14, which can be added by enzymes including Gcn5, PCAF, Esal, Tip60, SRC-1, TAF1, and p300 [44-50]. Gcn5 and p300/CBP are also known to acetylate H3K23 [42, 44, 51]. These same enzymes, along with ATF2 and Elp3, acetylate H4K5, H4K8, H4K12, and H4K16 to activate gene transcription as well [44-49, 52, 53].

Cellular functions relying on the epigenetic state

The epigenetic state of a cell is important for many cellular processes, including transcription, DNA repair, DNA replication, and the cell cycle. Being able to transcribe a gene requires the DNA to be accessible to the transcription machinery; conversely, closing the chromatin, thus denying access to the DNA, can facilitate repression of gene transcription. Since histone modifications dictate the open versus closed state of the chromatin, they play a key role in regulating transcription. Acetylation is associated with transcriptional activation for two main reasons. First, the acetyl group decreases the charge of the histone tail, weakening the histone's interaction with DNA and creating a more physically open state [54]. Second, acetylated histones are bound by chromatin-modifying factors that are proteins associated with RNA polymerase or that directly

recruit the polymerase for transcription [55]. The impact of methylation on transcription depends on the specific mark. H3K4 and H3K36, both correlated with activation, recruit activators to the nucleosomes, some of which have been co-purified with RNA polymerase II [37, 56-59]. H3K9, H3K20, and H3K27 induce a closed chromatin state while also recruiting transcriptional repressors, such as Polycomb Group proteins [60]. Ubiquitin modifications also vary in their transcriptional response, as the mark on H2A K119 represses while ubiquitin on H2B K120 activates transcription [61, 62]. Phosphorylation is associated with active transcription, particularly at H3S10 [63]. In addition, crosstalk of phosphorylated H3S10 can augment acetylation of H3K14, eliminate acetylated H3K9, and block methylated H3K9 [31, 64-66]. Transcription is unmistakably influenced by the epigenetic histone marks.

DNA repair is another process that requires an open chromatin state. The repair machinery must be able to access the broken DNA region, and nucleosomes and individual histones may need to be shifted to facilitate the repair process. One of the first steps in DNA repair is the phosphorylation of the H2A variant H2AX by PI3K at the damage site, which acts to recruit and maintain the necessary repair proteins [67-70]. This H2AX phosphorylation can also bring in HATs to acetylate other residues and relax the chromatin structure [69]. Acetylation is known to be essential for DNA repair, as cells are more sensitive to DNA damaging agents when they cannot acetylate H3K56 [71]. Methylation of yeast H4K20 is required in the recruitment of Crb2 to sites of DNA breakage, which is a critical protein to activate the cell cycle checkpoint; a similar mechanism is in place in humans with the Crb2-equivalent 53BP1 [72, 73]. Ubiquitination of histones H3 and H4 plays a valuable role during UV-induced DNA

damage in recruiting the XPC repair protein [61]. Lastly, phosphorylation of H4S1 occurs after the repair is complete, coinciding with the decrease in acetylation levels; this mechanism likely reinstates the normal chromatin state [74].

DNA replication is a process that must be highly accurate, for both the DNA sequence and the maintenance of the cell's epigenetic state, to provide the correct genetic information to both daughter cells. During replication, the DNA polymerase and proteins associated with the replication fork need to access the DNA, resulting in disruption of the nucleosomes. Following replication, the nucleosomes must be reassembled with their proper histone modifications. As with the other processes, acetylation is critical to opening the chromatin structure to make the DNA available. In yeast, higher levels of histone acetylation are correlated with an earlier firing of the replication origin [75]. Similarly in humans, the replication timing is linked to acetylation level [76]. Furthermore, the HAT complex HBO1 is associated with MCM2, a replication factor, as well as the origin recognition complex 1 [77, 78]. Without this complex or its HAT member, ING5, S phase progression is inhibited [79].

SUV39H1, an HMT with important cellular roles

One of the quintessential sets of experiments to identify genes encoding chromatin proteins occurred in *Drosophila*. This relied on the concept of position effect variegation (PEV), where chromosomal rearrangements place regions of euchromatin next to heterochromatin. PEV was first discovered in the 1930s in fruit flies, when radiation-induced mutants showed variable expression of the *white* gene, leading to mosaic eye color in the flies [80]. The phenomenon was later found to be caused by chromosomal breakage in heterochromatic regions, resulting in the

spreading of heterochromatin marks and silencing of the normally transcriptionally active euchromatin region [81, 82]. Two laboratories embarked on a large genetic screen, looking for enhancers and suppressors of PEV, with the idea that these mutations would identify genes involved in regulation of chromatin structure [83, 84]. They found over one hundred genes that could suppress or enhance PEV; most had dominant, dose-dependent effects where loss of one allele led to the suppressor or enhancer effect. However, a few suppressors also had dose-dependent effects in the presence of a duplication, where losing one copy reversed the enhancer effect and losing two copies led to the suppressive phenotype, and one of them was Su(var)3-9, or suppressor of variegation 3-9 [85].

Su(var)3-9 was of particular interest because it contained both a chromo domain and a SET domain, two highly evolutionarily conserved chromatin domains. The suppressor mutations isolated in Su(var)3-9 were dominant over nearly all of the enhancer mutations tested, suggesting this gene had an important role in gene inactivation [86]. Correspondingly, a gain-of-function mutation in Su(var)3-9 led to ectopic heterochromatin formation [87]. Additionally, an orthologous gene was found in the fission yeast *Schizosaccharomyces pombe* as *clr4*, involved in centromere silencing and mating type switching through the action of each of the critical domains [88-90]. Mammalian homologues were later isolated, which also play a role in heterochromatin and mitotic centromeres [91]. It was with the mammalian SUV39H1 that the Su(var)3-9 family of proteins were demonstrated to be H3K9me3 HMTs with the catalytic SET domain [31].

SUV39H1 and its corresponding H3K9 methylation have a variety of impacts on chromatin structure and cellular function as a major transmitter of heterochromatin. First, the silencing methylation itself depends on the H3K9 residue being deacetylated; this is why SUV39H1 is physically associated with HDAC1 across many species [92-94]. SUV39H1 has also been shown to interact with other HMTs, including SETDB1, G9a, and GLP, allowing for specificity among the enzymes as mono-, di-, and trimethyltransferases [95]. One of SUV39H1's important roles, along with the closely related SUV39H2, is to control the heterochromatic state of telomeres; cells lacking SUV39H1 and H2 have abnormal telomere elongation [96]. SUV39H1 is important during mammalian development and, in particular, the balance between proliferation and differentiation in cell fate decisions [97]. It interacts with heterochromatin protein 1 (HP1), and together these proteins are recruited by the retinoblastoma (Rb) protein as the mechanism in which Rb silences S phase genes [98-101]. In mice, Suv39h1-mediated H3K9 methylation acts to suppress a set of genes upon lineage specification in the murine blastocyst, implicating Suv39h1 and its regulation of chromatin structure in early development [102].

Chromatin in Zebrafish

Zebrafish as a model organism

Danio rerio, commonly known as the zebrafish, is a member of the teleost family, consisting of bony fish with rayed fins. Technically easy to work with, the zebrafish has traditionally been an excellent vertebrate model organism for

studying development. Zebrafish eggs are fertilized externally, development occurs entirely *ex vivo*, and embryos are optically clear, making their eggs accessible and amenable to genetic manipulation. They are competent for large-scale genetic screens since their small size requires less space than mammalian systems. In addition, females can produce one to two hundred eggs every week, and their generation time is short. The zebrafish genome is fully sequenced, facilitating the use of genetic tools.

Zebrafish development occurs rapidly over the course of a few days, all able to be visualized through the clear chorion. Immediately after fertilization, the zygote remains in the one-cell stage for forty-five minutes, permitting genetic manipulations as nucleic acid injections can be performed at this stage. Gastrulation to form the three primary germ layers commences around 5 hours post fertilization (hpf). Somites and organs begin to form beginning at 10 hpf. Shortly after 24 hpf, circulation and pigmentation commence; by 48 hpf the zebrafish begin hatching from their chorions and by 72 hpf are swimming freely [103].

Chromatin studies in zebrafish development

Numerous studies utilizing zebrafish as a model organism have demonstrated the important and varied roles that chromatin plays in vertebrate development. The first study noted that injected plasmids were methylated *de novo* early in embryonic development to repress transcription; expression of the injected transgene could be induced by treatment with 5-azacytidine to block this DNA methylation or sodium butyrate to block histone deacetylation [104]. Since then, the zebrafish has proven useful in other large-scale studies. By profiling H3K4 mono- and tri-methylation marks in

embryonic zebrafish, *cis* regulatory features were identified, leading to a more defined transcriptional network [105]. Epigenetic modifications have been shown to specifically mark genes needed during development, whether with marks solely associated with active transcription or also with repressive marks to ensure the genes are off but ready, which often occurs as early instructive signals before the onset of zygotic genome activation [106, 107]. Large scale profiling has also been used for cancer gene expression, noting that there is global genomic repression due to silencing histone methylation modifications in zebrafish melanoma, similar to what is observed in human melanomas [108].

Several HDACs have been shown to control critical steps in zebrafish embryonic development. In one study, HDAC1 was shown to be necessary for proper formation of craniofacial cartilage, the pectoral fins, and the retina, as both mutant fish and morpholino-injected fish exhibited deformities [109]. HDAC1 has also been shown to be required for blood stem cell development [110]. In another study, loss of HDAC1 caused defects in specification and differentiation of both the liver and exocrine pancreas while producing ectopic endocrine pancreatic clusters [111]. It was demonstrated that HDAC3 also plays a role in liver and exocrine pancreas development, as blocking it with valproic acid or with a anti-sense blocking morpholino impaired their organogenesis; these effects could be rescued by HDAC3 overexpression [112].

Other chromatin-modifying factors have been demonstrated to act as regulators of zebrafish myogenesis. Chd7, a chromodomain protein, controls left-right symmetry in the developing somites, as loss of the gene results in asymmetric segmentation of the mesoderm [113]. Knockdown of Brg1 leads to a stunted tail and disorganization of actin

in the muscle, phenocopying loss of Dicer; this observation led to the discovery that Brg1 regulates microRNAs important for the myogenic program [114]. DPF3, a novel epigenetic factor that can bind both acetylation and methylation marks, acts specifically in muscle and heart development, recruiting the BAF chromatin remodeling complex to complete cardiac looping and proper muscle fiber assembly [115]. The timing of myogenesis is controlled by Smarcd3, a SWI/SNF remodeler family member, as its expression triggers myod and myf5 expression [116].

Several other chromatin-modifying factors play key parts in zebrafish embryonic development. Brg1 is important for accurate retinal differentiation, craniofacial formation, brain patterning, sensory neuron formation, and neural crest induction [117]. The BAF complex, which includes members Brg1 and Brm to promote chromatin remodeling, exists in a delicate balance with cardiac transcription factors to moderate heart development; this repression caused by the BAF complex may be the mechanism underlying cardiac transcription factor haploinsufficiency [118]. A double morpholino injection for Hmgb1 and Hmgb2 revealed that these two factors play redundant roles in maintaining Wnt signals throughout the embryo and Shh expression in the developing pectoral fin; this parallels research in mice that the loss of these two genes reduces Shh levels, resulting in loss of the fifth digit [119].

The zebrafish embryo has acted as a platform for chromatin enzymatic discovery and characterization. Zebrafish were utilized for demonstration of the first reproducible DNA demethylase, involving the combination of a deaminase and glycosylase, with this interaction enhanced by Gadd45 [120]. After showing it could demethylate H3K9 and H3K27 *in vitro*, researchers moved novel demethylase KDM7 into the fish to display its

in vivo function as a remover of repressive marks; knockdown of KDM7 resulted in developmental defects in the brain, including reduced size and number of neurons [121]. Similarly, when the SMCX family was discovered as a family of H3K4me3 demethylases, the *in vivo* studies were performed in fish, showing a cell autonomous role for SMCX in neuronal survival and brain patterning [122]. Two sets of chromatin-modifying enzymes have been shown to act together as a cell fate network during development. Dnmt1 and SUV39H1 appear to work together to control differentiation of the intestine, exocrine pancreas, and retina, as individual knockdown of one gene phenocopies the other and overexpression of one can rescue knockdown of the other [123]. Likewise, Dnmt3 and G9a displayed a similar epistatic interaction for neurogenesis in the brain and retina, suggesting the pair of factors plays a key tissue-specific silencing role in cell fate determination [124].

Several chromatin factors have been revealed as regulators of Hox genes in the zebrafish embryo. Hoxb1-regulated promoters are highly acetylated, and binding of Meis to Pbx at these promoters inhibits HDACs from silencing the loci in fish [125]. Bromodomain-containing Brpf1 was shown to regulate anterior Hox gene expression important for segmental identity by promoting histone acetylation through MYST3 and remaining at the chromosomes throughout mitosis [126]. These studies, and the ones mentioned above, reveal how important the zebrafish has been as a model system to elucidate mechanisms surrounding chromatin structure and the factors that regulate it.

Cancer

Cancer is the second leading cause of death in the United States, causing one quarter of all deaths each year. Over 577,000 cancer-related deaths are predicted to occur in America in 2012 [127]. The lifetime probability in the US for developing an invasive cancer is 45% for men and 38% for women. As such a large public health issue, much scientific research is focused on cancer and its underlying pathways to illuminate the molecular mechanisms causing malignancy. This is leading to the development of improved diagnostic and therapeutic practices, as is evidenced by the modest yet growing decline in mortality over the past decade [127]. Much of this research occurs in animal models to obtain the most physiologically relevant results possible.

Previously, cancer was thought of as a genetic disease, consisting of an accumulation of various genetic lesions. This was first discovered when a single oncogene was not sufficient to transform fibroblasts but a combination of two was [128, 129]. The first model using this idea of multiple hits was proposed for colon carcinogenesis [130]. This was supported by the concept of clonal evolution, where the more mutations accumulate, the more unstable the cell becomes, thus making further mutations likely [131]. At its core, cancer is a disease of unrestrained cell division, where proper checkpoints fail and cell proliferation can run rampant. Genetic mutations certainly impact these processes, but more recent research has revealed that epigenetics has just as big of an impact. This will be reviewed further below.

Tumor suppressors are genes that keep the cell cycle tightly regulated and are often silenced en route to malignancy. To develop cancer, both copies need to be lost, whether both by somatic mutations or by one germline followed by one somatic lesion [132]. The most studied tumor suppressor gene is p53, as more than 50% of cancers

contain mutations in this gene [133]. The protein can be activated by DNA damage, hypoxia, and low ribonucleoside levels, arresting the cell in G1 so that aberrant DNA synthesis does not occur. Activated p53 can also induce apoptosis if the cell cannot fix the above issues [134]. When mutated or inactivated, these functions cannot occur, leaving the cell susceptible to uncontrolled growth under conditions of DNA damage, leading to further mutations. Patients with Li-Fraumeni syndrome are heterozygotes for p53 through germline transmission, leaving only one good copy of this critical gene; this puts them at a higher susceptibility for developing cancer [135].

Oncogenes are genes that promote cell growth and are generally overexpressed or activated during tumorigenesis. The first oncogene discovered was src, part of the chicken retrovirus Rous sarcoma virus required to maintain a transformed state [136]. Since then, numerous oncogenes have been discovered. One of the earliest ones found and most common ones is RAS, a small GTPase that is activated by growth factor signaling. The RAS gene was first found to be the main transforming unit in two rat sarcoma viruses; it was later connected to human cancer as an oncogene [137-140]. RAS proteins activate several fundamental cell signaling pathways, including the MAP kinases, PI3Ks, and RALGDS proteins [141]. All three family members, HRAS, KRAS, and NRAS, are mutated in cancer, most commonly gain of function mutations at codons 12, 13, and 61; the cancer types range from pancreatic, colon, and lung adenocarcinoma to thyroid tumors to myeloid leukemias [142].

Cancer Modeling in the Zebrafish

Genetic tools for cancer research in the zebrafish

Zebrafish, traditionally known for their role in developmental biology, have emerged as a thriving vertebrate model organism for cancer research. This is made possible by the myriad of genetic tools available for use in the zebrafish. For embryonic manipulations, specific genes can be overexpressed by microinjecting mRNA into a one to two cell stage embryo. Conversely, genes can be knocked down by microinjection of morpholinos, antisense oligonucleotides that can either block translation initiation or promote frameshift mutations through aberrant splicing [143]. Mutations in specific genes can be identified via TILLING (Targeted Induced Local Lesions in Genomes), leading to functional studies by reverse genetics [144, 145]. More recently, zinc finger nucleases have been used to target mutations to desired genes for reverse genetics purposes [146, 147]. The zebrafish is also amenable to transgenic technology, both in mosaic and stable forms, by microinjecting DNA vectors into one-cell stage embryos.

Other tools of particular interest to cancer researchers have also been developed. An array comparative genomic hybridization (CGH) platform was designed specifically for zebrafish to impartially detect genomic imbalances [148]. Transplantation has also emerged, enabling the assessment of migration and self-renewal capacities of a particular tumor [149]. Transplants have been used in leukemias and myeloproliferative disorders to assess serial transplantability, melanomas to determine metastatic potential, and rhabdomyosarcomas to isolate the cancer stem cell [150-155]. Two strains of fish have been developed to facilitate the transplantation assay. First, the double pigment mutant *casper* are nearly transparent fish that allow ease of visualization of transplanted tumor cells [154]. The *casper* mutants have been combined with confocal imaging and an *in vivo* flow cytometer to create a platform for simultaneous cell visualization and tracking

in the zebrafish [156]. Second, clonal, isogenic zebrafish lines were generated, permitting tumor cell transplantation into unirradiated recipients, thus minimizing the irradiation-related immune response on the recipient fish [157].

Screens for study of cancer in the zebrafish

The potential of the zebrafish as a cancer-modeling organism was first realized upon exposure of embryos and larvae to MNNG (N-methyl-N'-nitro-N-nitrosoguanidine), a carcinogen. Upon exposure to the drug, zebrafish developed a wide spectrum of tumors, including hepatocellular adenoma and carcinoma, chondroma, biliary adenoma and carcinoma, seminoma, pancreatic adenoma, and various mesenchymal neoplasias [158]. A similar group of diseases arose in fish treated with DMBA (7,12-dimethylbenz[a]anthracene) [159]. These studies proved that zebrafish could develop cancer histologically similar to that of humans.

A large-scale, insertional mutagenesis screen was completed in the zebrafish, looking at genes essential for early development, using a mouse retroviral as the mutating agent [160-162]. Of the over three hundred genes identified, twenty-eight were ribosomal proteins. However, when heterozygous fish for each of these ribosomal mutations were grown to adulthood, seventeen out of the twenty-eight developed malignant peripheral nervous sheath tumors (MPNSTs) [163, 164]. This displayed how forward screens could be used to identify cancer causing mutations.

Another large-scale screen for cell proliferation mutants revealed multiple cancer related genes. After treating zebrafish with mutagenizing ENU (ethylnitrosourea), progeny were stained for phospho-H3, a marker of mitotic cells. The first mutation characterized was in the *bmyb* gene, which was shown to play a role in mitotic

progression; the mutants were marked by misregulation of cyclin B, genomic instability, and susceptibility to cancer when treated with MNNG [165]. Another mutant from the screen, in the *separase* gene, displayed increased apoptosis, aneuploidy, and a similar higher susceptibility to cancer upon MNNG treatment [166]. Another mutation found by increased phospho-H3 staining was that of the *sil* (SCL-interrupting locus) gene; the mutants had disorganized mitotic spindles, leading to genomic instability [167]. A similar screen revealed other mutations that resulted in genomic instability and tumor predisposition [168].

Zebrafish mutant cancer models

Several zebrafish mutants have developed cancer similar to their human counterparts, proving again the efficacy of zebrafish as a cancer model organism. Zebrafish harboring a hypomorphic allele for p53 were resistant to apoptosis following irradiation and were prone to developing MPNSTs [169]. An additional p53 mutation leads to the formation of sarcomas in homozygous mutants and heterozygotes undergoing loss of heterozygosity [170]. Heterozygous zebrafish containing a mutation in the *APC* (adenomatous polyposis coli) gene, a tumor suppressor that regulates the Wnt/beta-catenin pathway, develop intestinal, hepatic, and pancreatic neoplasias upon carcinogen treatment [171]. Mutants for the *vhl* (von Hippel-Lindau) gene form retinal neoplasms, closely mimicking the human diseases associated with this mutation [172].

Zebrafish transgenic cancer models

The first transgenic cancer model developed in zebrafish was a model of T cell leukemia, where murine c-Myc was overexpressed in the T cell compartment by the *rag2* promoter [173]. This leukemia was clonal and serially transplantable, and this study

opened up a whole field of cancer modeling using the fish. A conditional variant of the *rag2*-mMyc was later developed as well as a novel model overexpressing NOTCH to induce malignancy [151, 174]. B cell leukemia was generated by driving the TEL-AML1 fusion protein with the ubiquitous *beta-actin* promoter [150]. The first model of acute myeloid leukemia (AML) drove the MYST3-NCOA2 fusion protein behind the *spi1* promoter to produce AML in a low percentage of adult fish [175].

Transgenic melanoma was created by combining the *mitf* promoter driving $hBRAF^{V600E}$ specifically in melanocytes with a p53 null background; adding the p53 mutation provided a needed second hit, as the transgenic alone formed benign moles but no malignant tumors [176]. A second transgenic melanoma model was made using $hNRAS^{Q61K}$ behind the *mitf* promoter, also in a p53 null background [177]. Another melanoma model utilized the *kita* promoter to drive oncogenic $hHRAS^{G12V}$; these zebrafish displayed expanded melanocytes as larvae and developed tumors at an earlier onset than the previous melanoma models, making this model particularly amenable to screening [178]. A model of rhabdomyosarcoma was developed utilizing mosaic transgenic animals, caused by expression of *rag2*-$hKRAS^{G12D}$ in the muscle satellite cells [155]. A model of pancreatic neuroendocrine carcinoma, using *myod* to drive MYC, was made that histologically resembled human tumors [179]. Using the *ptf1a* promoter to drive $hKRAS^{G12D}$, a model of invasive exocrine pancreatic carcinoma was created [180].

Having transgenic models such as these has proved useful for learning about novel modifier pathways that affect cancer development. The Tg($BRAF^{V600E}$); $p53^{-/-}$ melanoma model has been the target of two major screens. The first also put an mitf mutation into the background, so the fish would have no melanocytes. The researchers

injected back the mitf open reading frame driven by the *mitf* promoter, along with a candidate gene of interest also driven by *mitf*; the candidates came from a region on human chromosome one that is amplified in human melanoma. This strategy ensured that fish with melanocytes got the injected vector, and those melanocytes would contain the candidate gene. Using this elegant approach, the researchers were able to find that SETDB1, an H3K9 HMT, accelerated melanoma development [181]. This study showed that the zebrafish could successfully be utilized as a platform for oncogene discovery and that histone methyltransferase activity may be important in cancer development.

The second screen using this melanoma model instead utilized the embryos rather than the adult fish. Microarray analyses comparing the embryos to the tumors revealed a set of genes important for both melanocyte development and tumor development. Noting that one of these genes, *crestin*, a marker of neural crest progenitors, had a different pattern in the transgenic embryos compared to wild-type embryos, the researchers used this expression pattern as the assay for a chemical screen. They found that leflunomide, a DHODH (dihydroorotate dehydrogenase) inhibitor, suppressed the expression of *crestin*, and through its effects on transcriptional elongation, also suppressed tumor growth in cell lines and xenografts [182]. This study showed how the embryos of a zebrafish tumor model could be utilized to discover a novel *in vivo* effect of a drug and potentially unveil a novel melanoma therapeutic.

Chromatin and Cancer

It is evident that epigenetics may serve as a mechanism on the road to oncogenesis in addition to genetic mutations. An aberrant epigenetic state could

realistically affect many stages of cancer development, including initiation, promotion, invasion, metastasis, and drug resistance. This is apparent since the epigenetic state impacts transcription, DNA repair, DNA replication, and the cell cycle, all factors that alterations of could lead to cellular transformation. Since histone modifications keep these processes under tight control, altering the modifications could misregulate them, leading to transformation. There are countless examples of how epigenetic alterations could aid in tumorigenesis along with traditional genetic aberrations. Epigenetics may be easier to treat in patients because the modifications are reversible, unlike genetic alterations.

Specific histone modifications are linked to cancer development when their global levels are altered. These changes are predictive of clinical prognosis. For example, the precise levels of acetylated H3K9, acetylated H3K18, acetylated H4K12, dimethylated H4R3, and dimethylated H3K4 in combination can predict in low-grade prostate cancer whether the tumor will recur over the next ten years [183]. Additionally, a global loss of acetylated H4K16 and trimethylated H4K20 is an early event in carcinogenesis, indicative of tumor development in a wide variety of cancers [184].

A variety of HMTs are involved in cancer, with both tumor suppressive and oncogenic functions. SUV39H1 is known to act as a tumor suppressor. In a double knockout mice with SUV39H2, loss of SUV39H1 results in increased genomic instability and therefore increased tumor risk [185]. SUV39H1-mediated senescence protects mice from developing Ras-induced T cell lymphomas [186]. Conversely, SUV39H1 was found silencing the tumor suppressor genes p15 and E-

cadherin in AML cell lines, suggesting that when SUV39H1 is dysregulated, it can participate in tumor formation [187]. SETD2 has also been shown to play a tumor suppressive role in breast cancer and clear cell renal cell carcinoma [188-190]. G9a is listed in the Oncomine database as being overexpressed in a number of tumor types; its overexpression is also known to increase metastatic and invasive potential of lung cancer [191, 192]. SMYD2 is overexpressed in esophageal squamous cell carcinoma and maintains MLL-AF9-induced AML cells in an undifferentiated state [193, 194]. HMT EZH2 is highly expressed in metastatic human prostate cells as opposed to the primary tumor; silencing it along with fellow Polycomb Group protein BMI-1 reverts the metastatic potential of the cells and diminishes their apoptotic-resistant properties [195]. In fact, EZH2 is a marker of poor prognosis for prostate, breast, and endometrial cancers [196, 197]. In follicular and diffuse large B-cell lymphoma, a heterozygous EZH2 mutation acts dominantly to increase methylation levels and thereby induce tumorigenesis, yet requires the wild-type allele for its mechanism of action [198].

HDMs are also emerging as playing a role in cancer formation. LSD1, the first HDM discovered, is overexpressed in high-grade prostate cancers and may be useful for prognostic purposes [199]. It is also highly expressed in high-grade neuroblastomas and estrogen receptor negative breast cancers [43, 200]. Conversely, LSD1 has been shown to suppress breast cancer metastases through the TGFbeta1 pathway; the mechanism to explain these differences remains unknown, but it is evident that LSD1 can be involved in cancer [201]. JMJD2C, a trimethylation-specific HDM, was found to be overexpressed in esophageal

squamous cell carcinoma [202]. PLU-1, an H3K4 demethylase, is overexpressed in breast cancer cells; knockdown of the gene by RNAi decreases the proliferation of these cells [203, 204]. Inactivating mutations have been found in JARID1C, an H3K4 HDM, and UTX, an H3K27 HDM, in clear cell renal cell carcinoma [189, 205]. As more is known about HMTs and HDMs, they may become a popular drug target for cancer therapies, similar to HDACs and DNMTs currently.

Histone acetylations and the HATs that add acetyl groups to histones are widely known to play a role in cancer development. Mutations in TIP60 that compromise its acetyltransferase activity impair DNA repair, leading to genetic mutations and general genomic instability [48]. TIP60 also associates with the androgen receptor and is linked to the development of prostate cancer [206, 207]. HATs p300 and CBP are known to modify critical cellular regulators such as p53, Rb, myb, and HIFalpha [208]. They are frequently part of chromosomal rearrangements in leukemias, and mice with inactivated alleles for these genes develop blood tumors [209, 210]. ING3, ING4, and ING5, all HATs involved in different cellular complexes, act as tumor suppressors controlling cell proliferation that can impact cancer development, including glioblastomas [79, 211]. MOZ is a common translocation partner in AML, often with p300 or CBP; these translocations involving 2 HATs can lead to activation of Hox genes and their cofactor Meis1, which can initiate AML [212, 213]. Similarly, MORF is often found translocated in AML to CBP and p300; it is also found translocated in uterine leiomyomata [214, 215].

Histone deacetylases (HDACs) are commonly deregulated in human cancers. For example, since HDAC1 is known to interact with Rb and E2F, misregulation of

HDAC1 could have profound effects on the cell cycle [216-218]. HDAC1 was first found to play a role in leukemia, as part of the mechanism triggered by the PLZF-RARalpha fusion product that represses transcription of differentiation genes [219, 220]. HDAC1 overexpression has been seen in gastric, renal, bladder, colorectal, and breast cancers and underexpression in ovarian cancer and glioblastomas [221-223]. Frameshift mutations in HDAC2 in sporadic cancers can cause loss of deacetylase activity along with resistance to HDAC inhibitors; HDAC2 overexpression in APC-deficient colorectal tumors prevents cancer cell apoptosis [224, 225]. High HDAC6 levels in breast cancer can be correlated with smaller tumors, good prognosis, and possibly responsiveness to endocrine therapy [226].

DNA methylation is often found awry in cancer cells. Overall, cancer cells are hypomethylated, especially at the repetitive regions containing many CpG islands. This hypomethylation results in genomic instability as well as oncogene activation, both important factors in cancer risk. The oncogene myc is noted as a frequently hypomethylated gene in tumors [227]. However, CpG islands at specific promoters tend to be hypermethylated in cancers. These generally come at the promoters of genes that would keep the cell in check, such as those involved with DNA repair, cell cycle, apoptosis, detoxification, and p53, inactivating these anti-tumor genes. In addition, mutations have been found in the DNMT3A genes in patients with T-cell lymphoma and AML, implying that aberrant DNA methylation has a role in cancer [228-230].

There is often interplay between the DNA methylation and the histone modifications in cancer development. A high percentage of genes often methylated

in oncogenesis are premarked during development with H3K27-trimethylation [231]. This trimethylation is completed by Polycomb Repressive Complex 2 (PRC2) member EZH2, which may recruit the DNMTs to methylate the DNA; the authors found a correlation between *de novo* methylation on genes implicated in cancer and PRC2-association on these same genes early in development. Often, PRC2 acting in a stem cell would repress transcription factors needed for differentiation; these same genes appear to have promoter DNA methylation, locking in stem cell-like properties and predisposing the cell to cancer [232, 233]. This crosstalk between the PRC2 and DNMTs may occur aberrantly early in oncogenesis, when the distribution of PRC2 across the genome is similar to that of a stem cell.

Conclusion

The epigenetic state of the cell is vital for the regulation of cellular processes such as transcription, DNA repair, and DNA replication. When the chromatin state is not properly maintained, these functions can go awry, potentially leading to cancer development. The zebrafish has proven itself as an ideal vertebrate model organism for the study of the chromatin state, chromatin-modifying factors, embryonic development, and tumorigenesis. In fact, a cancer screen in the zebrafish has already revealed a chromatin-modifying factor, SETDB1, to be a driver of tumorigenesis [181]. The studies in this dissertation utilize the zebrafish as a screening tool for a set of chromatin-modifying factors in both cancer and hematopoiesis. Using a rhabdomyosarcoma model, the H3K9 HMT SUV39H1 was found to be a regulator of tumor initiation. We demonstrate that the suppressive

effect is dependent on the enzymatic SET domain of SUV39H1 and that the HMT likely acts through cell cycle regulation to suppress tumor initiation. This dissertation also describes the development of a F1 transgenic screening approach to uncover novel roles of chromatin factors in hematopoietic development. These studies have contributed to the current knowledge about the interplay of chromatin and cancer as well as zebrafish screening technology.

Chapter 2

The histone methyltransferase SUV39H1 suppresses rhabdomyosarcoma initiation in zebrafish

Attributions

I am the primary contributor to this project. I cloned candidate genes into the *rag2* destination vector and sequence verified the clones as well as cloned the *mylz2*-hSUV39H1 and *mcad*-hSUV39H1 vectors. I prepared all microinjection dilutions, performed microinjections into one-cell stage embryos, and scored injected fish for mosaicism at 2 dpf. I scored fish for tumor formation by eye and fluorescence and generated Kaplan-Meier curves. I analyzed fluorescent images of larval fish for tumor growth rates. I cryosectioned fish, performed EdU staining on cryosections, and performed nuclear counts for the EdU incorporation analysis. I isolated RNA from tumors, prepared it for microarray and qPCR experiments, and subsequently analyzed qPCR data. I synthesized probes for and performed *in situ* hybridizations. I completed confocal microscopy of 5 and 7 dpf samples and analyzed pictures. I prepared fish for FACS analysis and for cell cycle analysis.

Narie Storer designed, optimized, and performed the overexpression screen. She assisted with Gateway cloning and sequence verification of clones. I received technical assistance from Erin Langdon in this project, including microinjection of vectors, scoring of tumors, *in situ* hybridizations, and qPCR reactions. Dave Langenau performed GSEA of chromatin factors in human rhabdomyosarcoma samples. Ellen Durand, Alison Taylor, and Dave Raiser provided assistance with FACS analysis. Owen Tamplin, Ilya Shestapalov, and Lisa Cameron provided assistance with confocal analysis. Sectioning and staining of pathology slides was done at the Brigham and Women's Hospital Pathology core. Microarrays were completed at the Children's Hospital Boston Microarray Core, and analysis was

performed by Tony DiBiase. ChIP-seq of HA-SUV39H1 and H3K9me3 in C2C12 cells was performed by Julien Pontis of Slimane Ait-Si-Ali's laboratory.

The following text and supplemental figures were prepared for manuscript submission. The subsequent addendum includes experiments I have performed as part of this project that are not in the manuscript.

Introduction

Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is a pediatric cancer representing more than half of all soft tissue sarcomas in children. There are about 350 new cases each year in the United States, with two-thirds of those occurring in children under the age of ten [234-236]. It is generally a sporadic tumor and tends to occur more frequently in boys than girls [234]. In about 20% of cases, the disease is metastatic at presentation, and even with aggressive treatments, five-year survival rates hover around 20% [234]. However, the overall survival rate when including nonmetastatic cases is currently nearly 80%, as compared to only 25% in the 1970s [237]. This is likely due to advancements in molecular biology techniques that allow for improved diagnosis and imaging, leading to tailored therapies. Patients generally undergo an intensive treatment regimen to ensure any micrometastases are removed, involving a multimodal approach of surgery, chemotherapy, and radiation therapy [236-238]. Future research in understanding the biology underlying the disease could aid in improving the overall survival rate even more as well as the metastatic survival rate, particularly in the biological differences between metastatic and nonmetastatic tumors.

There are two main distinct histological subtypes of RMS. The embryonal RMS (ERMS) subtype consists of 80% of RMS cases, is mainly in the pediatric population, and typically has a better prognosis. ERMS tumors tend to arise on the head and neck, extremities, and in the genitourinary tract [234]. Histologically, these tumors look like developing skeletal muscle, with characteristic alternating zones of

dense hypercellular myogenesis and hypocellularity [239]. Molecularly, ERMS is characterized by mutations or dysregulation of the RAS pathway and loss of heterozygosity at BWR1A [155, 240-244]. The alveolar subtype (ARMS) is more likely to occur in adolescents, be metastatic, and have a poorer prognosis. This subtype is named because tumor histology resembles clusters of primitive cells floating in alveolar spaces; ARMS tend to occur in limbs and axial musculature [239]. Molecularly, it is caused by a chromosomal translocation between either Pax3 or Pax7 and forkhead transcription factors [234, 242, 245].

Rhabdomyosarcoma in Zebrafish

Two transgenic models of ERMS have previously been developed in the zebrafish, both involving injections of linearized transgenes [153, 155]. The first was accomplished by driving expression of oncogenic human $KRAS^{G12D}$ with the *rag2* promoter, which was unexpectedly shown to express in mononuclear muscle satellite cells. The fish begin developing tumors as larvae, equivalent to the pediatric patients seen in the clinic [155]. The second was an inducible model, with $hKRAS^{G12D}$ driven by the *beta-actin* promoter [153]. Both sets of tumors express the traditional clinical markers of ERMS, including *myogenin, myod*, and *desmin*.

The *rag2*-$hKRAS^{G12D}$ transgenic model was shown to be amenable to a co-injection strategy. A second linearized transgene, also utilizing the *rag2* promoter, will always segregate together with the oncogenic transgene when both are microinjected into a one-cell stage embryo [155, 246]. This approach was used to demonstrate that overexpression of *noxa*, a p53 target, could suppress RMS

formation [246]. This study suggests that other genes potentially involved in RMS formation could be evaluated for their function in a similar co-injection system.

The role of Chromatin in Rhabdomyosarcoma

Though little is known about the role of chromatin-modifying factors in RMS, a few studies are beginning to implicate specific factors. BAF47, the human SNF5 homolog and a key component of the SWI/SNF chromatin remodeling complex, was noted to be mutated or deleted in 25% of primary tumors and 10% of RMS cell lines analyzed [247, 248]. Polycomb Group member YY1 was found to be upregulated in RMS cell lines and primary tumors, leading to recruitment of EZH2 and HDAC1 to miR-29, silencing this microRNA, and thereby preventing muscle differentiation and facilitating tumor development [249]. Upon treatment with 12-O-Tetradecanoylphorbol-13-acetate (TPA), the ERMS cell line RD differentiates through a mechanism involving PCAF and the BRG1 subunit SWI/SNF complex being sequentially recruited to the *myogenin* promoter; this may represent a novel therapeutic strategy to get tumors to differentiate *in vivo* [250]. SUV39H1 expression increases in an ARMS cell line under differentiation conditions, blocking MyoD and tumor differentiation, similar to its role inhibiting normal muscle development [251, 252]. The histone demethylase LSD1 was shown to have high expression levels in malignant sarcomas, including ARMS (2/2) and ERMS (6/7) tumors; it may prove useful as a diagnostic marker or a novel drug target [253, 254]. Since the regulation of chromatin structure can play a determinative role in the formation and behavior of cancers of the muscle, it is likely that many more chromatin factors participate in RMS, but they remain to be discovered.

Here, using an injection-based screening approach, we screened nineteen chromatin-modifying factors for their role in RMS formation. One, SUV39H1, was determined to be a strong suppressor, and this effect was dependent on an active SET domain. SUV39H1 did not impact overall tumor characteristics when compared to control tumors, including histological and gene expression analyses. Studies of tumor initiation using a fluorescent monitoring system demonstrated that SUV39H1 acts between 5 and 7 dpf at the beginning of tumor formation. Gene expression studies demonstrate reduced cyclin B1 mRNA expression in SUV39H1 injected embryos, and the cyclin B1 gene was bound by SUV39H1 in muscle progenitor cells. This data suggests a model in which altered cyclin B1 and cell cycle defects caused by SUV39H1 overexpression is responsible for the defect in tumor initiation.

Methods

Zebrafish

Zebrafish were maintained and developmentally staged as previously described according to IACUC guidelines [255]. The Animal Care and Use Committee, Children's Hospital Boston approved all animal protocols.

Vectors and cloning

The *rag2*-hKRAS^{G12D} and *rag2*-GFP vectors were obtained from D. Langenau [155]. The *mylz2*-GFP/mCherry, *mcad*-GFP/dsRedexpress, *myog*-GFP, *rag2* destination, *rag2*-hSUV39H1^{H324K}, and *rag2*-hSUV39H1^{C326A} vectors were obtained from N. Storer [256, 257]. *rag2*-chromatin factor expression vectors were generated by Gateway recombination using human, full-length open reading frames from the Ultimate ORF Clone collection (Invitrogen). A pENTR-mPAX7 vector was obtained from Open Biosystems and was put behind the *rag2* promoter through Gateway recombination. To generate the *mylz2*-hSUV39H1 and *mcad*-hSUV39H1 vectors, SUV39H1 was amplified from *rag2*-hSUV39H1 DNA using 5'-AATTCCGGATCCCACCATGGCGGAAAATTTAAAAGGCT-3' and 5'-TCAGGACGGCGTTTATGGAGAAGATCTTCGAATTAAGG-3'. The forward primer contains a BamHI site and the reverse a HindIII site. The amplified SUV39H1, *mylz2*-GFP, and *mcad*-GFP vectors were digested with BamHI and HindIII, treated with Antarctic Phosphatase, and ligated.

Microinjection and tumor scoring

The *rag2*-hKRAS^{G12D}, *rag2*-chromatin factor, and all fluorescent protein/SUV39H1-containing vectors were linearized with XhoI, purified, and diluted in 0.5xTE + 0.1M

KCl. For co-injection of three transgenes, each was diluted to 40 ng/uL, and for co-injection of four transgenes, each was diluted to 30 ng/uL. For co-injection of five transgenes, each was diluted to 25 ng/uL. One nL of the vector dilutions was microinjected into one-cell stage AB strain zebrafish embryos. For the screen, fish were scored for visible tumor formation every 2-4 days commencing at 12 dpf. For younger larvae, fish were scored for tumor formation by presence of fluorescence every 2-3 days commencing at 6 dpf. For the tumor growth analysis, fluorescent photos of each fish were taken at the same zoom and magnification, and photos were analyzed for number of fluorescent particles on ImageJ.

Identification of known and putative chromatin modifying factors

Human chromatin modifying factors were identified using CREMOFAC, SMART domain, CDD, and Pfam databases.

GSEA

GSEA on published human microarray data sets was performed as described previously [155, 258].

Statistical analysis

Tumor-free survival over time is graphically represented as a Kaplan-Meier estimate of survival. The log-rank test was used to compare survival of experimental and control groups.

Quantitative RT-PCR analysis

RNA was isolated from whole tumors using RNeasy Mini Kit (Qiagen) using the animal tissue protocol and subsequently treated with DNase I. cDNA synthesis was performed using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR, and

quantitative RT-PCR was performed using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen). The following primers were used to detect gene expression: *EF1-alpha* 5'-CATCGAGAAGTTCGAGAAGGAAGC-3', 5'-GTCAATGGTGATACCACGCTCAC-3'; *hKRAS* 5'-TTGATGGAGAAACCTGTCTCTTGG-3', 5'-CAAATACACAAAGAAAGCCCTCCC-3'; *hSUV39H1* 5'-TGTGTGCGTATCCTCAAGCAAGTTC-3', 5'-CCTCATTCTCTACAGTGATGCGTCC-3'; *pax7* 5'-CAGTATTGACGGCATTCTGGGAG-*3', 5'*-TCTCTGCTTTCTCTTGAGCGGC-3'; *myf5* 5'-CCAGACAGTCCAAACAACAGACC-3', 5'-TGAGCAAGCAGTGTGAGTAAGCG-3'; *cdh15* 5-'CTAAGGAAAGATGCACCCCATTAC-3', 5'-TCAGAGCTGTGTCGTATGGTGG-3'; *myog* 5'-GTGGACAGCATAACGGGAACAG-3', 5'-TCTGAAGGTAACGGTGAGTCGG-3'; *desmin* 5'-CGAGATTGACTCTCTCAAGGGCAC-3', 5'-GGGCGATAGTGTCCTGATAACCAC-3'; *mylz2* 5'-TTGACCACTCAGTGCGACAGGTTC-3', 5'-AACATTGCCAGCCACATCTGGG-3'. Gene expression was normalized to *EF1-alpha*.

Microarray analysis

RNA was isolated from six tumors each of the *rag2*-mCherry and *rag2*-hSUV39H1 types with the RNeasy Mini Kit (Qiagen) using the animal tissue protocol and subsequently treated with DNase I. cDNA was prepared and hybridized to zebrafish Affymetrix arrays. Genes differentially regulated between the tumor types were identified (>2-fold change, $p<0.05$). For the larval samples, RNA was isolated in the same manner from approximately twenty sibling embryos per sample and time point, with three biological replicates for each. Microarrays were performed on Affymetrix chips.

EdU incorporation

Tumor-bearing fish at 28 to 30 dpf were injected intraperitoneally with 10ul of 2.5mg/ml EdU per 0.25g body weight. After 24 hours, fish were euthanized and frozen in Optimal Cutting Temperature (OCT) medium at -80°C overnight. 12um cryostat sections were prepared for each tumor, and EdU labeling was performed using the Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen). Labeled sections were imaged at 400x magnification using a compound fluorescent microscope, and the number of EdU-positive and DAPI-positive nuclei were counted in three separate 1.37×10^4 um^2 fields per tumor. The ratios of EdU-positive to DAPI-positive nuclei in the three fields were averaged to calculate an EdU/DAPI ratio for each tumor.

Histopathology

Fish were euthanized and fixed in 4% paraformaldehyde overnight at 4°C and then decalcified in 0.5M EDTA, pH8. Paraffin embedding, sectioning, H&E staining, and RNA *in situ* hybridization were performed according to standard techniques by the Brigham & Women's Pathology Core.

***In Situ* Hybridization**

Whole mount *in situ* hybridizations (ISHs) were performed as previously described [259, 260]. Antisense mRNA probes were synthesized from digested plasmids [260]. After linearization and phenol:chloroform extraction, the DIG-labeled probes were synthesized using T7 polymerase and purified using the RNeasy kit (Qiagen).

FACS Analysis

Quadruple injected SUV39H1 and mCherry control 5 and 7 dpf larvae, along with uninjected wild-type controls, were homogenized, resuspended in 0.9X PBS, and

filtered (40um). The samples were run on a FACS LSRII (Becton Dickinson) and analyzed with FloJo.

TUNEL

Whole-mount staining for TUNEL was completed on 5 and 7 dpf triple injected SUV39H1 and mCherry larvae. Larvae were fixed in 4% paraformaldehyde (PFA) overnight, bleached to remove melanocytes in a 3% H2O2/0.5% KOH solution for 45 minutes, then fixed again overnight in 4% PFA. Larvae were then stained as previously described using the ApopTag® Plus Peroxidase *In Situ* Apoptosis Kit (Millipore) [165].

Confocal Imaging Analysis

Imaging was performed on a Zeiss 710 NLO laser scanning confocal microscope (Zeiss). GFP and mCherry were excited by 488 and 594 nm laser lines, respectively. Larval fish were first scored for fluorescence, then anesthetized in 2% Tricaine and embedded in agarose. Images were analyzed with ZEN 2011 and ImageJ.

Cell cycle analysis

Well-injected embryos were homogenized in 0.9x PBS + 10% FBS using QIAshredder columns; the cells were filtered, spun down, washed, and fixed in 2% PFA in 0.95x PBS for 30 minutes. Then the cells were spun down, washed in 0.9x PBS, and incubated in 70% ethanol for 30 minutes. Following another spin, the cells were then incubated in a PI solution for 30 minutes, filtered, then run on a FACSCantoII machine (Becton Dickinson) and analyzed with FloJo.

Results

An in vivo overexpression screen in zebrafish to identify modifiers of embryonal rhabdomyosarcoma

Because of the importance of epigenetic regulation in muscle development and cancer, we sought to determine whether chromatin-modifying factors play a role in RMS formation. Using protein domain family databases, 333 human genes were identified that were predicted to encode proteins harboring domains associated with chromatin regulation (Figure 2.S1.A). While some of these genes have known roles in chromatin regulation, we hypothesized that other genes on the list would have roles in chromatin regulation that have not yet been characterized. Using gene set enrichment analysis (GSEA) on human RMS microarray data sets, we found that this list of putative or known chromatin modifiers was significantly upregulated in human ERMS and ARMS ($p<0.05$, Figure 2.S1.B,C). These data suggest that deregulation of chromatin modifying factors may play an important role in RMS pathogenesis.

To identify chromatin-modifying factors that act as modifiers of RMS, we utilized a previously characterized model of ERMS in the zebrafish [155]. This model was based on a microinjection strategy amenable to co-injection of different factors with their expression driven by the *rag2* promoter. Injection of a *rag2*-hKRAS^{G12D} construct drives tumor formation; additional genes expressed on separate, linearized plasmids, all also driven by *rag2*, would be co-integrated with and co-expressed in the *rag2*-hKRAS^{G12D} tumors. Therefore, we developed a strategy to identify suppressors or enhancers of RMS formation when the candidate gene, driven by *rag2*,

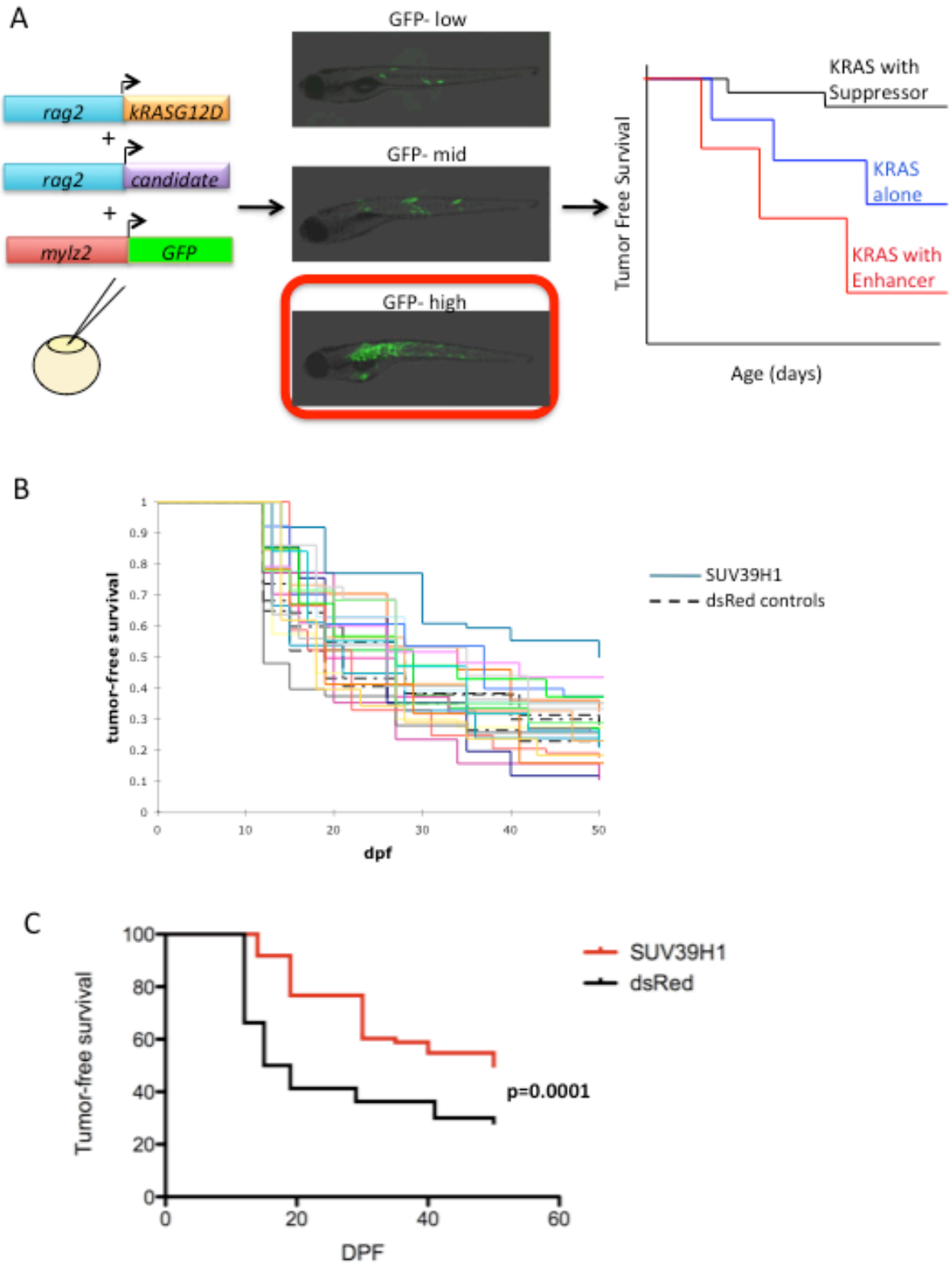
was co-injected with *rag2*-hKRAS^{G12D}. To eliminate variability, a third construct, *mylz2 (myosin light polypeptide 2)*-GFP, was co-injected. This allowed the microinjected embryos to be separated into categories of GFP-low, middle, and high mosaicism at 2 dpf. The level of GFP mosaicism directly correlated with successful microinjection of the hKRAS^{G12D} and therefore tumor levels (data not shown). Those with high GFP mosaicism were selected and analyzed for visual tumor formation every 3 days from 12 to 50 dpf (Figure 2.1.A). A list of nineteen factors were chosen for analysis, which represent proteins containing SET or chromo domains, as well as nine of the most highly upregulated genes in human ERMS versus normal muscle (Figure 2.1.B). Of these factors, SUV39H1 emerged as the strongest modifier, significantly suppressing tumor formation by the logrank test (p=0.0001, Figure 2.1.C).

Suppression by SUV39H1 occurs early and is dependent on enzymatic activity

SUV39H1-overexpressing tumors had a delay in tumor onset, so the Kaplan-Meier curve appears suppressed from the beginning of the assay at 12 dpf. To examine tumor formation with a more sensitive and quantitative assay, we used a quantitative fluorescent assay with a quadruple injection approach. As before, zebrafish were injected with *rag2*-hKRAS^{G12D} and either *rag2*-hSUV39H1 or, as a control, *rag2*-mCherry. The third construct was *rag2*-GFP to track the tumors by fluorescence as they arose, as it has been shown that 100% of *rag2*-GFP-positive foci go on to eventually form a tumor (X. Le, personal communication). The fourth construct was *mylz2*-mCherry to continue analyzing only successfully microinjected embryos. By analyzing GFP fluorescence in the musculature of highly mCherry

Figure 2.1. **An overexpression screen reveals SUV39H1 as a suppressor of rhabdomyosarcoma formation in zebrafish. (A)** Three linearized DNA constructs were injected into one-cell stage embryos, *rag2*-hKRASG21D, *rag2*-chromatin factor, and *mylz2*-GFP. At 2 dpf, embryos were scored for GFP mosaicism; only those that were GFP-high were kept for tumor evaluation. Tumor-free survival curves were then constructed for days 12-50 of life, looking for enhancers or suppressors of the curve produced by injected of *rag2*-hKRASG12D without a modifier gene. **(B)** Nineteen chromatin-modifying factors were analyzed for their effects on RMS formation. Most did not result in significant differences from the three historical control curves, seen as dotted black lines. **(C)** SUV39H1 significantly suppressed RMS formation, compared to a control curve where the modifier gene was dsRed (SUV39H1 n=73, dsRed n=80, p=0.0001).

Figure 2.1 (Continued)



mosaic larvae, SUV39H1 still significantly suppressed RMS formation (p=0.0003, Figure 2.2.A).

To determine if the HMT activity of SUV39H1 played a role in tumor suppression, we co-injected SUV39H1 with point mutations in the enzymatic SET domain along with *rag2*-hKRAS^{G12D} and analyzed early tumor formation by fluorescence. Both point mutations, H324K and C326A, resulted in tumor curves similar to the control mCherry-overexpression curve rather than the suppressed SUV39H1-overexpression curve (C326A p=0.0525, H324K p=0.7642, Figure 2.2.B,C). This result indicates that the tumor suppression depends on the HMT activity of the SET domain in SUV39H1, ruling out any scaffold effects since this enzyme is a part of a multiprotein complex.

Characterization of SUV39H1-overexpressing tumors

Since SUV39H1 is known to play a role in regulation of cell cycle and S phase genes [100, 101, 261, 262], we determined if there was any difference in cell cycle rate between SUV39H1-overexpressing tumors and control tumors overexpressing mCherry. 5-ethynyl-2'-deoxyuridine (EdU), a bromodeoxyuridine analog, was injected intraperitoneally into five fish of each tumor type. Twenty-four hours post injection, the fish were embedded in OCT and cryosectioned (Figure 2.3A). Staining for EdU in GFP-positive tumor sections revealed no difference in the percentage of cells dividing during labeling when normalized to cell number by DAPI staining, indicating no difference in cell cycle rate in the SUV39H1-overexpressing and control tumors (Student's t-test, p=0.78, Figure 2.3B).

Figure 2.2. Suppression by SUV39H1 depends on enzymatic SET domain. (A) Injections into one-cell stage embryos of *rag2*-hKRASG12D, *rag2*-hSUV39H1, *rag2*-GFP, and *mylz2*-mCherry, then selected for mCherry-high embryos, and monitored for tumor formation by GFP presence, results in a tumor curve that is significantly suppressed from *rag2*-mCherry control curves. (SUV39H1 n=56, mCherry n=58, p=0.0003). **(B)** Injection of the point mutant SUV39H1^{C326A} results in a tumor curve not significantly different from the mCherry control curve. However, this curve is significantly different from *rag2*-hSUV39H1 tumors (C326A n=57, mCherry n=48, p=0.0525). **(C)** Similar injections of the *rag2*-hSUV39H1^{H324K} point mutant also results in a tumor curve like the *rag2*-mCherry curve and significantly different from the *rag2*-hSUV39H1 curve (H324K n=17, mCherry n=19, p=0.7642).

Figure 2.2 (Continued)

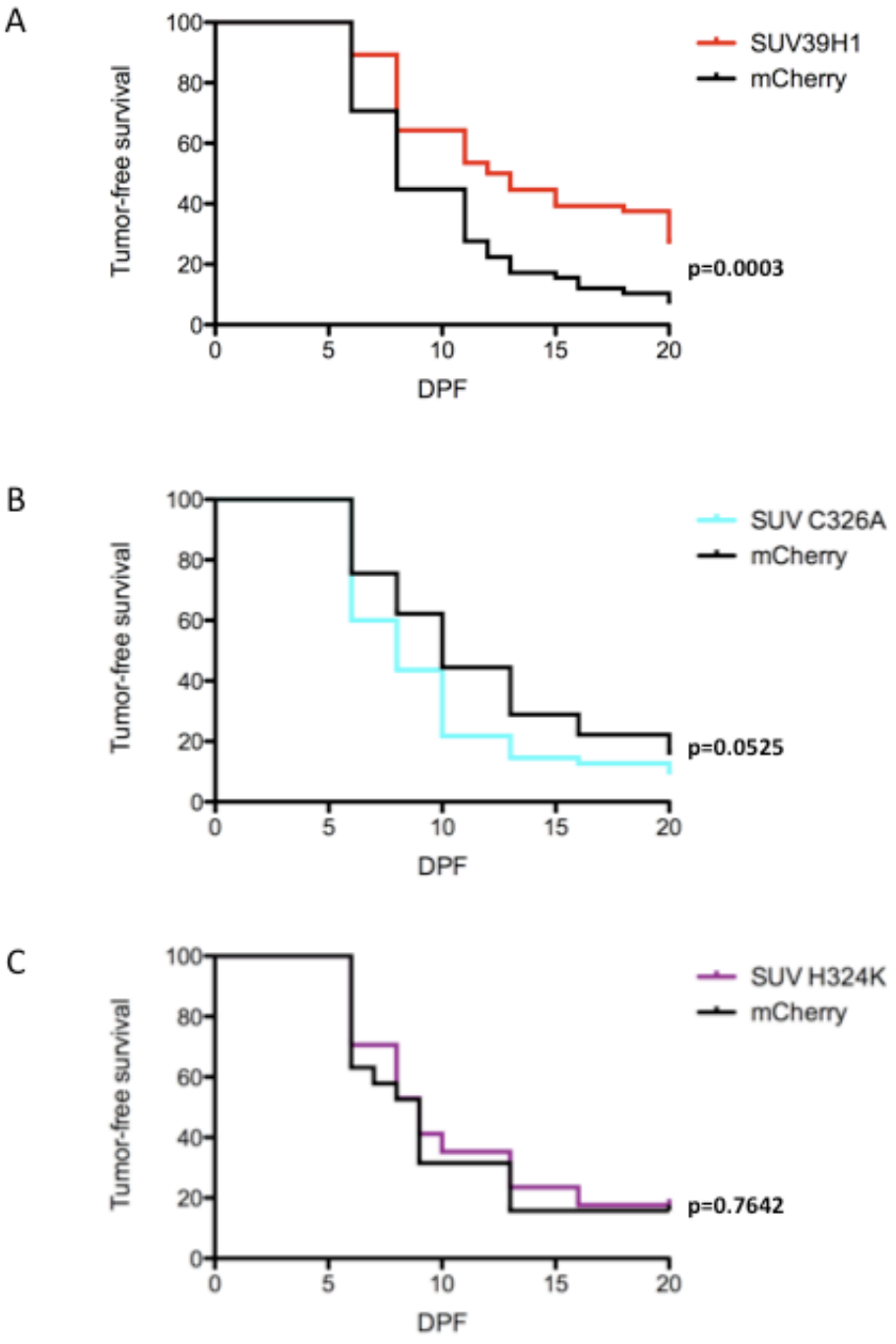
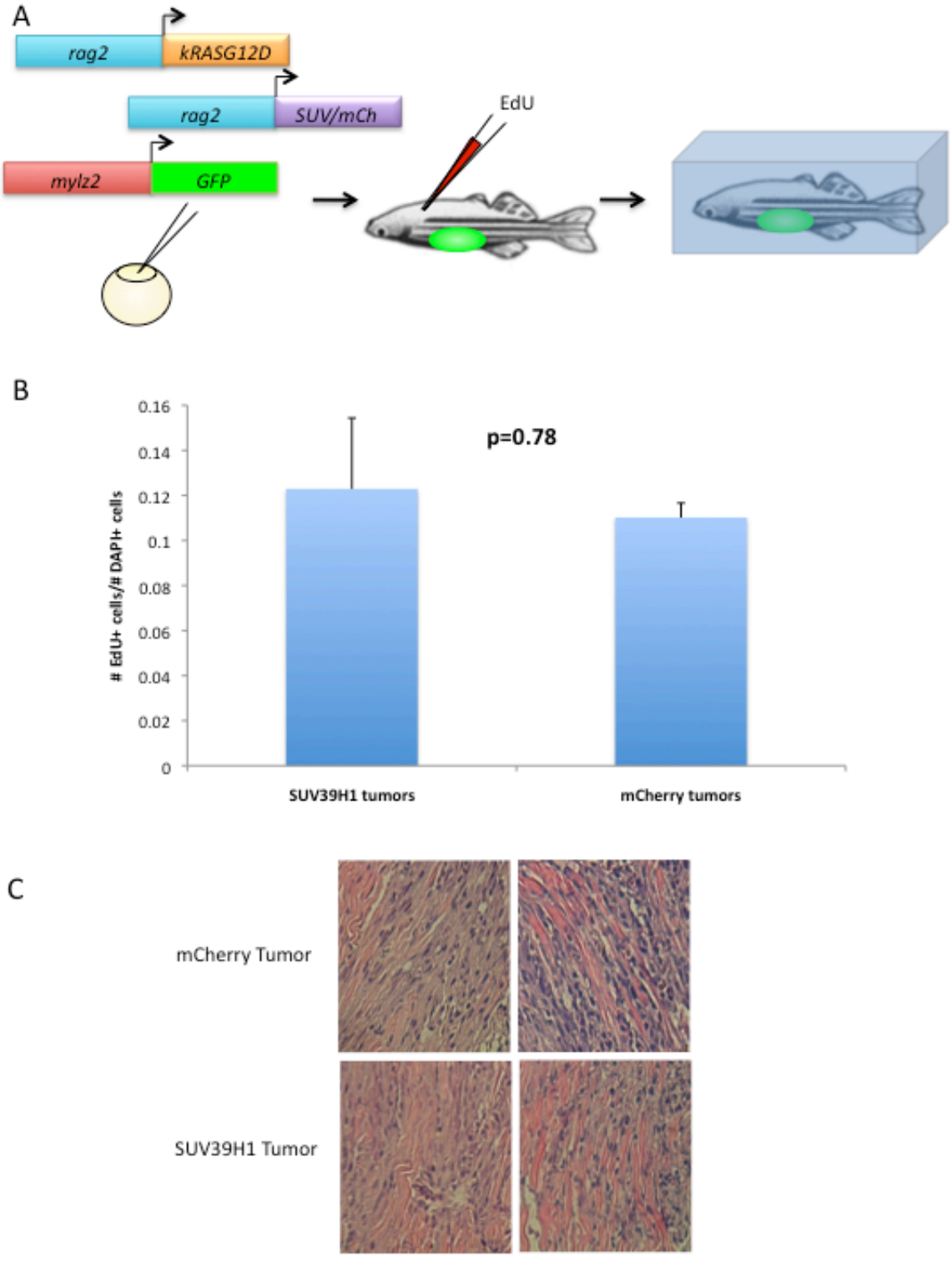


Figure 2.3. SUV39H1 overexpression does not impact cell cycle or muscle differentiation status of mature tumors. (A) Experimental design for *in vivo* cell cycle analysis. One-cell stage embryos were injected with three constructs *rag2*-hKRAS^{G12D}, either *rag2*-mCherry or *rag2*-hSUV39H1, and *mylz2*-GFP. High-GFP expressing fish were grown up, and those with tumors were injected IP with EdU. After 24 hours, the injected fish were sacked and cryopreserved. **(B)** Number of EdU-positive cells, stained on GFP-positive tumor sections, was normalized to number of total cells, determined by presence of DAPI. No difference was observed between the control and SUV39H1 tumors (n=5 for each group; p=0.78). **(C)** H&E staining of RMS tumors overexpression either mCherry or SUV39H1. Both sets of tumors are very poorly differentiated; they also look similar to each other, indicating no difference in differentiation state of the tumors (bars represent 50um, n=6 for each group).

Figure 2.3 (Continued)



We hypothesized that muscle differentiation may be affected in the modified tumors, since SUV39H1 is known to regulate the master regulator MyoD [252]. H&E staining on SUV39H1 and control tumors revealed no change in muscle differentiation status. Both sets of tumors were in an undifferentiated state, with higher cellularity and mostly mononucleated cells (Figure 2.3.C). Global gene expression analysis of control and SUV39H1 tumors did not result in any major differences (data not shown). These studies demonstrate that overexpression of SUV39H1 did not significantly affect cell cycle rate or the differentiation state of *rag2*-hKRAS^{G12D} RMS tumors, at least at the gross histological level.

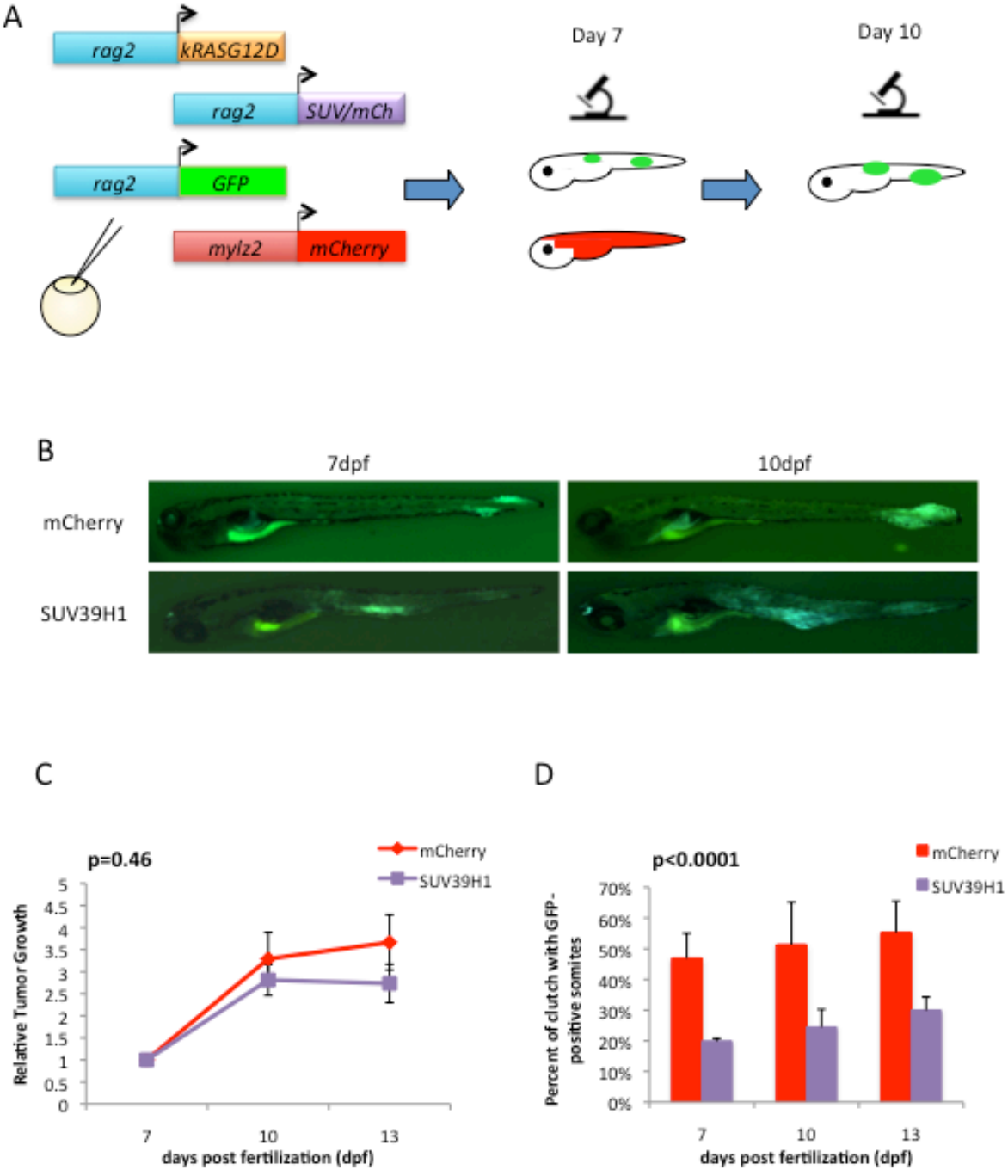
SUV39H1 suppress initiation of RMS tumors

The suppressive phenotype of SUV39H1 tumors in the Kaplan-Meier survival curves has already occurred at the first time point of 12 dpf (Figure 2.1.C). To examine earlier time points, we utilized the quadruple co-injection approach described above and shown in Figure 2.2. At 7 dpf, highly mCherry mosaic larval fish were examined for the presence of GFP in the musculature. Those that had GFP positive cells at 7 dpf were examined again at 10 and 13 dpf for the growth of these fluorescent patches into tumors (Figure 2.4.A). In 100% of cases, the GFP-positive patches went on to produce a tumor, some as early as 13 dpf (Figure 2.4.B). The size of the developing tumors can be quantified by measuring the number of fluorescent pixels in the image using the image analysis software ImageJ. Relative growth rates for SUV39H1 and control cohorts revealed that both sets of tumors grew at the same rate (Student's t-test, p=0.46, Figure 2.4.C). Within clutches containing equivalent levels of mCherry mosaicism, the number of larvae in a given clutch with GFP-

Figure 2.4. SUV39H1 impacts the initiation, not the growth rate, of the tumors

(A) Experimental design to view the tumors in larval stages by fluorescence. Four constructs are injected into one cell stage embryos, *rag2*-hKRAS^{G12D}, *rag2*-hSUV39H1 or control *rag2*-mCherry, *rag2*-GFP, and *mylz2*-mCherry. Fish highly mosaicism for *mylz2*-mCherry expression are analyzed on day 7 for presence of GFP, indicative of developing tumors. Analysis is also performed on day 10 to note tumor growth. **(B)** Representative images of 7 and 10 dpf larvae with GFP-positive cells in the musculature. There is visible growth between 7 and 10 dpf as they develop into tumors. **(C)** Tumor growth rates, relative to size of tumor on day 7. There is no significant difference between growth rates of the SUV39H1-overexpressing tumors compared to control tumors (SUV39H1 n=22, mCherry n=13, p=0.46). **(D)** Percentage of larvae within a clutch that contain GFP-positive cells in the musculature. More fish in the mCherry clutches have developing tumors when compared to SUV39H1 clutches (SUV39H1 n=200, mCherry n=136, $p<0.0001$).

Figure 2.4 (Continued)

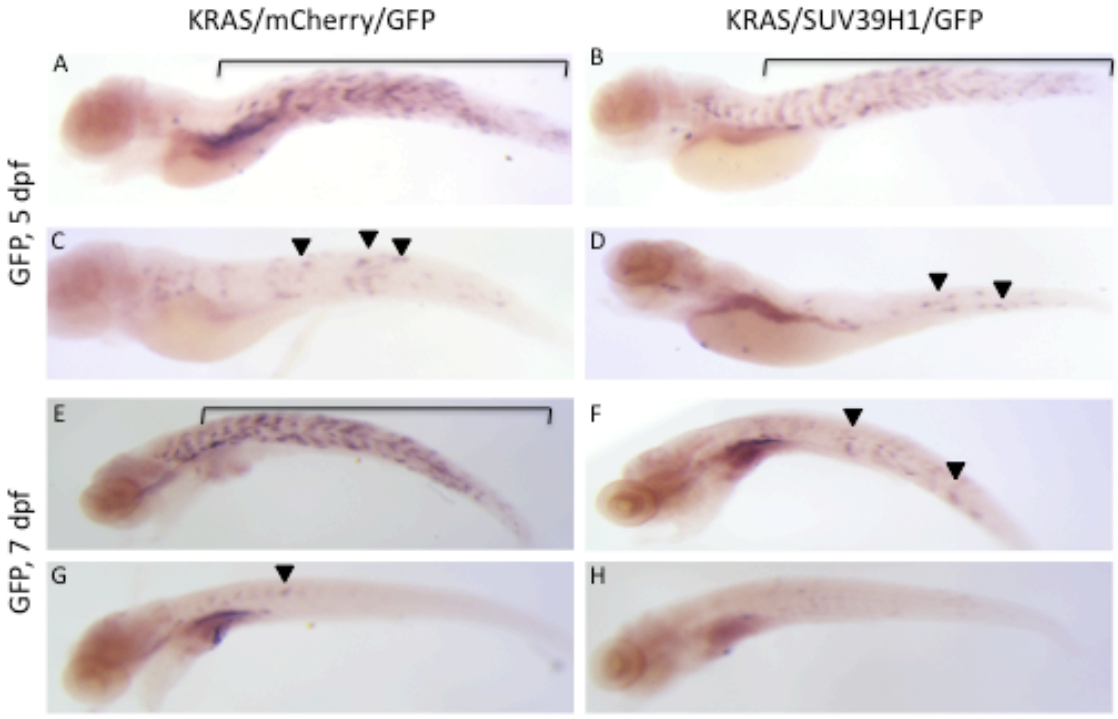


positive patches was evaluated in the musculature. Significantly fewer patches were found in the SUV39H1-overexpression clutches compared to controls (Fisher's exact test, $p<0.0001$, Figure 2.4.D). This study in larvae demonstrated that SUV39H1 overexpression impacts the initiation of tumors. Once the tumor initiates in the presence of SUV39H1 overexpression, it grows at the same rate; the main difference SUV39H1 causes is to prevent tumors from initiating.

To further analyze GFP expression, we took 5 and 7 dpf mosaic embryos that expressed high levels of *mylz2*-mCherry and performed *in situ* hybridizations (ISH) for GFP mRNA. This analysis by ISH was required because the levels of fluorescence were too low to detect by microscopy (data not shown). Analysis of these fish by flow cytometry revealed minimal expression of GFP in control and SUV39H1-overexpressing 5 and 7 dpf larvae, which confirms our visualization of GFP protein (Figure 2.S2). Surprisingly, some larval fish displayed GFP mRNA expression throughout the musculature. Though the levels of GFP varied between fish at 5 dpf, the SUV39H1-overexpressing and control larvae appeared to have similar ranges of GFP mRNA expression, with all fish having at least some expression (Figure 2.5.A-D,I). However, at 7 dpf, the SUV39H1-overexpressing larvae had much less GFP expression compared to controls, with about a third of fish (10/29) no longer expressing GFP at all (Figure 2.5.E-I). As expected, ISHs for KRAS or SUV39H1 mostly localized to putative tumors. KRAS expression levels were similar between 5 and 7 dpf in both SUV39H1 and control larvae, with fewer fish having tumors in the SUV39H1 cohort (Figure 2.S3). This result allows us to conclude that SUV39H1 is not simply suppressing $KRAS^{G12D}$ directly to block tumor initiation. Whole-mount

Figure 2.5. *rag2*-GFP mRNA analysis reveals difference between 5 and 7 dpf larval fish. (A-D) *In situ* hybridization for GFP mRNA in 5 dpf fish injected with of *rag2*-hKRAS^{G12D}, *rag2*-hSUV39H1 or mCherry, *rag2*-GFP, and *mylz2*-mCherry, then selected for mCherry-high embryos. Pictures represent the range of expression seen, which is similar between SUV39H1 and control fish. Brackets and arrowheads note patches of expression **(E-H)** *In situ* hybridization for GFP mRNA in 7 dpf siblings of 5 dpf injected fish. Pictures represent the range of expression seen, which is lower in the SUV39H1 fish compared to control fish (4X images). **(I)** Table with numerical representation of GFP expression. All 5 dpf and mCherry 7 dpf larvae have GFP expression; only in the 7 dpf SUV39H1 larvae is GFP not expressed in some fish, suggesting that SUV39H1 is acting between 5 and 7 dpf.

Figure 2.5 (Continued)



I

Embryos	High	Medium	Low	Negative	Proportion with expression
Ras/mCh: 5 dpf	4	15	10	0	29/29
Ras/SUV: 5 dpf	2	7	8	0	17/17
Ras/mCh: 7 dpf	3	9	24	0	36/36
Ras/SUV: 7 dpf	0	0	19	10	19/29

TUNEL analysis revealed no differences in apoptotic levels between SUV39H1 and control larvae (7 dpf, Figure 2.S4). We conclude that SUV39H1 is acting between days 5 and 7 as the first tumors initiate, likely repressing a tumor program to prevent tumor initiation, rather than a muscle-specific program.

To try to reveal what factors SUV39H1 could be repressing in the initiation of RMS, we performed global gene expression analysis on 5 and 7 dpf *rag2*-hKRAS^{G12D}, *rag2*-hSUV39H1/mCherry embryos highly mosaic for *mylz2*-mCherry. The 5 dpf larvae revealed no differences, as expected. The 7 dpf larvae revealed a gene set differentially expressed between SUV39H1-overexpressing and control fish (top downregulated genes in Figure 2.6.A). Of particular note is that cyclin B1 is downregulated in the SUV39H1-expressing fish (Figure 2.6.A). This may be indicative of SUV39H1 silencing a tumorigenic program through suppressing mitosis entry. To confirm this mechanism, we performed chromatin immunoprecipitation followed by massively parallel DNA sequencing (ChIP-seq) for HA in C2C12 cells transfected with HA-Suv39h1. Peaks revealed that Suv39h1 is bound in the first intron of cyclin B1 (Figure 2.6). Though the input has the same peak shape, the HA-Suv39h1 peaks in the first intron have been detected by peak calling with MACS ($p=10^{-3}$; 18000 peaks) and SICER (FDR=10^{-5}; 7000 peaks). Additional ChIP-seq for H3K9 trimethylation levels revealed the H3K9me3 mark in the first intron and 5 kb upstream of the cyclin B1 gene (Figure 2.6.B). Our studies establish that overexpression of SUV39H1 directly silences cyclin B1, and this cell cycle defect may cause the lower incidence of tumors that initiate with SUV39H1 overexpression.

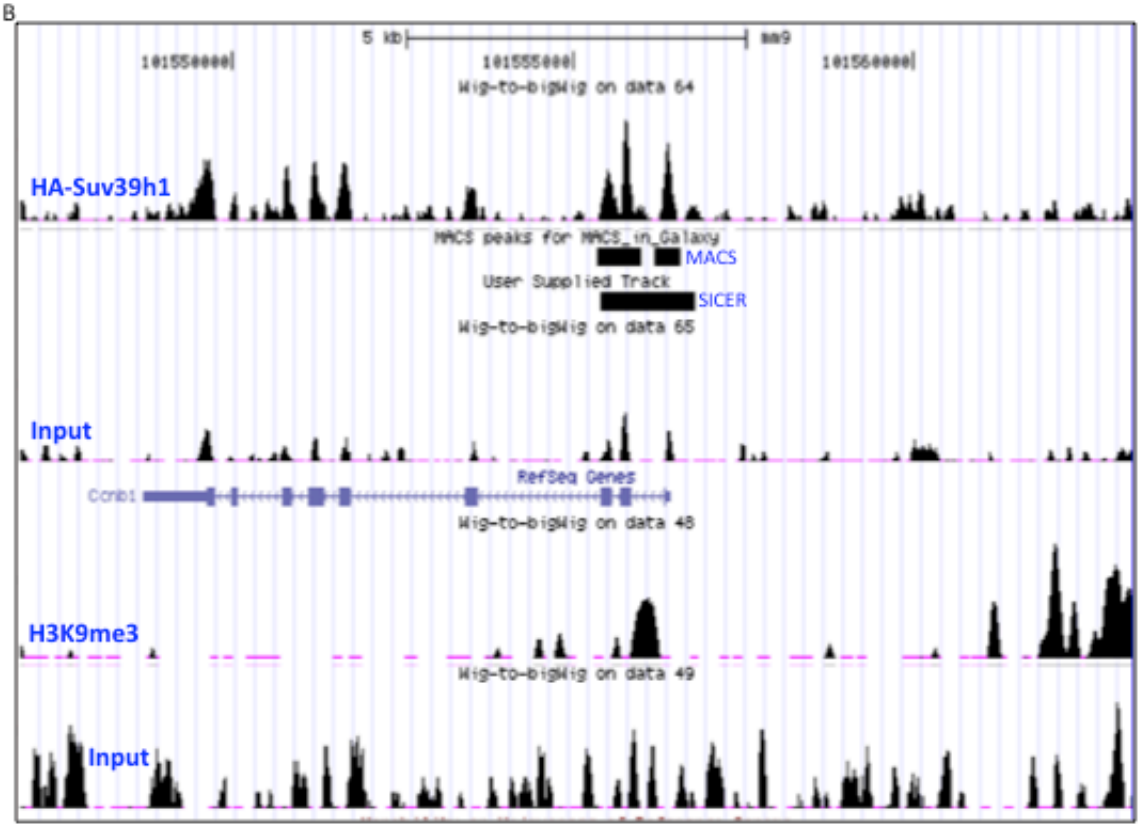
Figure 2.6. Global gene expression analysis and ChIP-seq reveals Suv39h1 acts through silencing of the cyclin B1 locus. (A) Table of top downregulated annotated genes in *rag2*-KRAS^{G12D}, *rag2*-SUV39H1 7 dpf larvae as compared to *rag2*-KRAS^{G12D}, *rag2*-mCherry control larvae. Of particular interest was cyclin B1. **(B)** HA-Suv39h1 was transfected into C2C12 muscle cells, and ChIP-seq was performed for HA. Peak calling determined peaks in the first intron of cyclin B1 (MACS: $p=10^{-3}$, 18000 peaks; SICER: $FDR=10^{-5}$, 7000 peaks). ChIP-seq was also performed for H3K9me3 in wild-type C2C12 cells. These peaks were found in the first intron and 5 kb upstream of the cyclin B1 gene.

Figure 2.6 (Continued)

A

Fold change	Gene	Fold change	Gene
-2.750	novel lectin C-type domain containing protein	-1.789	voltage-dependent anion channel 3
-2.608	coagulation factor XIII B chain precursor	-1.775	rab proteins geranylgeranyltransferase component A 1
-2.363	complement component c3b	-1.765	alanine--glyoxylate aminotransferase 2-like 1
-2.065	intelectin 3	-1.758	costars family protein C6orf115 homolog
-2.039	protocadherin alpha subfamily C, 1	-1.751	CLK4-associating serine/arginine rich protein
-1.973	tyrosine-protein kinase BAZ1B	-1.735	EGF-like fibronectin type III, laminin G domains
-1.934	UDP glucuronosyltransferase 2 family, polypeptide B6	-1.723	lactase-like protein precursor
-1.927	protocadherin 1 gamma 18	-1.723	fatty acid-binding protein, adipocyte
-1.906	cyclin B1	-1.714	protocadherin 2 alpha b 11
-1.857	protocadherin alpha subfamily C, 1	-1.714	reverse transcriptase
-1.848	major intrinsic protein of lens fiber a	-1.699	DEP domain-containing protein 7 isoform 1
-1.828	costars family protein C6orf115 homolog	-1.695	protocadherin alpha subfamily C, 1
-1.800	delta isoform of regulatory subunit B56, protein phosphatase 2A	-1.679	guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1
-1.794	peripherin-2	-1.675	protocadherin 1 gamma 22

B



Discussion

In this chapter, we describe an overexpression screen of chromatin-modifying factors that revealed SUV39H1 as a suppressor of rhabdomyosarcoma formation in zebrafish. This effect is specific to the histone methyltransferase activity of SUV39H1, as point mutations in the SET domain lacked the suppressive effect. The SUV39H1-overexpressing mature tumors have no differences in tumor proliferation and muscle differentiation status. Our studies establish that the larval fish with ectopic SUV39H1 initiate fewer tumors. This decreased initiation likely acts through a cell cycle pathway.

The requirement for the wild-type SET domain in our model is striking since this was not the case of HMTs in a zebrafish melanoma model. When SETDB1 was found to accelerate melanoma, two methyltransferase-deficient SETDB1 mutants also had an accelerated tumor incidence curve, likely because the complex still had methyltransferase activity [181]. This suggests that our result is more sensitive to the loss of SUV39H1 methyltransferase activity.

Previous studies involving SUV39H1 have demonstrated its role as a tumor suppressor involving senescence. SUV39H1 was demonstrated to associate with pRb as well as HDAC1, and a senescence response in melanocytes that acts through these two proteins is mediated by SUV39H1 heterochromatization [94, 99, 263]. In a murine model of Ras-driven T cell lymphoma, SUV39H1-dependent senescent growth arrest prevents the onset of tumorigenesis; this senescence is likely dependent on H3K9 methylation on specific growth genes [186]. Additionally, loss of SUV39H1 in Rb heterozygote mice leads to the development of C cell

adenocarcinomas, along with frequent expression of proliferation markers, suggesting that SUV39H1 suppresses tumors through senescence [264]. If oncogenic NRAS was transduced into cell lines derived from Rb deficient tumors, senescence was induced and SUV39H1 was recruited to chromatin [264]. Loss of SUV39H1 in a myc-driven model of murine B cell lymphomas led to faster onset of disease, whereas SUV39H1 wild-type mice displayed increased levels of senescence and growth arrest [265]. It has been suggested that the SUV39H1-mediated H3K9me3 mark on heterochromatin is a widespread mark of the senescence program and that this program could be targeted for cancer therapies [265]. SUV39H1 is also known to silence S phase genes as well as p21, both of which will induce growth arrest [100, 101, 261, 262, 266]. The mechanism demonstrated in our work on RMS is similar to these studies. There was a delayed onset of tumorigenesis with SUV39H1 overexpression, complimentary to the more aggressive onset observed with loss of SUV39H1 in these studies. These experiments are consistent with the notion that SUV39H1 may be a tumor suppressor [185, 186].

Global gene expression analysis comparing *rag2*-hKRAS^{G12D}/*rag2*-hSUV39H1 with *rag2*-hKRAS^{G12D}/*rag2*-mCherry 7dpf larvae revealed that cyclin B1 is downregulated in the SUV39H1-overexpressing tumors. SUV39H1 is already known to silence cyclins E and A [99]. Silencing of cyclin B1 may explain how SUV39H1 promotes senescence and growth arrest. Quiescence can often be mediated by cyclin B1 downregulation, as in CD34-negative hematopoietic stem cells and quiescent NIH3T3 cells [267, 268]. Sp1, a cell growth and survival transcription factor, has been shown to associate with SUV39H1 upon hydrogen peroxide treatment in an

epithelial carcinoma cell line, leading to growth arrest through the silencing of Sp1 target genes, including cyclin B1 [269]. SUV39H1 overexpression likely silences cyclin B1, leading to growth arrest and decreased tumor initiation.

A number of protocadherins, or cadherin-like cell adhesion proteins, were found to be downregulated in the SUV39H1-expressing fish upon larval microarray analysis. Though most protocadherins (PCDHs) seem to have tumor suppressive functions with reduced expression in tumors, PCDH11-gamma is known as a proto-oncogene [270]. It is upregulated in apoptosis-resistant cell variants of a prostate carcinoma line, and overexpression induces anchorage-independent growth and tumors [271]. Though PCDH11-gamma was not specifically on our list, the established precedent that protocadherins can function as oncogenes opens the possibility that the dysregulated protocadherins could have oncogenic properties, and suppression by SUV39H1 may contribute to blockage of RMS initiation in the zebrafish.

Being able to visualize the development of RMS with a fluorescent protein was critical to these studies. Identifying tumors as soon as they arose in the fish by fluorescence, rather than waiting for them to be large enough to be observed by eye, enabled us to hone in on the earliest steps of tumor initiation. It made the already rapid tumor model even quicker, with significant tumor curves able to be completed by 20 dpf. Using this early tumor model will have future applications as a screening tool, further expanding on the power of zebrafish genetics and numbers.

Performing ISHs for the developing tumors in larval fish revealed an unexpected finding. ISHs done against KRAS or SUV39H1 mRNA either had minimal

staining or staining in patches that represented the forming tumors (Figure 2.S3). However, ISHs performed for GFP mRNA driven by the *rag2* promoter resulted in staining throughout the musculature at various levels between individual fish, rather than just at the tumor location (Figure 2.5). This result was surprising since we could rarely visualize any fluorescence on a standard dissecting scope at 5 dpf, and often at 6 to 7 dpf it was only a few isolated cells in a distinct patch. FACS analysis for the presence of GFP protein confirmed the low to absent GFP fluorescence (Figure 2.S2). The ISHs demonstrate that *rag2*-GFP mRNA expresses in satellite cells at low levels throughout the musculature. Only when GFP protein levels accumulate, such as in a developing tumor, then fluorescence is detected.

In conclusion, we performed a screen of chromatin-modifying factors for their effects on tumorigenesis in RMS, using zebrafish as a model organism. SUV39H1 was determined to be a suppressor of RMS formation, dependent on its histone methyltransferase activity, suppressing tumor initiation likely by suppressing cyclin B1 and causing cell cycle arrest. These studies support previous work that SUV39H1 is a marker of senescence and will warrant further examination of the role of SUV39H1 in muscle differentiation.

Supplemental Figures

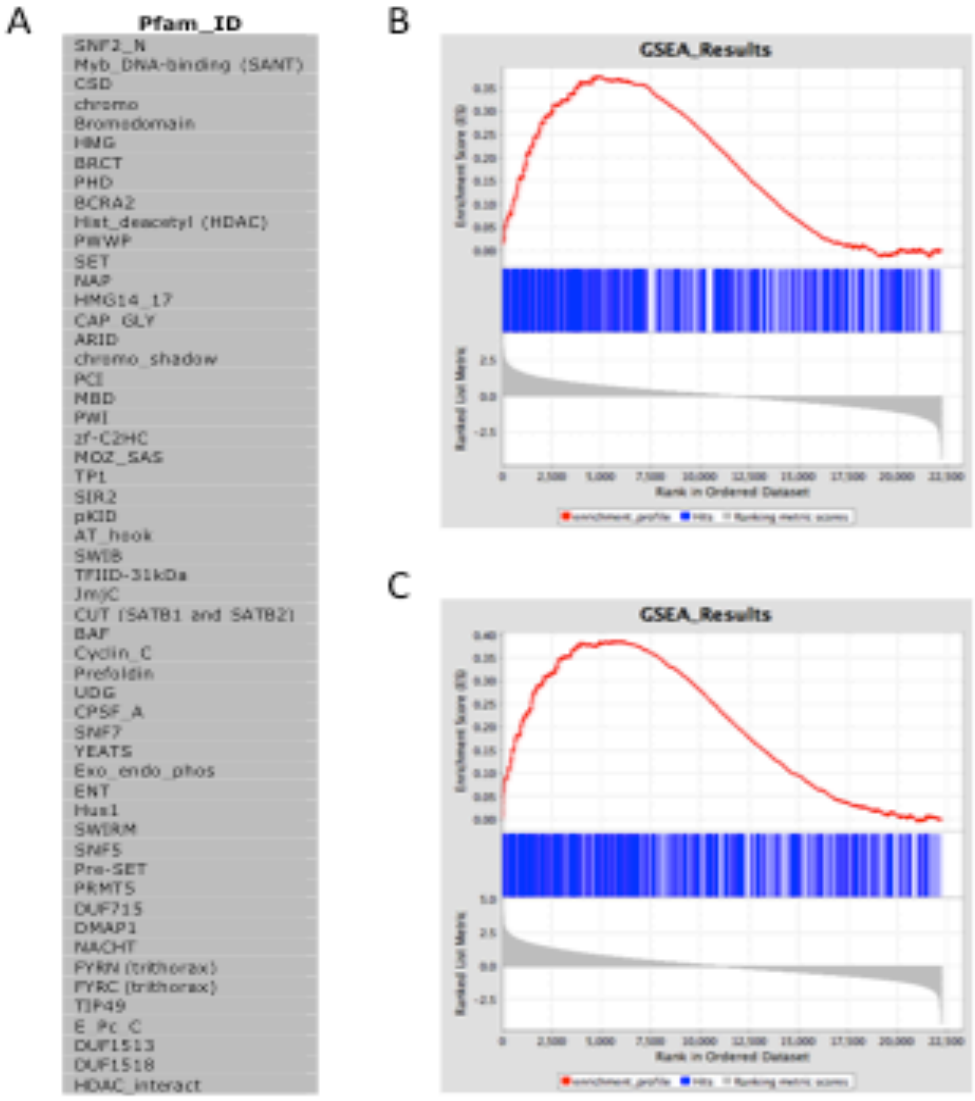


Figure 2.S1. **Human RMS samples contain upregulation of chromatin-modifying factors. (A)** List of protein domains resulting in a list of chromatin-modifying factors. **(B)** Gene set enrichment analysis results show significant enrichment for chromatin factor gene lists in human embryonal RMS versus normal human juvenile muscle. **(C)** Gene set enrichment analysis results show significant enrichment for chromatin factor gene lists in human alveolar RMS versus normal human juvenile muscle ($p<0.05$ for B,C).

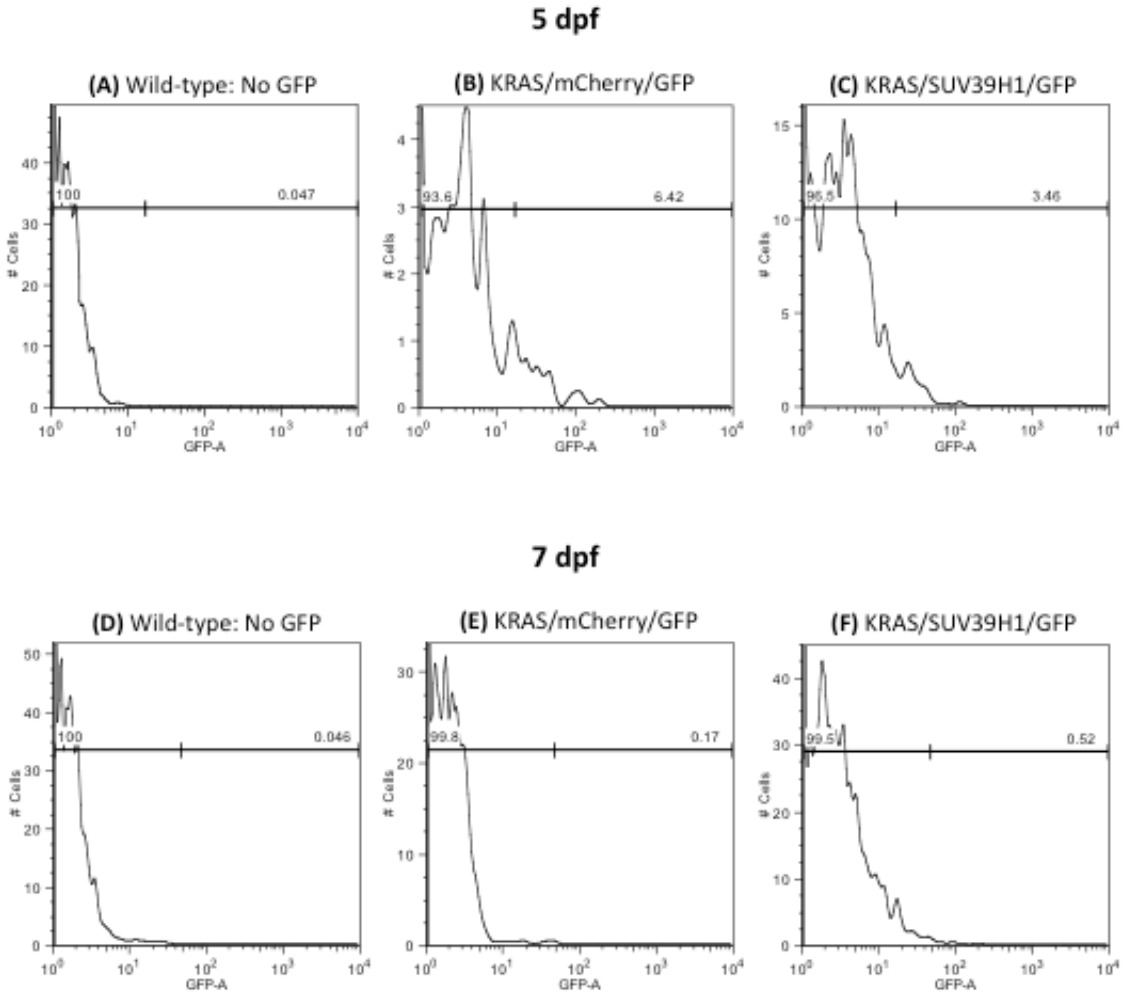
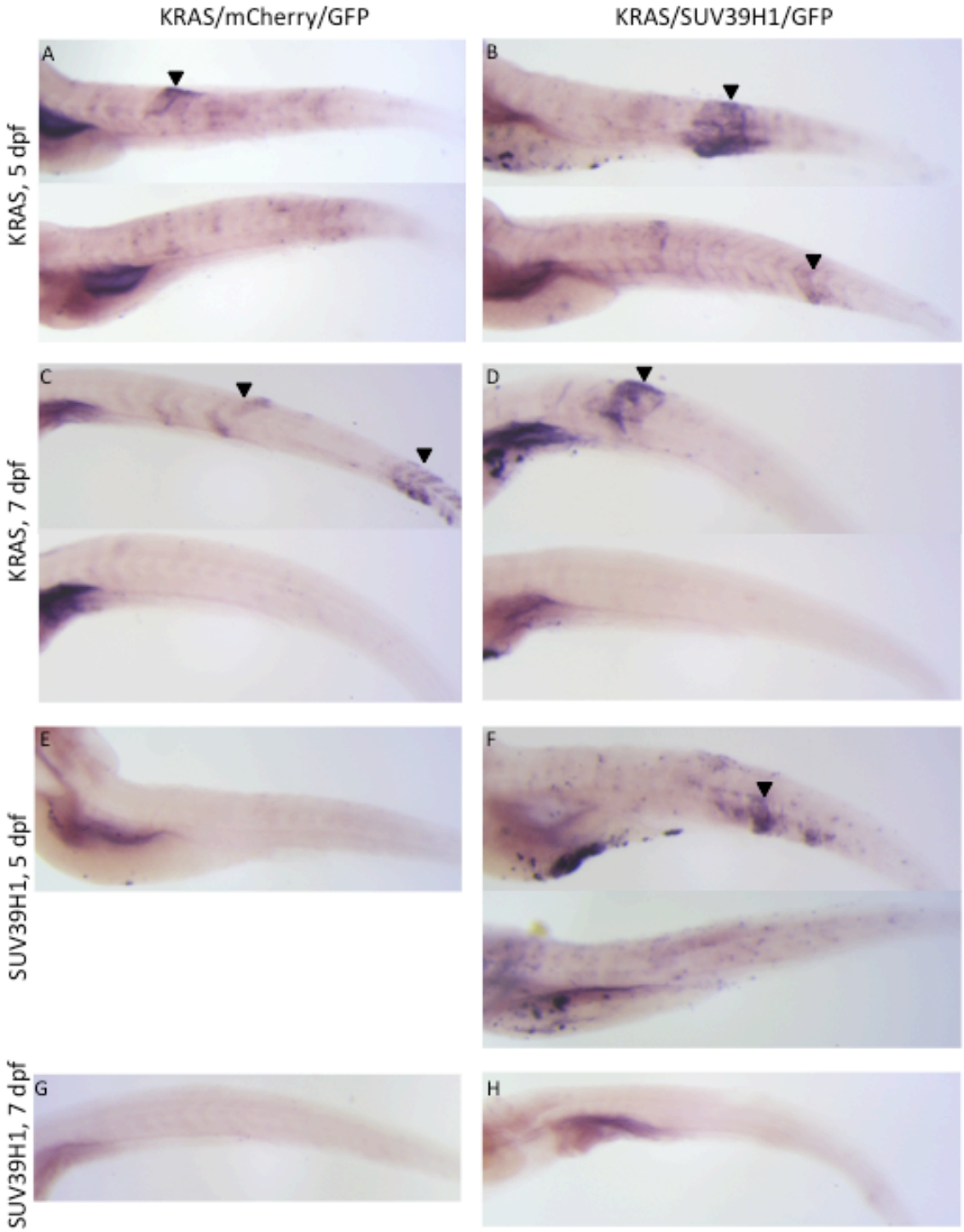


Figure 2.S2. GFP analysis by FACS reveals minimal GFP protein expression, with no differences between fish. (A-C) FACS analysis for GFP mRNA in 5 dpf fish injected with of *rag2*-hKRAS^{G12D}, *rag2*-hSUV39H1 or mCherry, *rag2*-GFP, and *mylz2*-mCherry, then selected for mCherry-high embryos, compared to wild-type fish. The analysis reveals a small number of dimly expressing cells present in both injected samples. **(D-F)** FACS analysis for GFP of sibling fish at 7 dpf. Both injected samples have very low amounts of GFP.

Figure 2.S3. KRAS and SUV39H1 expression generally localizes to developing tumors. **(A)** In *rag2*-hKRAS^{G12D}, *rag2*-mCherry, *rag2*-GFP, and *mylz2*-mCherry-positive 5 dpf fish, KRAS expression mostly localizes in distinct patches, likely where tumors are forming. **(B)** The same occurs for *rag2*-SUV39H1 5 dpf fish. **(C,D)** Some 5 dpf fish do not show much KRAS expression at all, as those fish are likely not forming tumors yet. **(E-H)** In 7 dpf larvae, KRAS expression is similar, with patches of expression in some fish, likely in developing tumors, and minimal expression in others. **(I, L)** As expected, there is no SUV39H1 mRNA expression in control fish. **(J,K,M)** Similar to KRAS expression, SUV39H1 expression is observed in patches of tumors forming or not visualized at all. In all panels, arrowheads note patches of expression (8X images).

Figure 2.S3 (Continued)



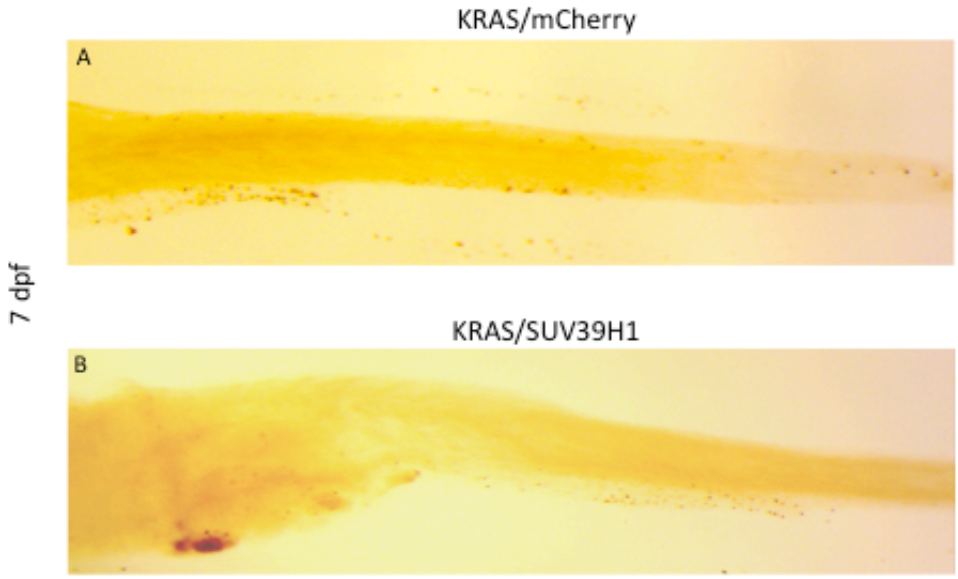


Figure 2.S4. SUV39H1 overexpression does not lead to increased apoptosis. (A) In *rag2*-hKRAS^{G12D}, *rag2*-mCherry, and *mylz2*-mCherry-positive 7 dpf fish, there is minimal programmed cell death, as noted through TUNEL staining. **(B)** Similar levels are seen in *rag2*-hKRAS^{G12D}, *rag2*-hSUV39H1, and *mylz2*-mCherry-positive 7 dpf fish, suggesting the tumor initiating cells are not simply dying off (5X images).

Addendum

The role of chromatin in muscle development

Epigenetic structure is known to be important during muscle development. It begins on the DNA level, as DNA demethylation is a critical step committing cells to the myogenic lineage. If DNA methylation is blocked using either 5-azacytidine or antisense nucleotides against *dnmt1*, this can convert fibroblasts to muscle cells [272, 273]. Genome-wide demethylation occurs in myoblasts upon differentiation induction, but some of the specific loci likely targeted are the regulatory regions of MyoD and myogenin [274-276]. Once the cells are committed muscle progenitors, many of the differentiation genes remain repressed through their chromatin structure, ensuring no premature differentiation occurs. In precursors, MyoD and its associated transcription factor MEF2 interact with histone deacetylases (HDACs), the repressive YY1 protein, and HMTs associated with silencing [277-283]. Overexpression of EZH2, an H3K27 HMT, can inhibit muscle differentiation by keeping muscle creatine kinase and myosin heavy chain repressed [282]. Overexpression of SUV39H1 directly represses MyoD and therefore differentiation, and G9a, an H3K9me2 HMT, similarly blocks differentiation through methylation on MyoD target promoters and MyoD itself [252, 283].

The chromatin landscape changes dramatically once muscle differentiation is ready to commence. MyoD and MEF2 no longer associate with repressive chromatin factors [284]. Repressive chromatin structure keeps myogenin and muscle creatine kinase transcriptionally inactive until MyoD opens the structure [285]. This is mediated through recruitment of the SWI/SNF chromatin remodeling complex by

MyoD, loss of which prevents muscle differentiation [286]. Additionally, MyoD directly binds the HAT p300, forming a complex with p300 and PCAF, another HAT [287-289]. To ensure that MyoD can fully activate its target genes, p300 acetylates histones for an open chromatin structure while PCAF acetylates MyoD to enhance its DNA binding activity [290, 291]. The epigenetic landscape aids in the regulation and timing of muscle differentiation.

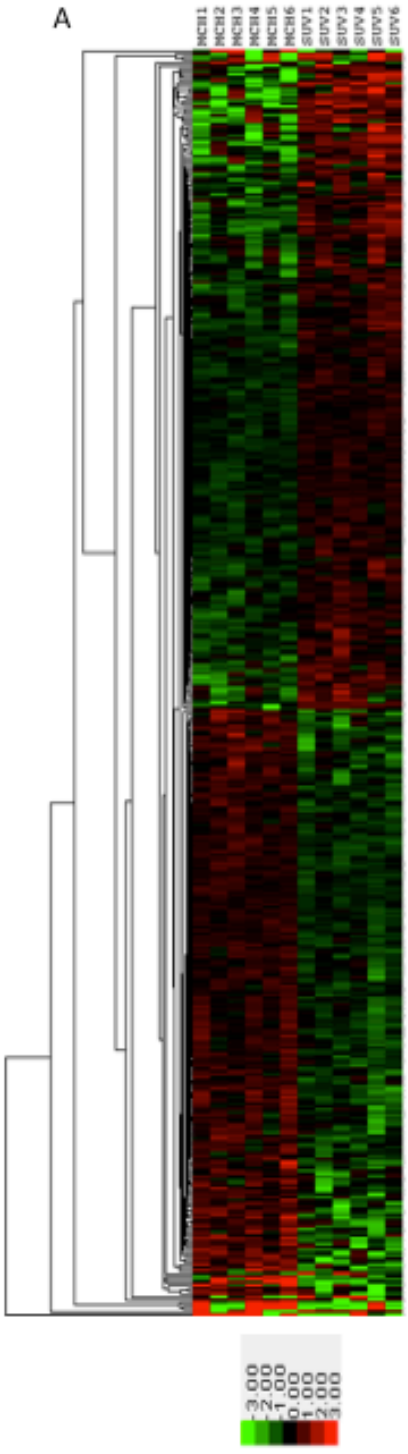
Molecular characterization of SUV39H1 in RMS

To understand the gene expression changes that occur in our different RMS tumor types, microarray analysis was performed on six SUV39H1-overexpressing tumors and six control mCherry-overexpressing tumors. A variety of genes were differentially expressed between the two tumor types, with definable clusters associating with the different tumors types (Figure 2.A1.A). The most striking set of genes were a set of transcription factors whose expression was down in the SUV39H1-overexpression tumors and would be consistent with overexpressed SUV39H1 functioning as a transcriptional repressor (Figure 2.A1.B). The most notable factor on this list was Pax7, a known player in muscle differentiation and translocation partner in causing ARMS. Also, quantitative PCR analysis of a panel of endogenous muscle genes, along with $hKRAS^{G12D}$ and hSUV39H1, revealed that expression of the muscle program was decreased in SUV39H1-overexpressing tumors as compared to mCherry control tumors (Figure 2.A2.A). However, when this panel was examined in the SUV39H1 enzymatic point mutants, C326A and H324K, expression of some muscle genes was increased in comparison with the SUV39H1 tumors (Figure 2.A2.B,C). This fits with previously known data that

Figure 2.A1. Global gene expression analysis reveals transcription factor targets. (A) Heat maps for 6 SUV39H1-overexpressing and 6 mCherry-overexpressing tumors reveal clusters of gene that are differentially expressed between the two tumor types. **(B)** A list of transcription factors that are downregulated in the SUV39H1-overexpressing tumors, including the muscle transcription factor Pax7.

Figure 2.A1 (Continued)

A



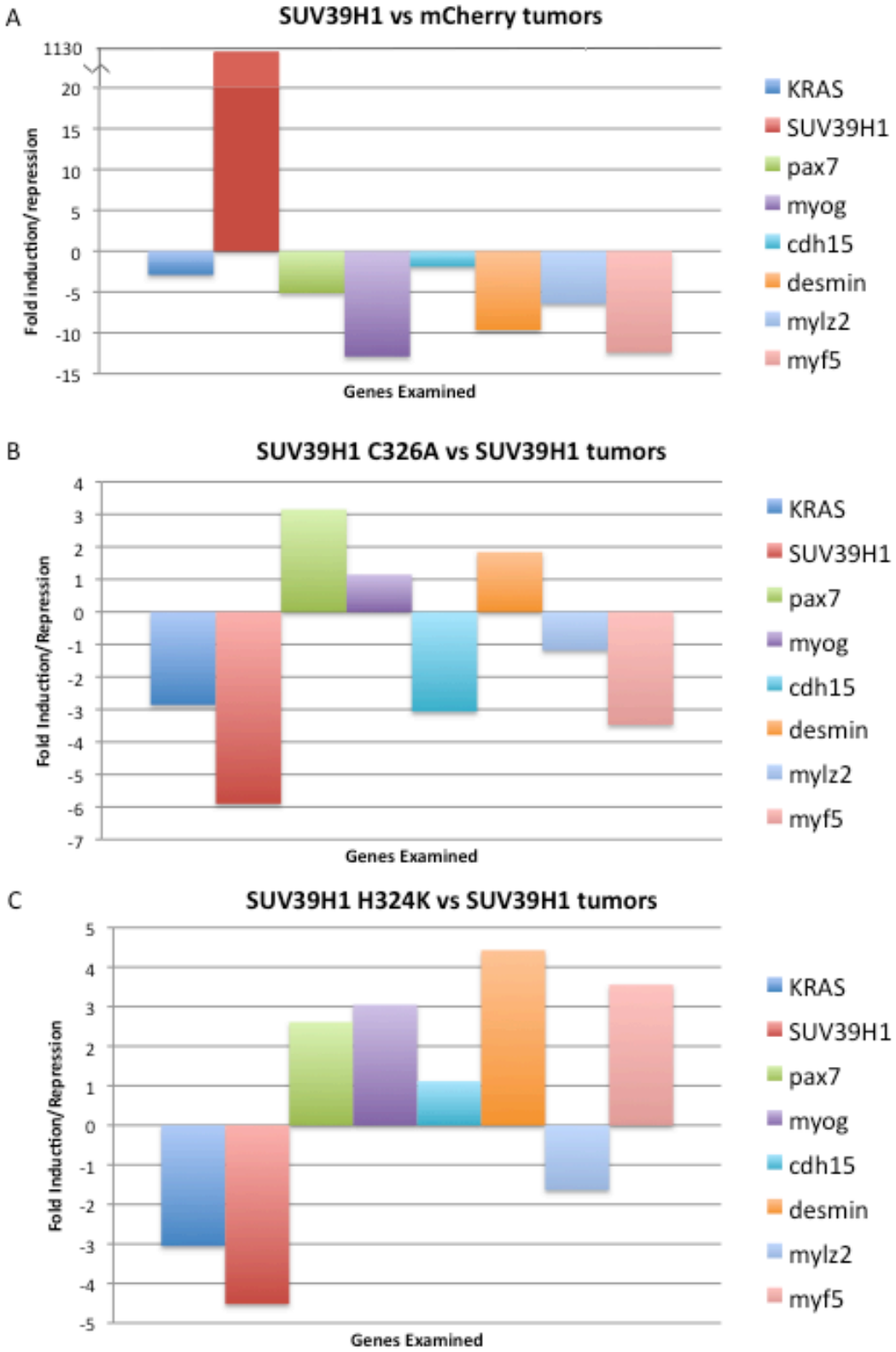
B

Transcription factors that are downregulated in the SUV39H1 tumors:

Gene	Fold change
SOX9	-3.051189
FOXP4	-1.238242
SOX11	-1.200574
SIX4	-1.108439
PAX7	-1.025738
ID2	-1.01725
SPRY2	-1.006189
HLF	-1.002747
DBX2	-0.988144
MXD4	-0.933126
PBX1	-0.901805
SPRY4	-0.838193

Figure 2.A2. Gene expression analysis of muscle genes in SUV39H1-overexpressing tumors. (A) qRT-PCR analysis from SUV39H1-overexpressing tumors compared to mCherry tumors reveals that some of the muscle program may be transcriptionally down in the SUV39H1 tumors. **(B)** When compared to SUV39H1-overexpressing tumors, some of the muscle program is upregulated in SUV39H1^{C326A} tumors. **(C)** When compared to SUV39H1-overexpressing tumors, most of the muscle program is upregulated in SUV39H1^{H324K} tumors.

Figure 2.A2 (Continued)



SUV39H1 suppresses MyoD and differentiation and suggested that SUV39H1 is impacting the muscle program [251, 252]. An interesting observation here is the RAS levels across tumor types. Though RAS levels appear to be slightly lower in the SUV39H1 tumor compared to controls (Figure 2.A2.A), they also appear to be lower in the point mutants compared to wild-type SUV39H1 tumors, yet the C326A/H324K-overexpressing fish get more tumors than their wild-type SUV39H1 counterparts (Figure 2.A2.B,C). Since RAS levels do not correlate with tumor incidence, the conclusion is that RAS levels are not responsible for the tumor suppressive phenotype.

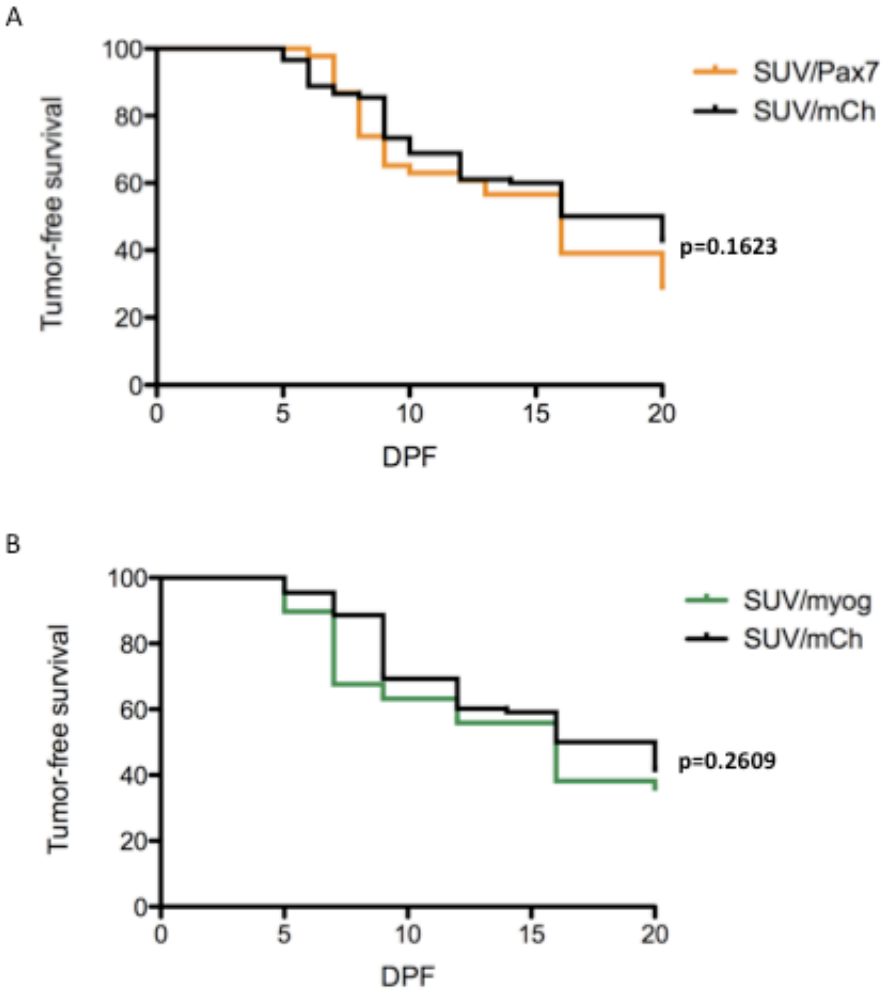
To determine if SUV39H1 repression of Pax7 was responsible for the suppressed tumor phenotype, we co-injected *rag2*-hKRAS^{G12D}, *rag2*-mPax7 with either *rag2*-hSUV39H1 or *rag2*-mCherry in our fluorescent tumor assay (*rag2*-GFP) along with the marker *mylz2*-mCherry. The Pax7 overexpression led to no significant difference between the suppressed SUV39H1 tumor curve with a control vector and the tumor curve with SUV39H1 and Pax7 ($p=0.1623$, Figure 2.A3.A), suggesting that Pax7 did not mediate the suppression phenotype. We repeated these curves with myogenin as well, which also showed no difference from the suppressed SUV39H1-overexpression curve ($p=0.2609$; Figure 2.A3.B). These data led us to conclude that the muscle program was not the sole cause of tumor suppression by SUV39H1.

Discussion

SUV39H1 appears to play a role in muscle differentiation. It is known to directly silence MyoD and differentiation through H3K9me3 [252]. SUV39H1 is

Figure 2.A3. Overexpression of muscle transcription factors cannot revert the SUV39H1-mediated suppressive phenotype. (A) Injections into one-cell stage embryos of *rag2*-hKRAS^{G12D}, *rag2*-hSUV39H1, *rag2*-mPax7, *rag2*-GFP, and *mylz2*-mCherry, then selected for mCherry-high embryos, and monitored for tumor formation by GFP presence, results in a suppressed tumor curve that is the same as *rag2*-hSUV39H1; *rag2*-mCherry control curves (Pax7 n=46, mCherry n=90, p=0.1623). **(B)** Injections into one-cell stage embryos of *rag2*-hKRAS^{G12D}, *rag2*-hSUV39H1, *rag2*-myog, *rag2*-GFP, and *mylz2*-mCherry, then selected for mCherry-high embryos, and monitored for tumor formation by GFP presence, results in a suppressed tumor curve that is the same as *rag2*-hSUV39H1; *rag2*-mCherry control curves (myog n=68, mCherry n=88, p=0.2609).

Figure 2.A3 (Continued)



required for S phase gene silencing in differentiating cells; myoblastic C2C12 cells under differentiation conditions were treated with SUV39H1 siRNA and were unable to differentiate [101]. Furthermore, in an ARMS cell line under differentiation conditions, SUV39H1 expression increases, inhibiting MyoD and differentiation; knockdown by siRNA restores differentiation as well as decreases tumorigenicity [251].

Initially, it seemed like a reasonable mechanism that overexpression of SUV39H1 would promote muscle differentiation and therefore suppress tumor formation. However, our data do not support this hypothesis. Though some experiments, such as the qPCR on a series of muscle-specific genes, were in line with this hypothesis, several factors were not. First, the histology of the control tumors and SUV39H1-overexpressing tumors was identical, and both sets of tumors were of the most primitive differentiation state seen in zebrafish RMS, indicative of tumors originating from muscle satellite cells [257]. This did not support the SUV39H1-overexpressing tumors becoming more differentiated. Additionally, co-injection of other muscle regulatory genes, including Pax7 and myogenin, did not alter the suppressive effects of SUV39H1 on RMS. If SUV39H1 was truly just silencing MyoD, overexpression of the downstream myogenin should have rescued the more aggressive tumor phenotype. Therefore, we concluded that SUV39H1 was not simply impacting the muscle program in the zebrafish RMS model, as the literature may have predicted.

Chapter 3

The development of a transgenic overexpression approach to screen for chromatin-modifying factors involved in hematopoietic development

Attributions

I am the primary contributor to this project. I designed the F0 and F1 overexpression screens. I prepared vector dilutions for microinjections, performed all microinjections of vectors into one cell stage embryos, and scored embryos for fluorescence. I scored larval and adult zebrafish for tumor formation by eye and fluorescence. I crossed transient transgenic animals and screened their progeny by fluorescence microscopy for presence of GFP-positive hearts. I staged all embryos and prepared them for *in situ* hybridization. I cloned the LCR constructs with a core LCR sequence and developed all Tol2 *lmo2*, *rag2*, and *ubiq* vectors. I cloned the non-Tol2 *rag2*-chromatin factor constructs and cloned all pCS2-ORF vectors. I made mRNA for all chromatin factor ORFs and transposase for the Tol2 injections. I synthesized probes for various blood markers and chromatin factors, performed all whole mount *in situ* hybridizations and double fluorescent *in situ* hybridizations, and photographed all fish. I prepared fish for sectioning.

Narie Storer made the non-Tol2 *rag2* destination vector. She assisted with cloning of the non-Tol2 *rag2*-chromatin factor constructs. Nelson Hsia provided sequence data on the globin locus control region. Joseph Lee provided technical assistance with microinjections and GFP scoring. Yi Zhou and Peter Song identified the list of 333 chromatin-modifying factors. The Brigham and Women's Pathology Core performed sectioning and staining of pathology slides.

Introduction

Vertebrate Hematopoiesis

All vertebrates require blood flowing in their bodies to carry oxygen and other nutrients to cells of all tissues and to provide defenses against foreign pathogens. Hematopoiesis, or the differentiation of the mature blood lineages from immature progenitors, occurs throughout the lifetime of an organism to regularly replenish the various cellular components of the blood. It occurs in two successive waves known as the primitive and definitive waves. The primitive wave produces hundreds of erythrocytes, or red blood cells (RBCs), in the intermediate cell mass (ICM) and some macrophages to support the developing embryo [292, 293]. The RBCs are defined by expression of embryonic *globin*s and *gata1*, the erythroid master regulator transcription factor, whereas the macrophages are characterized by expression of *pu.1*, a myeloid master transcription factor [294, 295]. The definitive wave results in the production of all blood cells through the hematopoietic stem cells.

Hematopoietic stem cells (HSCs) are rare cells that generate the entire blood system of an organism, comprised of lymphocytes, RBCs, myeloid cells, and thrombocytes. In addition to this ability, HSCs also have the capacity to self renew, maintaining their own population for the life of that organism. In the hematopoietic system, self-renewal is traditionally assessed by long-term transplantation capability [296-298]. A single HSC transplanted into a lethally irradiated recipient can rescue the entire immune and nervous systems [299]. Serial transplants, where HSCs from a primary transplanted organism are then transplanted into a new

recipient, are also used to demonstrate the self-renewal capacity of an HSC [300, 301]. Extrinsic regulators in the niche can impact the self-renewal of an HSC, including Wnt, Notch, Hedgehog, BMP, angiopoietin-like factors, thrombopoietin, retinoic acid, and prostaglandins [302-313]. Though many extrinsic pathways responsible for determining whether an HSC undergoes self-renewal have been revealed, the stem cell-intrinsic pathways are still not completely understood.

The developing HSCs in a mammalian embryo are initially found in the aorta-gonad-mesonephros (AGM) region [314, 315]. Similarly, in a zebrafish embryo, the first definitive HSCs are found in the ventral wall of the dorsal aorta by 36 hours post fertilization (hpf) [316, 317]. These can be detected as clusters of cells expressing *runx1* and *c-myb* [318-320]. The HSCs then bud off from the floor of the aorta and enter circulation to migrate to their next location [321, 322]. For many, this will be the caudal hematopoietic tissue, or CHT, which is roughly equivalent to the mammalian fetal liver [323, 324]. Here the HSCs will differentiate and expand while also producing the progenitor cells that will seed the thymus and kidney, the adult sites of hematopoiesis [323, 325]. In zebrafish, the kidney marrow is the equivalent of the mammalian bone marrow, with the HSCs residing near the reticular stromal cells among renal tubules and blood vessels. By 5 days post fertilization (dpf), nearly all HSCs are found in the kidney marrow rather than the AGM, producing HSCs and blood cells for the life of the zebrafish [293]. Flow cytometry analysis comparing cells by size (forward scatter) and granularity (side scatter) can be performed on the zebrafish kidney marrow to reveal four distinct populations, erythroid, myeloid, lymphoid, and precursors [326].

With the HSCs in the kidney marrow by 5 dpf, definite hematopoiesis is well underway by this timepoint in the zebrafish. Definitive erythropoiesis produces RBCs to replace the primitive ones in circulation [327]. Myelopoiesis generates two lineages by 7 dpf, one resembling mammalian neutrophils and one resembling a combination of mammalian eosinophils and basophils [293, 328]. Starting at 3-4 dpf, T cells derive from the kidney and develop in the bilateral thymi [293, 329]. B cells develop in the kidney marrow, commencing at 19 dpf [330]. Lastly, thrombocyte production begins around 36 hpf, resulting in cells similar to mammalian platelets [331].

HSC Self-Renewal and Chromatin

Chromatin-modifying factors are likely candidates to control cell intrinsic pathways regulating self-renewal in HSCs. One of the most well known families of chromatin factors that have a role in hematopoiesis is the Polycomb Group (PcG) of proteins. The PcG proteins were first discovered in *Drosophila melanogaster* as regulators of homeotic genes to form the various body segments of the fruit fly [332, 333]. Similarly, two main complexes of PcG proteins exist in vertebrates that play a role in repressing chromatin. Polycomb repressive complex 1 (PRC1) consists of chromodomain proteins HPC1, 2, and 3; HPH1, 2, and 3; E3 ubiquitin ligases RING1A and 1B; and the two homologues BMI-1 and MEL18. This complex is typically responsible for maintaining the repressive chromatin structure [334]. PRC2 is composed of the WD40 domain protein EED, HMT EZH2, zinc finger domain protein SUZ12, and PHD domain proteins PHF19 and MTF2. PRC2 establishes the repressive mark in the chromatin [335]. Two other complexes exist, PRC3 and PRC4,

which are less well studied; their components are similar to PRC2, with different EED isoforms [336, 337].

It has been proposed that the balance between the two major PcG complexes, PRC1 and PRC2, may impact whether a stem cell chooses to remain quiescent or to divide and differentiate [338, 339]. It is evident that these complexes play a role in HSCs, as PRC2 members EZH2 and SUZ12 are highly expressed in zebrafish and murine HSCs [340, 341]. Additionally, overexpression of BMI-1, of the PRC1 complex, in murine HSCs promotes symmetrical divisions and a higher repopulation potential; this increased self-renewal capacity is not seen when BMI-1 is overexpressed in more mature progenitor cells instead [342].

Two studies characterized the role of BMI-1 in the self-renewal of murine HSCs. The first showed that BMI-$1^{-/-}$ mice contain half as many adult HSCs as opposed to heterozygote or wild type mice, as determined by flow cytometry. The few HSCs found in BMI-$1^{-/-}$ mice have no detectable self-renewal since they cannot reconstitute the blood lineages upon transplantation [343]. A second study showed that AML cells, formed from BMI-$1^{-/-}$ fetal liver cells, were derived from only two to four clones; the idea that proliferative capacity was unaffected but there were fewer leukemic stem cells (L-HSCs) due to a self-renewal defect was confirmed when this leukemia did not transplant into another recipient [344]. Additionally, BMI-1 acts to protect HSCs from exhaustion caused by expression of Ink4a and Arf, as knocking out Ink4a and Arf in an HSC-depleted BMI-1 knockout mouse can rescue HSC self-renewal [345]. It is apparent that BMI-1 plays a role in the self-renewal of HSCs.

Other members of the PcG have been implicated to play a role in HSC self-renewal as well. RAE28, an HPH protein and member of PRC1, appears to play an integral part in self-renewal. Murine fetal liver cells lacking RAE28 cannot reconstitute the blood system upon transplantation, implying a self-renewal defect; mice without RAE28 also seem to have decreased numbers of HSCs and decreased stem cell activity [346, 347]. An additional PRC1 member, MEL18, seems to have contrasting effects. Loss of MEL18 increases HSC quiescence and promotes self-renewal; thus, MEL18 appears to negatively regulate HSC reconstitution capabilities [348]. Many PcG factors are impacting HSC self-renewal, both positively and negatively.

Trithorax proteins, whose functions generally oppose that of the PcG proteins by activating transcription, are also known to play a role in HSC self-renewal [349]. For example, MLL, the *trithorax* homolog, is the mixed lineage leukemia gene that acts as a transcriptional activator, with one of its major targets being the homeobox genes. MLL is often found in leukemic cells as part of a fusion gene generated by chromosomal translocations during oncogenesis; it has over fifty translocation partners. Over half of all infant acute leukemias and 10% of common AMLs contain MLL translocations or duplications [350]. The MLL-AF9 translocation, for example, can initiate transformation in a myeloid progenitor cell by turning on a self-renewal associated gene signature and inducing self-renewal capacities in the progenitor cell; therefore, overexpression of MLL can increase self-renewal [351]. Conversely, embryo and chimera studies demonstrated that MLL-deficient cells have decreased self-renewal and cannot contribute to the pool of HSCs in an organism; without MLL,

the cells enter the cell cycle and are depleted, so the role of MLL is likely to maintain quiescence [352, 353]. It is clear that MLL plays a key role in regulating HSC self-renewal.

Methylation also participates in the intrinsic control of self-renewal in HSCs. Combinatorial knockdown of both Dnmt3a and Dnmt3b, *de novo* DNA methyltransferases, impaired the self-renewal of murine HSCs, as assayed through long-term transplantation, despite the knockout HSCs having normal differentiation capacities [354]. A more recent study demonstrated the opposite phenotype by conditionally depleting Dnmt3a alone. They showed that Dnmt3a plays a role in silencing HSC regulatory genes to keep the balance between self-renewal and differentiation [355]. Despite the technical differences in the studies, which may have led to the differing results, both groups would agree that the Dnmt3 factors play some role in HSC self-renewal. This is also confirmed since Dnmt3a mutations are found in about 20% of AML cases [229, 230]. Disruption of Dnmt1 in the hematopoietic system leads to impaired HSCs that cannot self-renew or produce the appropriate progenitor cells for standard hematopoiesis to take place [356, 357]. Histone methylation is also likely important for HSC self-renewal, as murine HSCs express Fbxl10, or F-box and leucine-rich repeat protein 10, a histone H3K36 demethylase; forced expression of Fbxl10 produced more multipotent cells in colony-forming assays and in long-term serial transplantation assays [358].

Many other types of chromatin-modifying factors also have been shown to impact HSC self-renewal. Hmgb3, a high mobility group protein that can bind DNA, is preferentially expressed in HSCs; knockdown of the gene in mice produces more

HSCs at the expense of differentiation, due to activation of the Wnt pathway, suggesting it plays a key role in the regulation of self-renewal [359]. Conditional knockout of Mi-2beta, an ATPase and component of the Nucleosome Remodeling Deacetylase (NuRD) complex, leads HSCs to enter the cell cycle and produce only erythroid progenitors, revealing that Mi-2beta contributes to both self-renewal and lineage priming [360]. Deletion of murine MOZ, a histone acetyltransferase known to interact with key blood transcription factors Runx1 and PU.1, results in embryonic death, caused by lack of HSCs and therefore all hematopoiesis [361, 362]. MOZ is also a frequent translocation partner found in leukemias, and its translocation with TIF2 can induce self-renewal in hematopoietic progenitor cells during oncogenesis [363].

Chromatin-modifying factors are increasingly becoming known as regulators of hematopoiesis and HSC self-renewal. However, since the few factors tested come from a wide variety of families, it is likely that many more with important roles remain to be discovered. Here we describe a series of novel screening approaches to determine what factors, when overexpressed in the hematopoietic system, have an impact on HSCs and hematopoiesis. A F1 transgenic screening strategy was determined to be the best approach in targeting the desired tissue. Our results will have future applications to target the hematopoietic system as well as other tissues.

Zebrafish

Zebrafish were maintained and developmentally staged as previously described according to IACUC guidelines [255]. The Animal Care and Use Committee, Children's Hospital Boston approved all animal protocols.

Vectors and Cloning

The plasmid containing the zebrafish globin locus control region (LCR), globin alpha-A1 promoter, and GFP was obtained from N. Hsia [364]. To obtain this plasmid containing just the core 200 bp of the LCR, the following primers were used to amplify the core sequence by High Fidelity PCR and add the respective restriction enzyme sites: 5'- GGGGTACCCCAAGTGCAGAGTCATGGAGGGCT-3' and 5' GGACTAGTCCAAGTGATGAGTCAGCTGTTTGT-3'. After gel extraction of this PCR reaction, the core fragment and the original plasmid were digested using KpnI and SpeI enzymes and treated with Antarctic Phosphatase, and the core fragment was ligated in place of the original 6.2 kb LCR. The non-Tol2 *beta-actin*-GFP and dsRed plasmids were obtained from D. Langenau. The p5E-*lmo2* construct was obtained from O. Tamplin. The p5E-*ubiquitin* construct was obtained from C. Kaufman [365]. The non-Tol2 *rag2* destination vector was obtained from N. Storer. The Tol2-p*lmo2* Gateway destination vector (#367) as well as the pCS2 Gateway destination vector (#201) was obtained from the Lawson lab. The Tol2 multisite destination vectors without and with *cmlc2*-GFP (#394, 395), the p5E-*beta actin* (#299), the p5E-multiple cloning site (#228), the pME vectors with GFP and mCherry (#383, 386), and the p3E-polyA vector (#302) were obtained from the Tol2Kit [366]. The Tol2

beta-actin-GFP and mCherry plasmids were cloned through multisite Gateway reactions using LR Clonase II Plus (Invitrogen) with vectors 394, 299, 383/386, and 302. The p5E-*rag2* construct was cloned by digesting vector 228 and a non-Tol2 *rag2*-BMI-1 with XhoI and BamHI enzymes and treating with Antarctic Phosphatase. Quick ligation of the components put the *rag2* promoter into the p5E vector. The Tol2-*rag2*-ORF plasmids were cloned through multisite Gateway reactions using LR Clonase II Plus (Invitrogen) with vectors 395, p5E-*rag2*, the desired ORF, and 302. The Tol2-*lmo2*-ORF plasmids were cloned through multisite Gateway reactions using LR Clonase II Plus (Invitrogen) with vectors 395, p5E-*lmo2*, the desired ORF, and 302. All open reading frames were obtained from the Ultimate ORF Clone collection (Invitrogen), with the exception of mMyc (C. Xu), MLL-AF9 (M. Stubbs from the Armstrong lab), runx1 (E. Trompouki), and BMI-1 (N. Storer).

Microinjection

All non-Tol2 constructs were linearized with XhoI, purified by phenol:chloroform extraction, and precipitated in ethanol. These purified constructs were diluted to 50 ng/uL in 0.5x TE + 0.1 M KCl for injections, when one nL of this stock would be injected into one-cell stage embryos. All Tol2 constructs were injected at a total of 25 ng/uL with 15 ng/uL transposase mRNA in 0.5x TE + 0.1 M KCl; one nL of this dilution was injected into one cell stage embryos. Transposase was made by linearizing the pCS-TPase vector with NotI, synthesizing mRNA with the SP6 mMessage mMachine kit (Ambion), and purifying with the RNeasy mini kit (Qiagen). All pCS constructs for chromatin factor mRNA injections were linearized with NotI, then gel extracted. This extracted DNA served as a template for the SP6 synthesis

reaction using the mMessage mMachine kit (Ambion); the synthesized mRNA was purified using the RNeasy mini kit (Qiagen). The purified mRNA was diluted with water to a concentration of 40 ng/uL for injections of 1 nL, 2 nL, or 3 nL to yield 40 pg, 80 pg, and 120 pg. All zebrafish used for microinjection were of the AB strain.

Identification of known and putative chromatin modifying factors

Human chromatin modifying factors were identified using CREMOFAC, SMART domain, CDD, and Pfam databases.

***In Situ* Hybridization and Histopathology**

Whole mount *in situ* hybridizations (ISHs) were performed as previously described [259, 260]. Antisense mRNA probes were synthesized from digested plasmids [260]. For the chromatin factors, the antisense probes were synthesized from the pCS2-ORF vectors for each factor. The vectors were linearized with either EcoRI or BamHI, using one enzyme if the other site was present in the ORF. After phenol:chloroform extraction, the DIG-labeled probes were synthesized using T7 polymerase and purified using the RNeasy kit (Qiagen). Whole-mount double fluorescent ISH was performed as previously described using the TSA Plus Fluorescein and TSA Plus Cy3 kits for tyramide amplification (Perkin Elmer) [367]. For ISH on the adult thymus, fish were euthanized and fixed in 4% paraformaldehyde overnight at 4°C. Paraffin embedding, sectioning, H&E staining, and RNA *in situ* hybridization were performed according to standard techniques by the Brigham & Women's Pathology Core. RNA *in situ* probes were synthesized in the same manner as for whole mount ISHs.

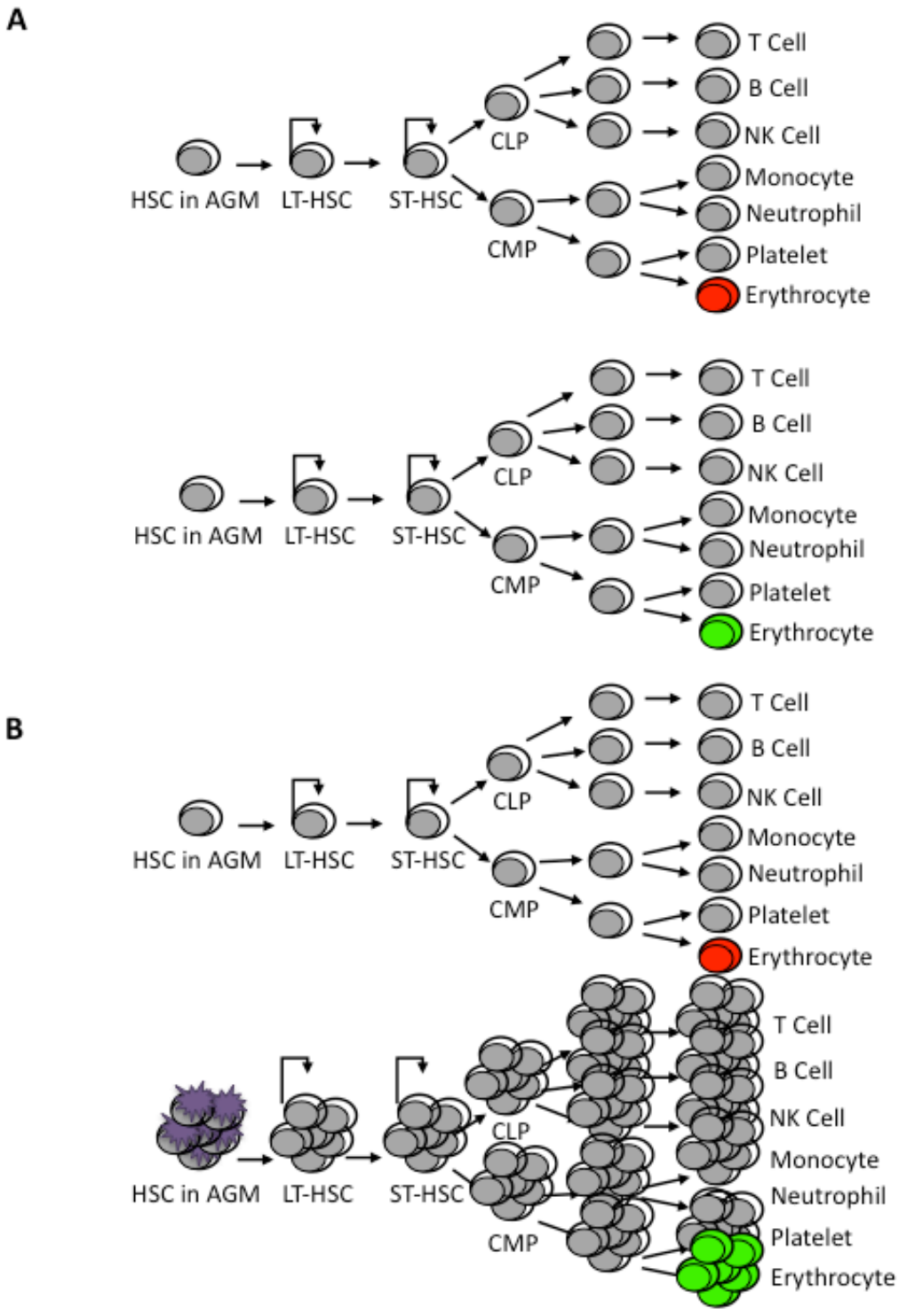
In vivo Hematopoietic Stem Cell Competition Assay

The gold standard for assessing self-renewal capabilities of an HSC is the transplantation assay. However, when performing a transplant, several other factors are involved in determining whether the HSC successfully repopulates the recipient's blood system. First, the HSC must successfully home and migrate to the marrow; second, it must interact properly with the niche in the marrow, allowing for engraftment. Homing, migration, and niche interactions represent an additional set of factors, each with their own regulation. Furthermore, performing transplants can be laborious and tedious, not ideal for high-throughput screening. In order to focus on self-renewal in an efficient *in vivo* setting, we proposed creating a competitive assay to monitor HSC competition during embryonic development. This assay would be parallel to transplants but in the context of development instead, as the HSCs would compete with each other in the AGM rather than the marrow and engraftment would still be read out in the differentiated lineages. If an HSC has a competitive advantage over other HSCs as they arise in the AGM, we would read out this increased self-renewal in the downstream lineages, such as the RBCs (Figure 3.1). This concept was feasible because definitive HSCs are rare within the fish, such that if the self-renewal of a few HSCs increased, the downstream effects would present in a large proportion of the blood lineage.

We set out to construct a transgenic system to overexpress an array of chromatin-modifying factors in individual HSCs within the same animal and then determine which factors are important for self-renewal. This system required a

Figure 3.1. Conceptual schematic for *in vivo* stem cell competition assay. (A) Different HSCs present in the AGM of a developing zebrafish each can differentiate into a full set of mature blood cells. If one of these HSCs were carrying a construct driving GFP expression by the globin locus, it would result in GFP-positive erythrocytes. A different HSC could contain a construct with mCherry driven by the globin locus, resulting in mCherry-positive erythrocytes instead. **(B)** With the base strategy presented in (A), the HSC capable of producing GFP-positive erythrocytes could also contain a factor that would give this HSC a competitive advantage over the other. This would lead to an increased number of these HSCs, relative to the other, which could be read out as an increase in GFP-positive erythrocytes as opposed to the mCherry-positive erythrocytes.

Figure 3.1 (Continued)



method to express the factor of choice in the HSC as well as a method to assess "engraftment" downstream. Using an HSC/progenitor specific promoter, the expression of chromatin factors would be driven in the HSCs. Utilizing an RBC-specific promoter to drive two or more fluorescent proteins in this differentiated lineage served as the engraftment readout. Ideally these two components would be placed on a single vector to ensure the HSC receiving the chromatin factor always also received the same fluorescent color for accurate readout. Then, the different RBCs, formed from different HSCs, would be marked *in vivo* by different fluorescent proteins. The presence of multicolor peripheral blood in an adult fish would thus imply equal "engraftment" among the competing HSCs during development; however, if one color is more prominent than the other(s), this would suggest an impact on competition during development and therefore self-renewal. For example, we would start with a two color system using GFP and mCherry in the RBCs, where GFP marked RBCs derived from an HSC overexpressing a factor and mCherry marked RBCs derived from an HSC with overexpressing a control factor. If this factor of interest were to enhance self-renewal, a clonal expansion of those HSCs would occur; this would lead to a clonal expansion of the fluorescent color it was co-expressed with (here, GFP) further down in the blood lineage. A simple way to monitor this expansion is to analyze fluorescence expression by FACS or simply by looking at circulation *in vivo* in the tail of a live zebrafish. Factors with no HSC impact should have equal numbers of green and red RBCs. However, if the candidate factor confers a competitive advantage or enhances self-renewal of the HSC, the green RBCs will expand relative to the red ones (Figure 3.1). Similarly, if

the proportion of GFP-positive erythrocytes were less than half, this would suggest that the candidate factor had a negative influence on HSC development. The applications of this assay include an assessment of the role of various genes in the self-renewal of stem cells, the elimination of the need to perform time-consuming transplants, and a true assessment of self-renewal without migration, homing, and niche considerations.

We began developing a multi-fluorescence based system to readout RBCs arising from genetically modified HSCs. This utilized a globin LCR vector, consisting of the hemoglobin locus control region adjacent to the globin alpha A1 (adult 1) promoter driving a fluorophore of interest [364]. When transiently injected into one-cell stage zebrafish embryos, this promoter and regulatory element drives robust fluorophore expression in the erythrocyte lineage. The original vector was 6.2 kb and therefore lacked maneuverability, so we characterized a core 200 bp sequence of the LCR that retained robust erythrocyte expression that was used for subsequent studies. This 200 bp includes an important DNase hypersensitive site as well as several GATA1 and NFE2 binding motifs.

In addition to needing fluorescent transgenes to mark different HSC-specific progeny, expression of modifiers in the HSCs was also needed. Potential modifiers of HSC function would be expressed in the HSC under the control of the *lmo2* promoter. We obtained Tol2 transposon vectors containing the *lmo2* promoter in front of Gateway recombination sites. The Gateway system would allow ease of cloning in a set of factors of interest to be screened in the *in vivo* competition assay over traditional restriction digests and ligations. The Tol2 transposon system

would permit efficient transgenesis of up to 11 kb of cargo through single-copy integrations when the transposons were co-injected with transposase mRNA [368]. We also obtained around one hundred open reading frames of human chromatin-modifying factors from Invitrogen that were Gateway compatible.

With all the pieces in place, we needed to show that this *in vivo* competition assay would be technically feasible. To test both linearized plasmid and transposon constructs for segregation, we injected vectors of each type with the *beta-actin* promoter driving GFP and dsRed/mCherry. In the case of co-injections of both linearized plasmids and transposons, there was colocalization of the red and green color (Figure 3.2). This meant that if the cells received one vector, they were getting both vectors. The concept for the *in vivo* competition assay relied on the different fluorophore vectors landing in different cells. This proof of principle experiment displayed that the assay would not work as planned, and we would need a new assay to assess effects on HSC self-renewal *in vivo*.

Test of F0 Ubiquitous Overexpression

In looking for another approach to assess the impact of overexpressing chromatin-modifying factors on HSC biology, we first examined the possibilities of ubiquitous overexpression in the F0 generation of embryos. There were some immediate concerns that ubiquitous overexpression of a chromatin-modifying factor would result in an embryo too universally affected to evaluate. We tested two methods of ubiquitous overexpression, expression of the factor by injecting full-length mRNA and by injecting a DNA construct driving expression of the factor with

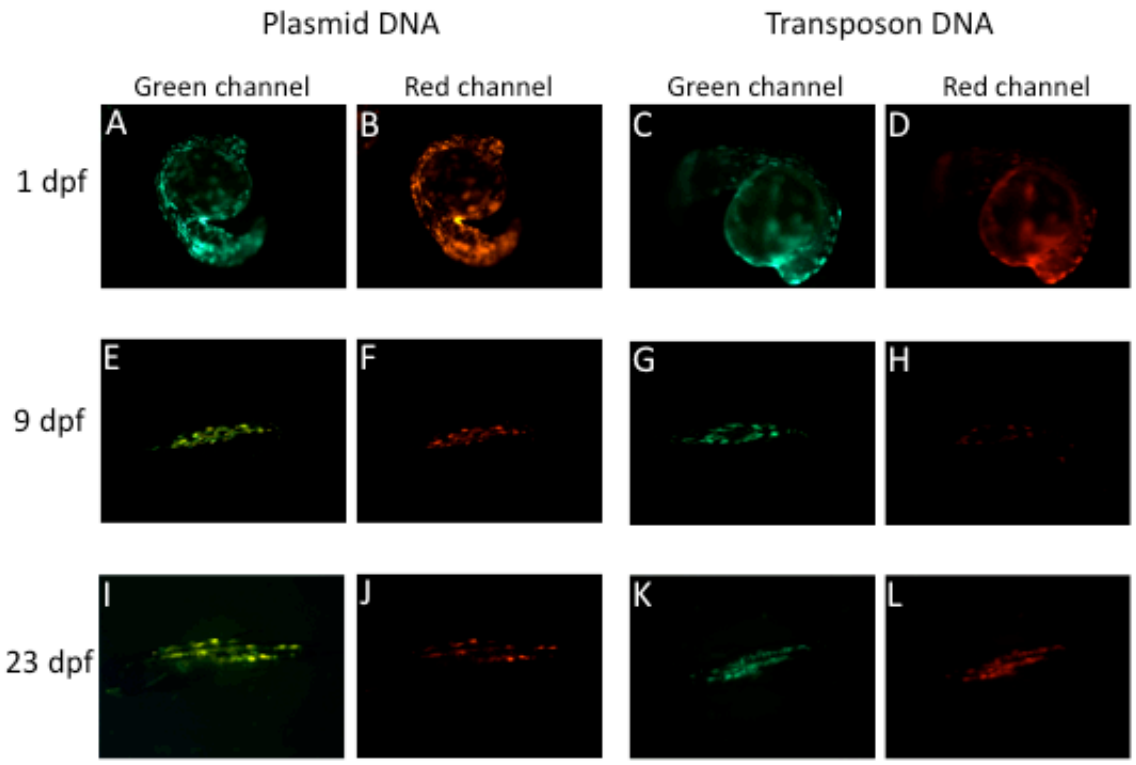


Figure 3.2. **Ubiquitous DNA vectors colocalize in the zebrafish embryo, regardless of vector type**. *beta-actin*-GFP and *beta-actin*-dsRed linearized plasmids **(panels A,B,E,F,I,J)** showed colocalization when co-injected. Similarly, Tol2-*beta-actin*-GFP and Tol2-*beta-actin*-mCherry constructs co-injected, along with transposase mRNA, also showed colocalization of fluorescent cells **(panels C,D,G,H,K,L)**. Fish were examined for fluorescence at 1 dpf (A-D), 9 dpf (E-H), and 23 dpf (I-L).

the *ubiquitin* promoter. The chromatin factor mRNAs were injected at 40, 80, and 120 pg into the yolk of one and two cell stage embryos, since each mRNA would have a different optimal dose. The embryos were then fixed at 36 hpf in 4% paraformaldehyde, and *in situ* hybridization was performed on them for *c-myb* and *runx1*, markers of the HSCs at this timepoint. The following factors were screened for effects on *c-myb/runx1* expression by mRNA overexpression: HMGA1, CBX1, ING3, ING4, MBD3, BMI-1, C20orf20, CBX5, ING5, TNFAIP6, TERF1, BRD7, BRD2, CBX8, BRPF3, ARID3A, ARID3B, SUV39H1, SUV39H2, CBX3, CBX6, CBX7, CDYL2, RFX5, ZHX1, ELF3, N-PAC, BRPF1, PCAF, HMGN1, and MBD1.

The *ubiquitin*-driving chromatin factor constructs were made as Tol2 transposons. It was thought that using the transposon system would lead to more efficient expression that linearized vectors. The transposon carrying *ubiquitin*-chromatin factor and the transposase mRNA were injected into the cell of one cell stage embryos at 25 ng/uL and 15 ng/uL, respectively. Similar to the mRNA injections, these embryos were fixed at 36 hpf and stained by *in situ* hybridization (ISH) for *c-myb* and *runx1* probes. The following factors were screened for effects on *c-myb/runx1* expression by *ubiquitin* overexpression: BMI-1, CBX6, SCL, HDAC3, HDAC8, HDAC10, HDAC11, ING3, ING4, MLL-AF9, runx1, CBX3, CBX5, CBX7, CBX8, CDYL2, and CBX1.

We examined these options more closely using BMI-1 as the factor of choice. Upon performing the injections with either BMI-1 mRNA or a Tol2 based *ubiquitin*-BMI-1 construct with transposase, these embryos were fixed as usual at 36 hpf. Rather than staining for the blood markers, however, we performed the ISHs using

probe for BMI-1 (Figure 3.3). The mRNA-injected embryos showed a diffuse, ubiquitous overexpression of BMI-1, with the higher doses having slightly darker staining than the low dose of 40 pg (Figure 3.3.C-D). The *ubiquitin*-based injections, being a DNA construct, were more mosaic, revealing distinct but random patches of BMI-1 expression over the entire embryo (Figure 3.3.B). Based on the overexpression quality, the mRNA strategy was more likely to be getting into every cell, including the blood cells we wanted to study. However, we had some concerns that producing quality mRNA in a high-throughout manner for each factor we wanted to screen would be troublesome. On the other hand, producing quality DNA vector, such as the *ubiquitin* transposons, was not difficult at all, as a standard miniprep was of sufficient quality; some larger ORFs we were unable to synthesize as mRNA but could easily produce a DNA-based vector. Yet, this strategy did not guarantee the desired factor would be expressed in a blood cell. The one thing both strategies had in common was that neither was cell intrinsic; if we saw a phenotype, we would be unable to determine if it was due to an effect in the HSC, progenitor cell, mature blood cell, the niche, or any other cell in the embryo. Neither option presented itself as the best way to screen for a self-renewal phenotype, which was the confirmation we needed to perform the screen in the F1 generation.

F1 Transgenic Screen in Hematopoietic Stem and Progenitor Cells

In order to best address the question of how overexpression of a particular factor affects the self-renewal of HSCs in a vertebrate, we developed an F1 transgenic screening approach. Having an F1 approach allows the factor of interest to be overexpressed stably in the desired tissue of choice, eliminating issues of

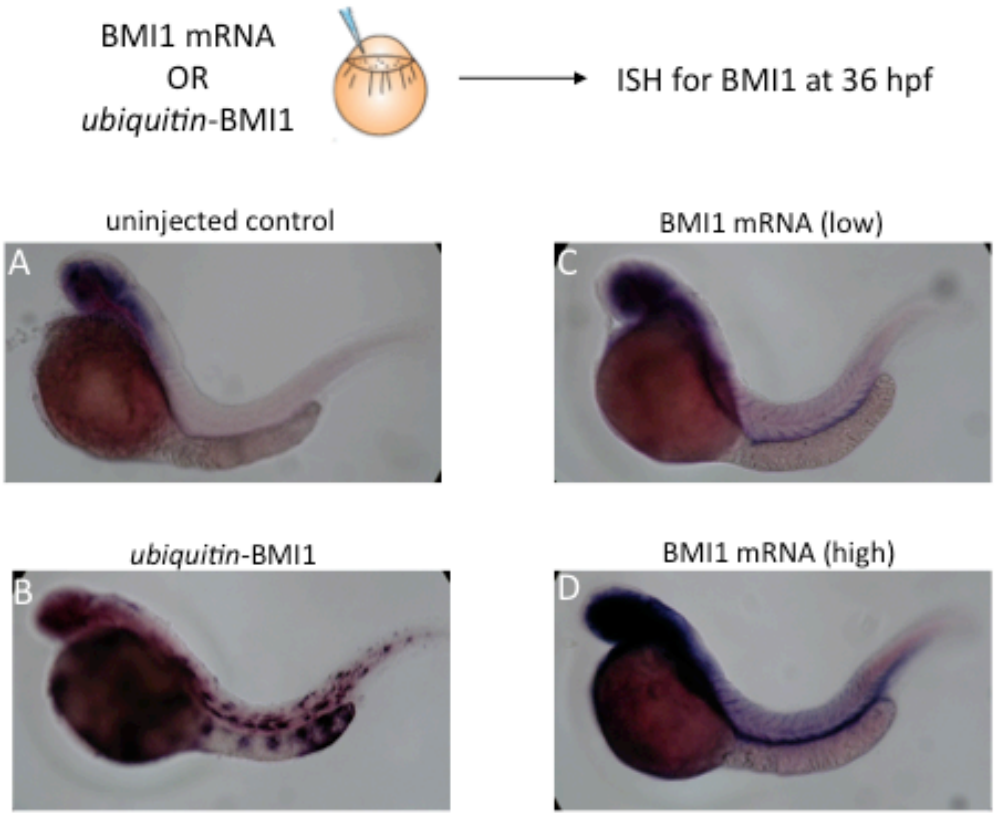


Figure 3.3. **Transient injections to overexpress chromatin factors.** Embryos were injected with either BMI-1 mRNA or a DNA *ubiquitin*-BMI-1 construct. At 36 hpf, ISH was performed to BMI-1. When compared to uninjected controls **(A)**, the DNA construct **(B)** resulted in a patchy, mosaic expression pattern. In contrast, two different doses of mRNA **(C,D)** revealed a diffuse, ubiquitous expression pattern.

variability and inconsistent expression. Additionally, targeting the expression of the factor of interest to the desired cells allows for a cell intrinsic assessment, thus eliminating the need to do cell autonomy studies later. The major disadvantage to this system as a screening tool is the lag time waiting for the next generation. However, this loss in time is gained later when a factor proves worthy of further study, and more embryos can be obtained weekly by setting up the founders. Any studies that would require the development of a transgenic line can commence sooner because the line has already been made.

The F1 screen we performed was in the HSC/progenitor compartment. This screen was designed utilizing the Tol2Kit vector #395, a Tol2 destination vector containing the *cardiac myosin light chain 2* (*cmlc2*) promoter driving GFP as well as multisite Gateway recombination sites [366]. This vector allowed the use of the *cmlc2*-GFP as a transgenesis marker; fish carrying the transgene had GFP-positive hearts. The Gateway sites allowed the recombination into the vector of the 5' *lmo2* promoter, the middle chromatin-modifying factor ORF of interest, and the 3' poly-A tail to be in tandem with the *cmlc2*-GFP.

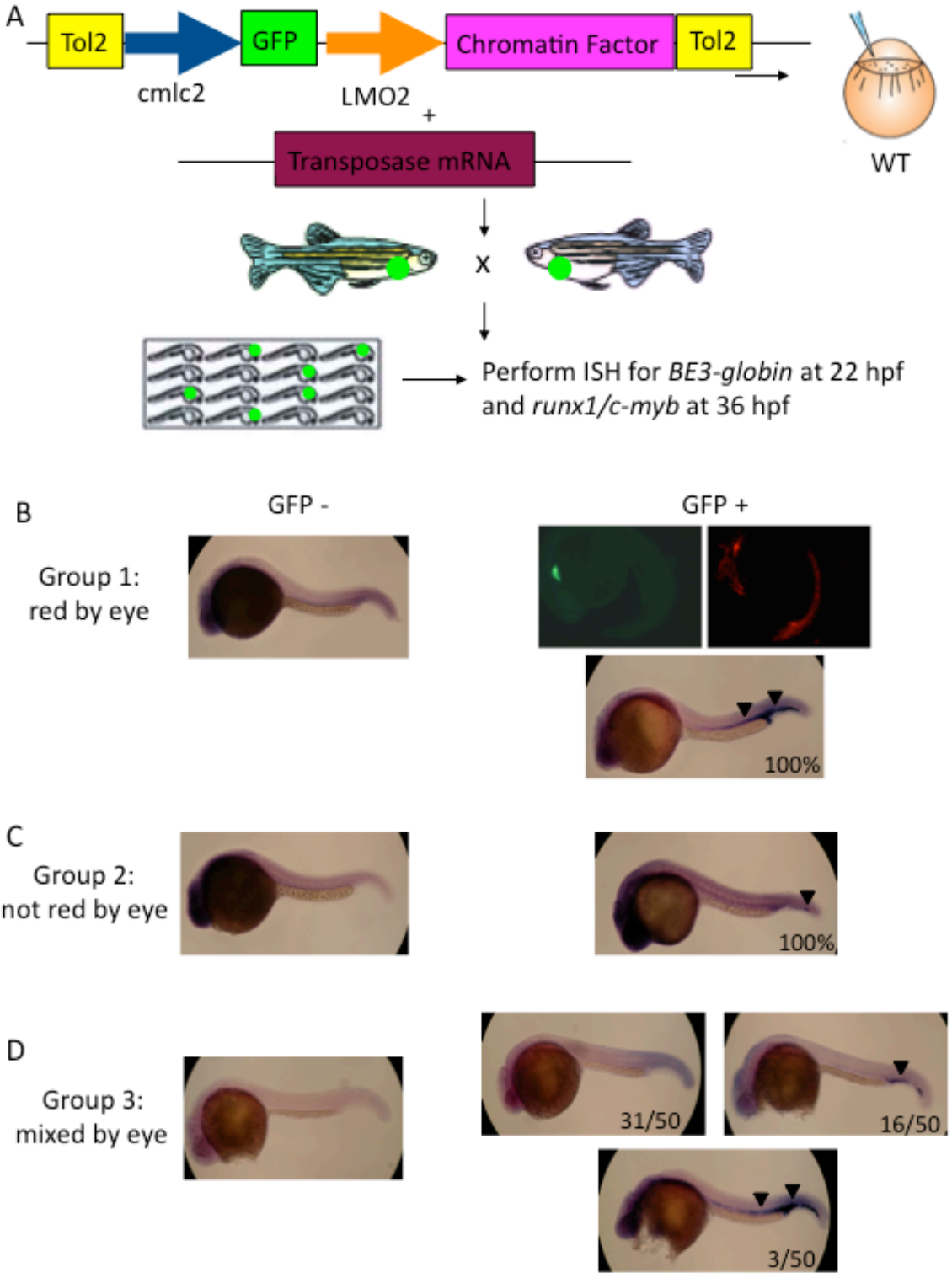
A proof of principle was executed first with a vector carrying *cmlc2*-GFP, *lmo2*-mCherry, where we anticipated that embryos with GFP-positive hearts would have mCherry-positive blood. The first clutch from an F0 incross yielded 58 GFP-positive embryos, none of which were mCherry-positive by eye. Some of these embryos were fixed at 36 hpf and were examined by *in situ* hybridization for mCherry expression. 12/14 were negative, while 2/14 did show some expression in the hematopoietic compartment. More F0 incrosses yielded more GFP-positive

embryos. From this larger cohort of clutches, we saw three groups of embryos (Figure 3.4). In the first set, we observed that all the GFP-positive embryos were also mCherry-positive in the blood, as expected. These also stained well for mCherry mRNA by ISH. In the second set, none of the GFP-positive embryos were mCherry-positive. When these embryos underwent ISH, they had a very small number of cells in the posterior blood island (PBI) stain positive for mCherry mRNA. The third set contained a mixture; most GFP-positive embryos were mCherry-negative, yet a few were mCherry-positive. Upon performing the ISH on this clutch, a few had strong mCherry staining, likely the ones that could be visualized. About one-third had some moderate staining, and the rest had essentially no staining at all. The correlation between GFP and mCherry expression did not seem to be as clear cut as desired, so investigation continued with a few selected *cmlc2*-GFP, *lmo2*-chromatin-modifying factor lines that had already been injected.

The following four lines were examined more in depth: *cmlc2*-GFP, *lmo2*-CBX1; *cmlc2*-GFP, *lmo2*-CBX7; *cmlc2*-GFP, *lmo2*-ING3; and *cmlc2*-GFP, *lmo2*-CDYL2. For each transgenic line, F0 fish were incrossed, GFP-positive embryos were segregated, and those embryos were fixed at 22-24 hpf. GFP-negative siblings were also fixed at the same time as negative controls. ISHs were completed on these embryos with probes to their respective chromatin-modifying factor. In general, at 22-24 hpf, it appears that expression of both CBX1 and CBX7 was highly correlated with GFP expression. All embryos had some expression in the expected pattern, though sometimes the levels of expression varied greatly between weak and robust

Figure 3.4. **HSC F1 screen strategy and proof of principle. (A)** A Tol2-based vector, containing the transgenesis marker *cmlc2*-GFP with *lmo2* driving the chromatin factor of interest, was co-injected with transposase mRNA into wild-type embryos. These F0 fish were grown up to adulthood, incrossed, and the subsequent F1 embryos were scored for GFP-positive hearts. Those with GFP-positive hearts were fixed at either 22 hpf or 36 hpf for subsequent ISH for *beta-E3-globin* or *runx1/c-myb*, respectively. **(B-D)** This was first tested with a proof of principle using mCherry as the factor of interest. Three groups of F1 GFP-positive embryos would appear: mCherry-positive by eye (B), mCherry-negative by eye (C), and some of each (D). ISH for mCherry on these three groups revealed robust expression when mCherry was seen by eye and little to none when it was not visualized. Arrowheads indicate patches of expression.

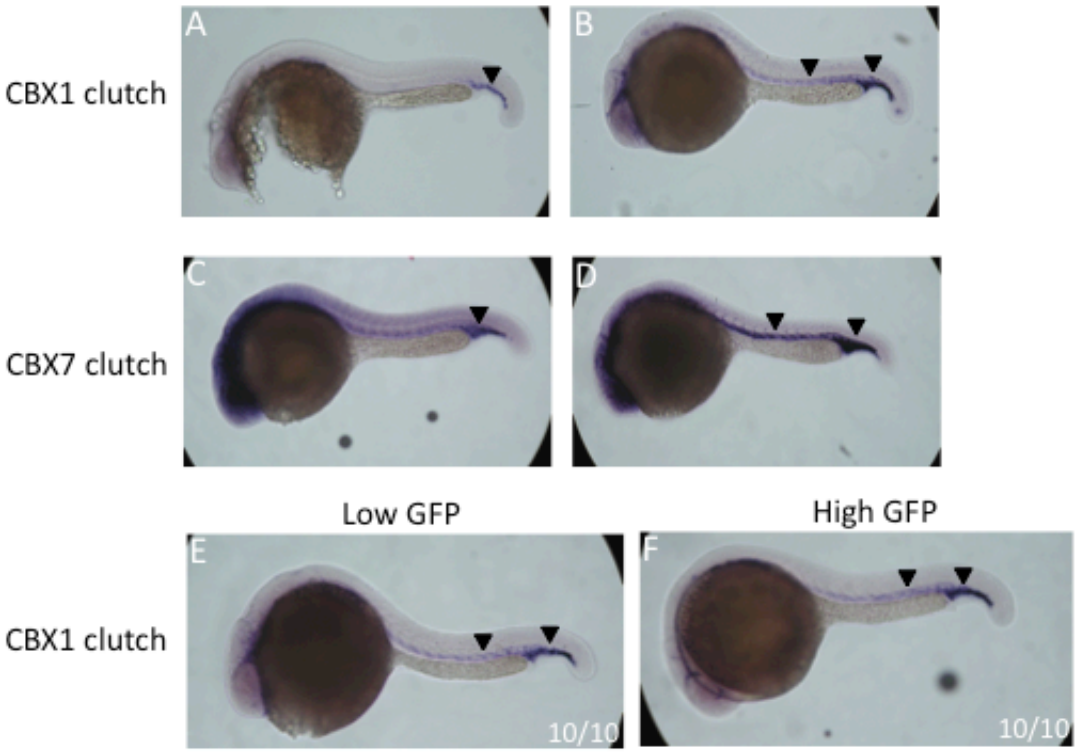
Figure 3.4 (Continued)



(Figure 3.5.A-D). However, almost all GFP-positive had ICM expression of CBX1 or CBX7, as would be expected at this time point. We also wondered if the variation in expression levels correlated to GFP intensity, as we observed differences in the GFP intensity in the hearts of these embryos. There was minimal correlation between higher expressing GFP levels in the heart and higher/lower chromatin factor expression levels (Figure 3.5.E-F). The two other lines, *cmlc2*-GFP, *lmo2*-ING3 and *cmlc2*-GFP, *lmo2*-CDYL2, displayed similar results, with greater than 95% of the embryos with GFP-positive hearts having some expression of the chromatin factor by ISH. However, only 25-50% of those embryos were classified as having good expression, which was defined as robust expression throughout both the PBI and intermediate cell mass (ICM; Figure 3.5.G). It was unknown how much of this was implicit in the transgenic fish and how much was ISH and probe-dependent. Since we were seeing expression of some level in nearly 100% of embryos, we concluded that the screen of chromatin factor expression in the blood could officially commence. However, we decided to modify the approach so that we would know the general levels of expression of the overexpression factor within each clutch. Therefore, each clutch was segregated into GFP-positive and GFP-negative embryos, and each of those groups was fixed in thirds for three different ISHs. One ISH occurred on 22 hpf embryos for *betaE3 globin* (primitive RBCs), the second ISH occurred on 36 hpf embryos for *runx1/c-myb* (definitive HSCs), and the last ISH occurred on 22 hpf embryos with a probe for the chromatin factor being tested (Figure 3.6.A-F). The GFP-negative siblings acted as a control for each clutch, and

Figure 3.5. HSC F1 screen for first few factors is more promising than mCherry. ISH were performed for the injected chromatin factor to examine expression levels. Representative pictures from CBX1 ISHs **(A,B)** and CBX7 ISHs **(C,D)** are shown; there is expression, but levels vary. **(E,F)** Expression levels do not correlate with GFP levels in the heart by eye, as embryos separated into low and high GFP look similar. Arrowheads indicate patches of expression. **(G)** The table shows all data from the first four chromatin factors examined for their expression levels; most have some expression, but it is not necessarily high levels of expression.

Figure 3.5 (Continued)

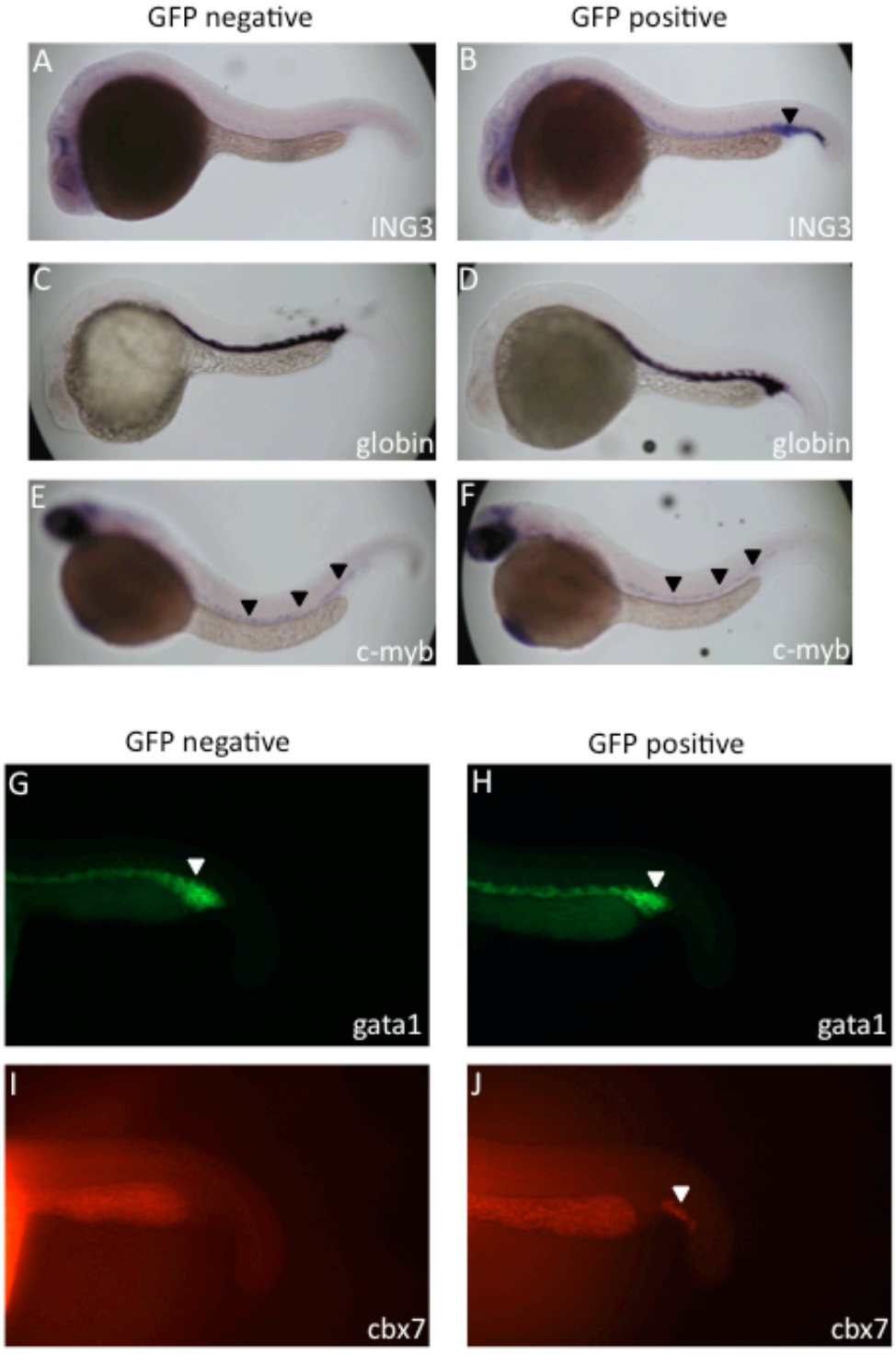


G

Gene	Number with positive expression	Number with good expression
mCherry	84/133	61/84
CBX1	152/165	75/152
CBX7	83/84	71/83
ING3	60/62	13/60
CDYL2	41/43	20/41

Figure 3.6. HSC F1 Screening. Representative images from a full set of data from a representative chromatin factor, ING3. For each factor, clutches were split into thirds to complete ISH for the factor itself (**A,B**; 22 hpf), *beta-E3-globin*(**C,D**; 22 hpf), and *runx1/c-myb* (**E,F**; 36 hpf). **(G-J)** Double fluorescent ISH were also tested to avoid having to split clutches and perform the ISH for the blood marker and factor in a single embryo, shown here for CBX7. Arrowheads indicate patches of expression.

Figure 3.6 (Continued)



we felt it was important to know the expression levels of the chromatin factor in each clutch since it was variable.

To eliminate the need to perform three separate ISH for each set of embryos, we explored the possibility of performing double fluorescent ISH [367]. The double ISH would enable the visualization of both the blood marker and the chromatin factor in the same embryo. The fluorescent ISH would permit the visualization of each probe with a different fluorophore, since both genes were being expressed in the same region and could not be separated with traditional two-color ISH. This was tried with several chromatin factor probes that were successful in traditional ISH, including CBX1 and CBX7. The only chromatin factor probe to produce any signal in the fluorescent ISH was CBX7, probably the strongest chromatin factor probe we had. However, in the double fluorescent ISH, it stained the ICM very minimally (Figure 3.6G-J). The procedure works best for probes that are strong, so we concluded that this set of probes was likely too weak to perform the fluorescent ISH in a high-throughput manner. Therefore, we continued the screen as previously, splitting each clutch to perform three sets of traditional ISH.

Twenty-seven different chromatin-modifying factors were fully examined in the F1 generation as embryos for effects on blood development. They were: CBX1, CBX7, ING3, CDYL2, ID2, HDAC9, BMI-1, CBX3, HMGA1, ARID3B, ARID3A, ING4, PCAF, HDAC10, MBD3, ING5, CBX5, CITED2, SUV39H1, HDAC3, C20orf20, CCDC71, CTCF, SATB2, BRPF3, N-PAC, and CHRAC1. Several others, including CBX6, UHRF2, and MLL-AF9, had no blood phenotype but the ISH for the factor itself was unsuccessful (Figure 3.7). The only factor to display any phenotype was CBX5,

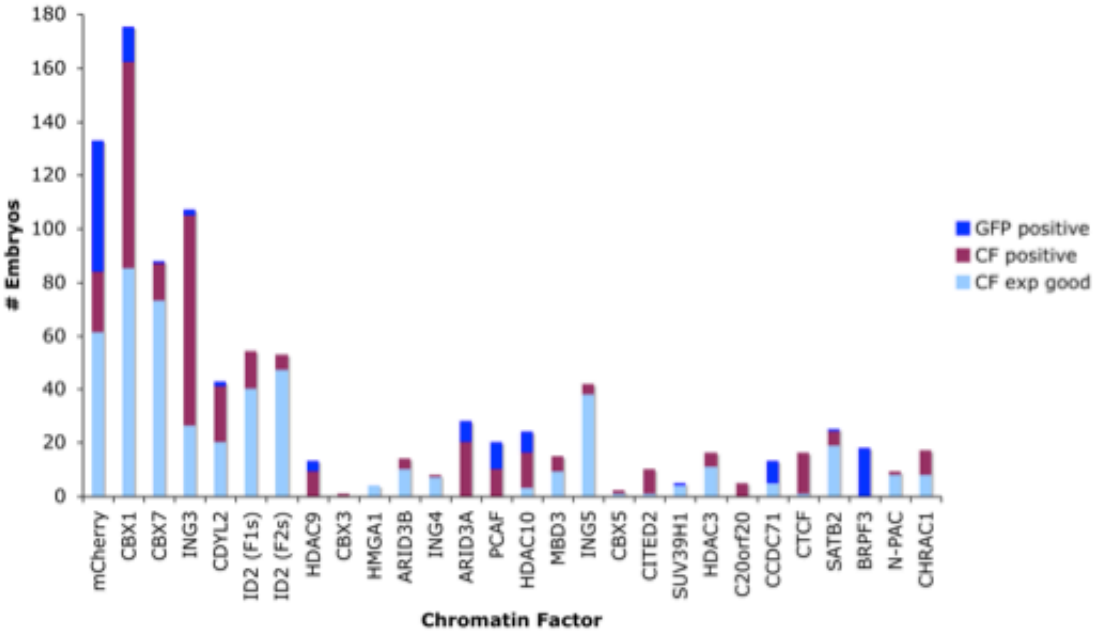


Figure 3.7. Expression levels for each chromatin factor examined in the F1 screen. Of the GFP-positive embryos collected per factor (GFP positive), there was a wide variety of percentages for whether the factors were expressed highly (CF exp good) or even at all (CF positive).

which looked to have fewer HSCs by *runx1/c-myb* staining on the first few GFP-positive embryos compared to negative siblings (Figure 3.8.A-D). This seemed interesting because CBX5, along with Myb and Hmgb3, is known to be important for leukemic stem cell maintenance [369]. However, the phenotype did not repeat from new founders, leading us to conclude that the effect was not specific to CBX5. At this point, we decided not to continue screening the remaining thirty factors that had been injected as F0s because it seemed unlikely that we would see an interesting phenotype.

F0 Transgenic Screen in Lymphoid Cells

In parallel with the F0 ubiquitous overexpression studies, we also attempted an F0 screen in the lymphoid progenitor compartment. This screen was designed using the *rag2* promoter, the *recombination activating gene 2* that expresses in developing lymphocytes (Figure 3.9.A-E). This approach was desirable as *rag2* expresses in the common lymphocyte progenitor, maintaining this as a stem and progenitor cell screen. It was also desirable because we had a positive control with *rag2*-mMyc, known to induce T-ALL in zebrafish; since this was our best hypothesis at the phenotype we could expect this screen was also a screen looking for leukemias/lymphomas [173]. The chromatin-modifying factors were recombined using the Gateway system behind the *rag2* promoter, then sequenced for verification. Each *rag2*-chromatin factor linearized construct was co-injected into one cell stage AB wild-type fish along with a linearized construct of *rag2*-GFP, with each construct injected at 50 ng/uL. Injected constructs of good quality ORFs included the following factors: BMI-1, MLL-AF9, HDAC9, CBX1, CBX6, CBX7,

Figure 3.8. CBX5 overexpression may reduce HSCs in the AGM. (A-D) From one founder, CBX5 overexpression in the HSC compartment resulted in a reduction of HSCs, as shown by *runx1/c-myb* staining at 36 hpf over two clutches (clutch 1, A,B; clutch 2, C,D). Arrowheads indicate patches of expression. **(E-G)** Even from this one founder, levels of CBX5 expression varied greatly, as assessed by CBX5 staining at 22 hpf. However, these results did not repeat with another founder.

Figure 3.8 (Continued)

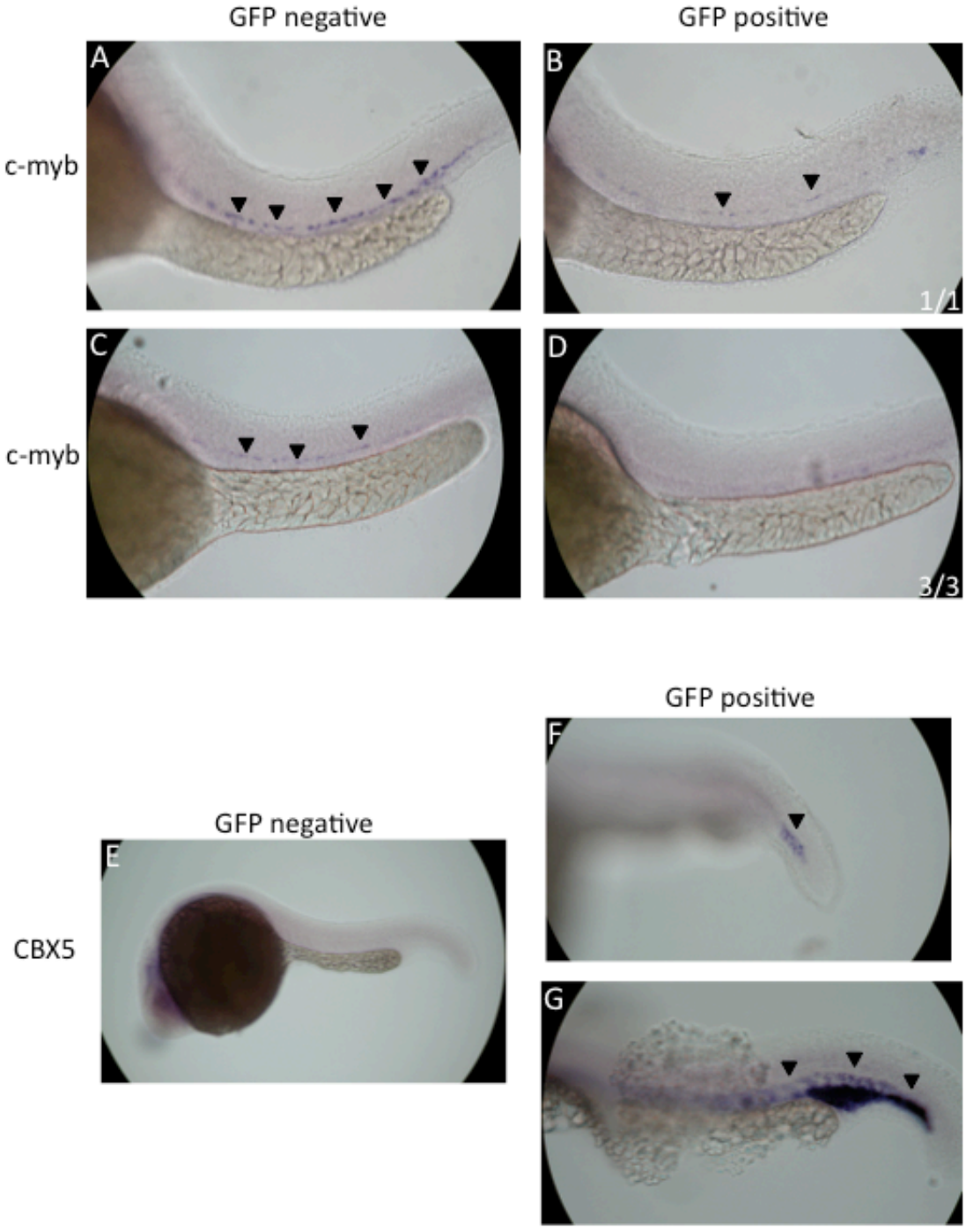
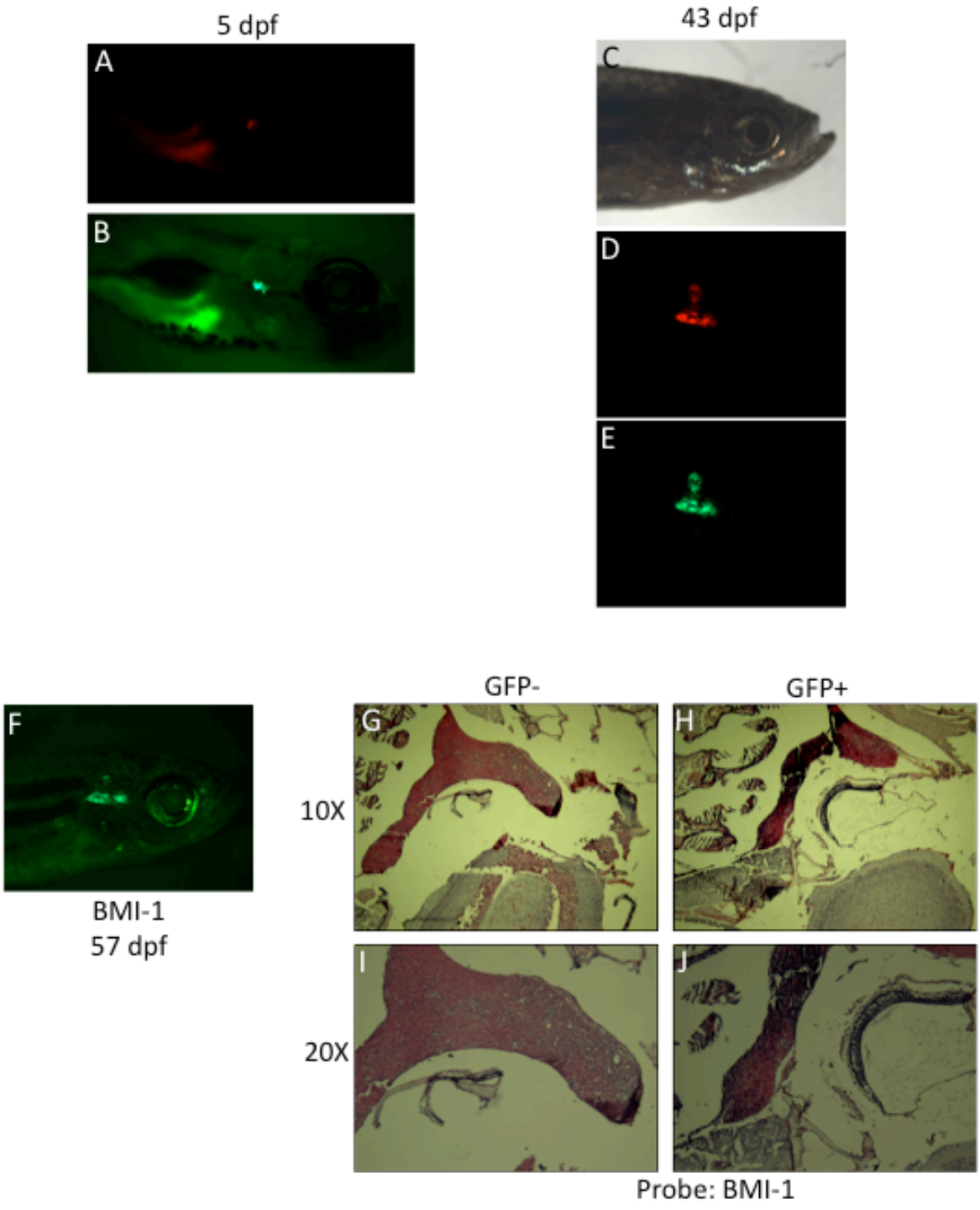


Figure 3.9. F0 transient co-injection approach utilizing the *rag2* promoter leads to co-expression in T cells. (A,B) Co-injection of linearized constructs *rag2*-GFP and *rag2*-mCherry demonstrates that by 5 dpf, both proteins are expressed in T cells in the thymus. **(C-E)** In adulthood, both constructs are still expressed in T cells in the thymus. **(F)** An adult fish, injected as an embryo with *rag2*-BMI-1 and *rag2*-GFP. The T cells look normal by fluorescent visualization. **(G-J)** *In situ* hybridization of the fish shown in (F) for BMI-1, which is expressed in the GFP-positive but not GFP-negative section. This shows that GFP-positive cells do in fact express the factor co-injected with GFP.

Figure 3.9 (Continued)



SUV39H1, WHSC1L1/NSD3, WHSC1/NSD2, CBX5, CBX3, CBX8, HRX/MLLT11, MLL5, EZH2, PRDM1, CHD2, CDYL1, CDYL2, and SUV39H2. A subset of these was also injected into $p53^{-/-}$ fish as well as wild-type. At 5 dpf, the injected embryos were examined for presence of the GFP in the thymus, and those with GFP-positive cells in the thymic region were grown to adulthood. To prove that the presence of GFP also meant the presence of the co-injected chromatin factor, we first injected *rag2*-mCherry with *rag2*-GFP and showed that both colors were present (Figures 3.9.A-E). We also performed ISH staining for BMI-1 on adults co-injected with *rag2*-BMI-1 and *rag2*-GFP that had GFP-positive thymi. BMI-1 mRNA was detected in the thymi of fish with GFP-positive thymi but not in those that were negative (Figure 3.9.F-J).

When the F0 fish reached adulthood, they were screened for fluorescence once every 3-4 weeks for a lymphocyte expansion or a putative tumor. The positive control for this assay was mMyc [173]. When co-injected with *rag2*-GFP, the GFP-positive T cells invade the entire fish by just a few weeks of life, causing T-ALL (Figure 3.10). Based on this, our hypothesis was that some chromatin-modifying factors might also lead to a lymphocyte expansion or even outright tumor development. The factors most likely were BMI-1 and the oncogenic fusion protein MLL-AF9.

The F0 transient screen has several underlying issues. The largest one was simply obtaining a useful number of fish. Only about 5% of injected embryos would have GFP-positive T cells, which is reasonable in transience for a subset of the blood compartment, already such a small percentage of the embryo at 60/8000, or 0.75%,

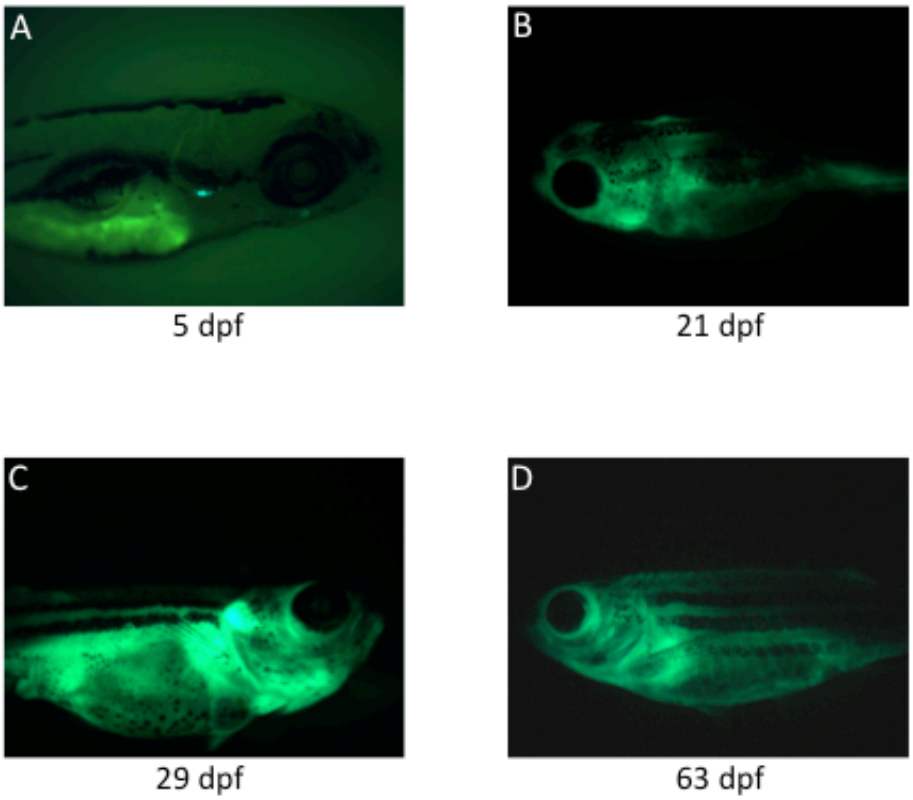


Figure 3.10. Proof of principle experiment with mMyc. (A) Embryos injected with *rag2*-mMyc and *rag2*-GFP express GFP by 5 dpf. **(B)** Some zebrafish show signs of T-cell acute lymphoblastic lymphoma by 21 dpf. **(C,D)** The disease continues to take over the larval and adult fish.

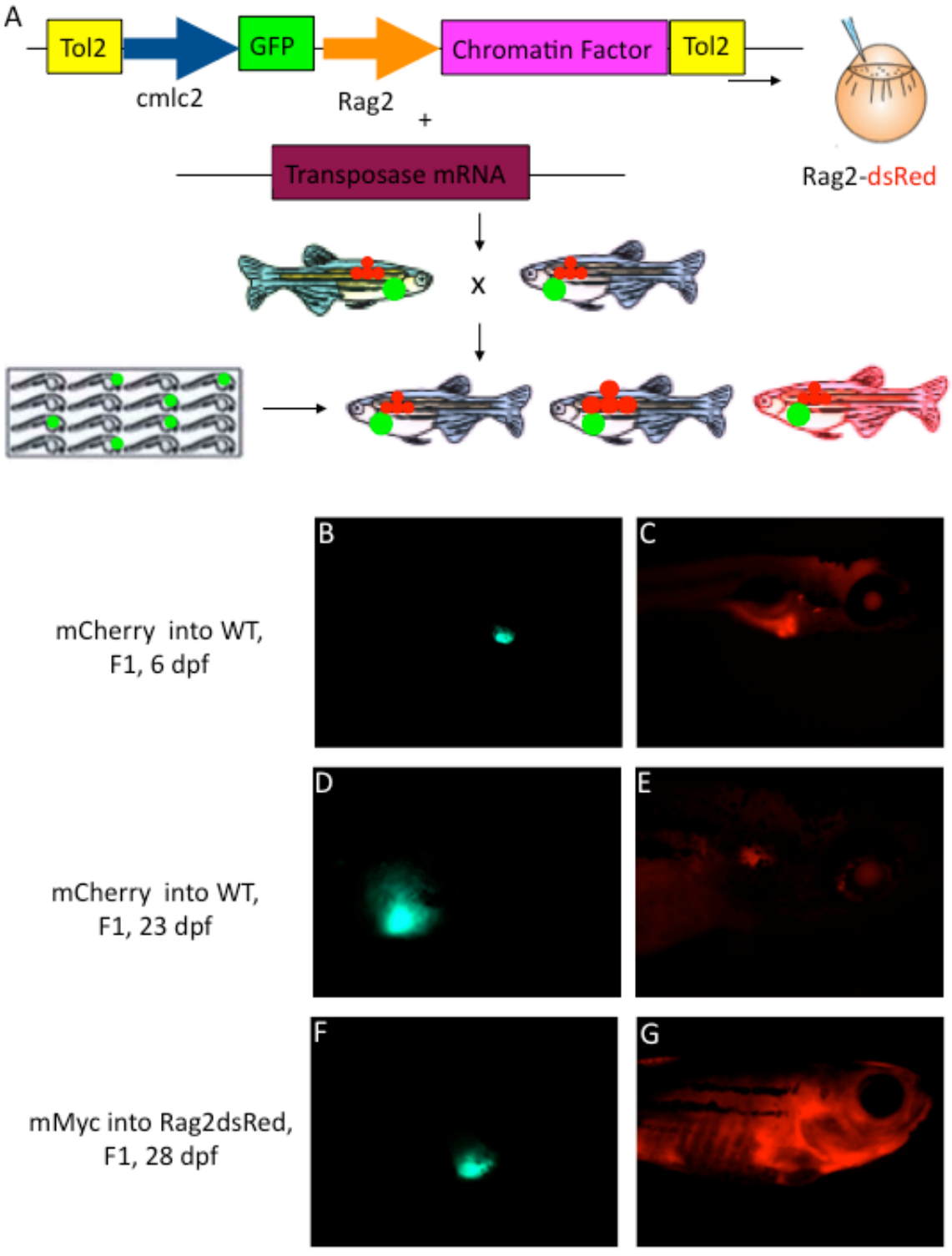
early gastrula cells [370]. This means that 95% of the injected embryos were discarded at 5 dpf. Of the 5% remaining, the vast majority of these would no longer have GFP-positive thymi at 8 weeks of life, when they were next scored for fluorescence. This correlates with the fact that *rag2* is expressed in developing lymphocytes only, rather than mature ones. However, for the screen, it meant that we were usually scoring less than five fish from the initial injection of five hundred embryos, which were often all non-fluorescent, in hopes that they developed an expansion of developing lymphocytes due to the chromatin factor's presence. Such a low number of GFP-positive fish, even as embryos, really takes away the advantage of numbers that zebrafish offers. The other major issue with this screen was that it was more of an oncogenesis screen than self-renewal screen. We were screening for an expansion of cells, but what we were really looking for was cancer. This was too much to ask of a chromatin factor on its own to do; even injections of BMI-1 and MLL-AF9, with known oncogenic roles, were unable to result in a phenotype.

F1 Transgenic Screen in Lymphoid Cells

Based on the above conclusions on the F0 lymphoid screen, we moved to an F1 transgenic approach, paralleled to the HSC F1 screen described above. It was based on the same transgenic technology, utilizing the 395 Tol2kit vector. This time, rather than the *lmo2* promoter, we used the *rag2* promoter to drive expression of the desired chromatin factors in the lymphoid progenitor and developing T cells of the fish embryo (Figure 3.11.A). Twenty-five factors were subcloned into the 395 vector and subsequently injected into *rag2*-dsRed stable line along with transposase

Figure 3.11. T cell F1 screen strategy and proof of principle. (A) A Tol2-based vector, containing the transgenesis marker *cmlc2*-GFP with *rag2* driving the chromatin factor of interest, was co-injected with transposase mRNA into Rag2-dsRed embryos. These F0 fish were grown up to adulthood, incrossed, and the subsequent F1 embryos were scored for GFP-positive hearts. Those with GFP-positive hearts were grown up and examined for effects on the dsRed-positive T cells. **(B-E)** This was first tested as a proof of principle with mCherry as the factor of interest (injected into wild-type embryos), to show that GFP-positive hearts did correlate with T cell expression of the mCherry at 6 dpf (B,C) and 23 dpf (D,E). **(F,G)** The second proof of principle put mMyc in as the injected factor, and the F1 fish with GFP-positive hearts did indeed contract T-ALL by 28 dpf.

Figure 3.11 (Continued)



mRNA. These fish have red thymi, thus enabling the tracking of the thymus in the event of a lymphocytic expansion. GFP-positive embryos were analyzed at 7 dpf for impacts on the red thymi and were examined into adulthood for the development of a lymphoproliferative disease or cancer as noted by the fluorescent thymi (Figure 3.11.A).

The Tol2-F1 system was tested for proof of principle during the vector development stage. The first vector injected was *cmlc2*-GFP; *rag2*-mCherry into wild-type fish as a proof of principle. An F0 incross of fish injected with this vector yielded 26 GFP-positive embryos. Normally, T cells tagged with a fluorescent protein from the *rag2* promoter can be visualized beginning at 4 dpf; however, when these GFP-positive fish were examined at 6 dpf, no red fluorescence was seen. We looked at these same F1 fish again when they were 20 to 30 dpf, and about fifty percent of the larvae had mCherry-positive thymi. By the time the fish were about 6 weeks old, 24/26 of the F1 clutch had red thymi (Figure 3.11.B-E). Several other clutches followed suit, all with near 100% correlation between GFP and mCherry expression. It was assumed that mCherry was too dim to visualize in such a small number of cells in the 6 dpf embryo, and that the *cmlc2*-GFP transgenesis marker would be a successful informant of the embryos carrying the *rag2*-chromatin-modifying factor.

The second proof of principle experiment that was completed was to inject the 395 vector carrying *cmlc2*-GFP; *rag2*-mMyc, a positive control known to induce T cell lymphomas commencing by 21 dpf in F0 fish, into the *rag2*-dsRed stable line. When these injected F0s were first incrossed, a clutch containing five GFP-positive

embryos was grown up. At 28 dpf, three of the five larvae had developed myc-induced lymphoma (Figure 3.11.F-G). Combined with the aforementioned mCherry experiments, it seemed that the vector development was successful and the screen of chromatin-modifying factors could commence.

Injections into the *rag2*-dsRed stable line with twenty-seven different chromatin factors commenced. The following factors were injected: HMGA1, CBX1, ING3, ING4, MBD3, BMI-1, C20orf20, CBX5, HDAC9, HDAC8, SMARCD1, ING5, TNFAIP6, TERF1, BRD7, CBX8, ARID3A, ARID3B, SMARCD3, SMARCAL1, SMARCAD1, MBD1, SATB2, MLLT11, MLLT3, HDAC11, and CBX7. This list of factors were selected because either their expression is up or down in *lmo2*-positive and *cd41*-positive cells (A. DiBiase, personal communication) or they are members of the SWI/SNF complex, known to play a regulatory role in T lymphocyte development [371]. F0 injections were completed, and F1 embryos were screened for GFP-positive hearts. These F1 fish were examined into adulthood. None ever displayed any interesting phenotypes of lymphoproliferation or lymphoid tumors (Figure 3.12).

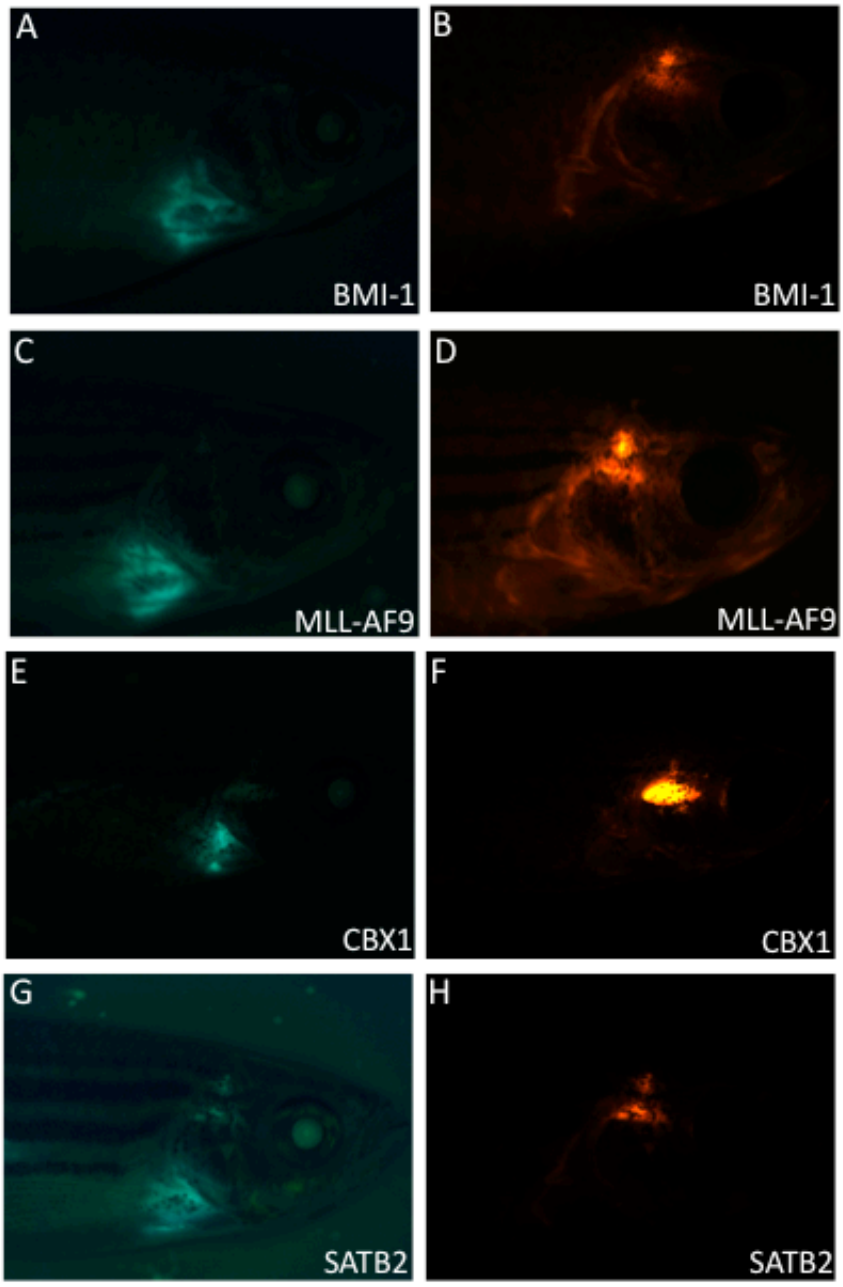


Figure 3.12. **Representative factors screened in the F1 assay for T cells.** Representative F1 adults for BMI-1 (**A,B**, 72 dpf), MLL-AF9 (**C,D**, 72 dpf), CBX1 (**E,F**, 44 dpf), and SATB2 (**G,H**, 51 dpf) that had GFP-positive hearts and were therefore expressing the factor of interest in T cells. However, all of these had no abnormal phenotype in their dsRed-positive T cells.

Discussion

In this chapter, we describe the development of a novel F1 transgenic screening approach that was used as a method of overexpression screening. The goal was to develop an *in vivo* assay that could act as a surrogate for transplantations as a self-renewal assay. When this approach was not possible technically due to cosegregation of plasmids, we turned to F0 transgenics; lastly, to overcome expression issues with F0 transgenics, we developed an F1 system. This approach capitalizes on the ease of generating F1 transgenics in zebrafish, particularly in a tissue like the blood that consists of relatively few cells. We used it in the context of both HSCs and lymphoid progenitors.

The only strong modifier detected by the F1 HSC screen initially was CBX5, which decreased the number of *runx1/c-myb* positive cells. This HSC phenotype did not consistently repeat with CBX5 overexpression from new founder fish yet still remains an interesting putative hit. CBX5, or HP1alpha, is an epigenetic repressor of the heterochromatin protein family. It is enriched in heterochromatin and associated with centromeres. CBX5 has an N terminal chromo domain, so it binds methylated lysines on histones, likely responsible for its transcriptional repressive properties. It also has a C terminal chromoshadow domain that allows it to homodimerize and interacts with other proteins; key interactions with essential kinetochore proteins suggest how CBX5 has a role in the formation of functional kinetochores. As a strong repressor associated with heterochromatin, it seems logical that overexpression of CBX5 could repress either *runx1/c-myb* directly or indirectly. Expression of CBX5 turns on gradually through early development, likely

keeping heterochromatin sustained throughout cell fate decisions; it is also highly expressed in embryonic stem cells. It has been suggested that CBX5 acts as a marker of cell type commitment, increasing the heterochromatin in cells once they have reached their appropriate fate so that the differentiation genes are repressed [372]. By overexpressing CBX5 in stem and progenitor cells, we may have disrupted the cell fate balance, leading to fewer HSCs. However, CBX5 is also known to play a critical role in the maintenance of leukemic stem cells (LSCs), part of an embryonic stem cell-like set of genes that maintain self-renewal. Along with Myb and Hmgb3, Cbx5 overexpression induces bone marrow stem and progenitor cell immortalization without requiring overexpression of the Hoxa/Meis pathway [369]. Though this study showed that overexpression led to self-renewal rather than the decrease we observed in HSC numbers in the zebrafish embryo, it suggests that CBX5 does play a role in self-renewal that our screen may have found.

This F1 transgenic overexpression screening approach has several applications. In this chapter, we showed how it could be used for overexpression screening of factors in a tissue that was difficult to inject into as an F0. This could be applied to other difficult to reach tissues, such as connective tissues, neural crest, or germ cells. Having this as a tool to stably overexpress a gene of interest in a cell autonomous manner would permit interesting hypothesis-driven studies. This could be done as an alternative to transplants as the only cell autonomy assay. Knockdown studies are often complemented by overexpression studies, and this is a straightforward system to overexpress a factor *in vivo*. This system could also be used to study toxic factors, like dominant negatives, whose ubiquitous

overexpression in an F0 embryo would be lethal. However, getting the transgene in the germline of the F0 would not necessarily be lethal, thus permitting study in the F1 generation. Similarly, this could also be performed in lethal mutants, if the transgene was injected into heterozygotes, as a method to rescue the mutants and allow study at an age when the embryos would normally not be alive anymore. This could replace the main rescue technique of mRNA injections, whose rescue is only transient due to mRNA instability and not cell intrinsic. As we were finishing up this project, a paper demonstrating this was published; they rescued the *vlad tepes* mutant (*gata1*) using a Tol2 transposon system [373]. However, they also only used transient transgenics, and it's likely their results would have been more consistent using embryos from the next generation.

Having these stable lines developed has already proved useful. Fluorescence-activated cell sorting was performed on the kidney marrows of adult F1 fish for about ten of the chromatin factors (H. Huang, personal communication). One, HMGA1, had an interesting phenotype of an increased population of precursors cells in the marrow. This is consistent with a previous study that found that knocking down HMGA1 in embryonic stem cells resulted in a decrease in T cell precursors. Loss of HMGA1 also altered the differentiation of precursors, with a reduction in monocytes and macrophages and an increase in megakaryocyte precursors and erythrocytes [374]. Another study additionally revealed that expression of HMGA1 in lymphoid cells undergoing tumorigenesis led to expression of hematopoietic pathways; HMGA1-expressing tumor cells were also enriched for stem cell genes [375]. These results suggest that HMGA1 may play a role in

maintaining HSCs or hematopoietic precursors that may have been detectable using the F1 transgenic fish created here.

In conclusion, we developed a F1 transgenic screening system that successfully expressed the factors of interest in the tissue of interest. This will have future applications as we targeted the hematopoietic system, a highly studied system that makes up a small proportion of the developing embryo. Being able to simply target this tissue that can be difficult to access by transient microinjection will be useful for further study. Though we were unable to use it along with *in situ* hybridizations to identify chromatin modifying-factors that strongly impacted *globin* and *runx1/c-myb* mRNA expression, the system could still be successful for future screens.

Chapter 4

Discussion and Future Directions

The cellular chromatin state contributes to the critical regulation of processes such as transcription, DNA repair, and DNA replication. When the epigenetic state is altered, these functions can be deregulated and, in extreme cases, lead to tumorigenesis. The studies in this dissertation utilized the zebrafish as a screening tool for a set of chromatin-modifying factors in both cancer and hematopoiesis. Using a rhabdomyosarcoma (RMS) model, the H3K9 histone methyltransferase (HMT) SUV39H1 was found to be a suppressor of tumor initiation. This effect appears to act through a cell cycle or growth arrest pathway rather than the muscle differentiation program. This dissertation also describes the development of a F1 transgenic screening approach to uncover novel roles of chromatin factors in hematopoietic development. These studies have contributed to the current knowledge about the interplay of chromatin and cancer as well as zebrafish screening technology.

Zebrafish Assay Development

Cancer Initiation Assay

To perform a screen for the involvement of chromatin factors in RMS formation, a triple co-injection strategy was used to minimize variability. This screen took advantage of the ease of creating transient transgenics in the zebrafish. This system is particularly unusual since we drive expression of hKRAS^{G12D} using a large exogenous fragment containing the *rag2* promoter, which has been shown to express in muscle satellite cells even though the endogenous *rag2* promoter is not

expressed in these cells [155]. Being able to knock-in oncogenic KRAS into its own locus would resolve this issue and represent a system more like human cancer, but unfortunately that technology does not exist in the zebrafish. In the mouse, knock-in technology has permitted the development of an ARMS mouse expressing a Pax3-FKHR translocation conditionally from the Pax3 locus and an RMS mouse expressing $KRAS^{G12V}$ from the endogenous KRAS locus [376, 377].

In this dissertation we transitioned from making longer tumor curves to 50 dpf based on visual tumor formation to using a larval tumor curve monitored by fluorescence to 20 dpf. Our previous method of scoring until 50 dpf limited how many factors could be screened. In contrast, by shortening the screening process from 50 to 20 dpf, we have established a larval tumor development assay based on initial RMS tumor development that will prove useful for future cancer drug discovery. Rather than trying to treat adult fish with tumors, RMS screening could be completed in the larval stages, enabling a larger cohort of fish to be screened at one time. In fact, a small-scale assay on a few chemicals looking for effects on tumor growth was performed as a secondary screen and demonstrated that two chemicals suppressed growth (X. Le, personal communication). A method to expand this to a large-scale screen will be discussed further below.

In vivo HSC competition assay for defining the role of chromatin factors

The first concept to screen chromatin-modifying factors in the blood system was to develop an *in vivo* HSC competition assay. This was technically unfeasible because injected plasmids always co-segregated, whether using linearized plasmids or the Tol2 transposon system. This would not allow for plasmids injected together

to integrate in separate HSCs and thus to be able to read out different HSCs *in vivo* (Figure 3.1). It was already known that plasmids injected together co-segregated in tumors [246]; however, it was not necessarily known in regular tissues. We opted to move on to a different strategy rather than develop a method to get plasmids to undergo independent segregation. This may have been possible using another transposon system, such as the Tdr2 [378]. It also may have been feasible by injecting one HSC factor with one RBC color into one cell of a two-cell stage embryo and injecting the control factor and other RBC color into the other cell. Some studies have previously been successful with this technique, obtaining a fish embryo with different phenotypes on the different lateral sides of the fish [379]. However, the assay would be tedious to perform, and the transient injections would likely prove difficult to hit HSCs in both halves of the fish. To do this as a screen with enough doubly fluorescent injected fish would have been challenging.

F0 Transgenic Approaches

Generating transient F0 transgenics in the blood is a process severely limited by numbers; even the best micro-injection technique will only deliver transgene to the developing blood cells a low percentage of the time, as only 60 out of the 8000 early gastrula cells, or 0.75%, are fated to become blood [370]. Transient transgenics also often contain inconsistent expression levels of the gene of interest from fish to fish, since each individual fish is a unique injection and likely has integrated the transgene in a different genomic location. Examining the F1 generation instead solved these issues. The transgene must integrate into the germline rather than the tissue of choice, which for the hematopoietic system was

an easier task. Even with only a few founders, this system is preferable as fish can be mated weekly to produce more F1 offspring. In transient fish, if only 5% of the F0 clutch has hematopoietic expression, then those are the only embryos available for analysis until the next round of injections. Also, taking multiple clutches from the same founder is preferable because they will all have the transgene integrated in the same place and, therefore, have similar expression levels. Driven behind the promoter of choice, the desired factor is expressed cell intrinsically, whereas F0 transient injections more often contain ectopic expression in larger tissues such as the musculature or nervous system. The main downside to the F1 strategy is the generation lag time, having to wait until the F0 potential founders are sexually mature. In zebrafish, this can be as low as 45 days of life, shorter than many species, and is worth waiting for a better experiment. It is still shorter than making full stable lines, yet if a factor is found that requires a stable line, a benefit is that founders have already been identified, thus saving a step.

F1 Transgenic Approaches

The F1 transgenic screen performed in T cells analyzed twenty-seven different chromatin factors; none resulted in the T cell expansion phenotype we hypothesized would occur. There are several possible reasons for this outcome. First, we may not have screened enough or the best candidate genes. We chose to screen from a list of genes that were up- or down-regulated in hematopoietic progenitor cells. However, we were screening for a lymphoid phenotype, and this set of genes may not have been particularly critical to lymphoid development. The other screened genes are from the SWI/SNF complex, which is known to play a

regulatory role in T cell development [371]. We may not have been expressing these genes at the right stage of development to see a phenotype. Second, this screen may have served well as a modifier screen instead. We simply injected these chromatin factors and then screened for a cancer-like phenotype. Without co-injecting an oncogene, injecting into $p53^{-/-}$ fish, or exposing the fish to another cancer-inducing agent, the fish were very unlikely to spontaneously develop tumors from the overexpression of a single chromatin factor. It is reasonable to think that in the proper background, with overexpression of an oncogene or loss of a tumor suppressor, overexpression of one of these chromatin factors could have provided the additional hit needed to induce T cell expansion or generate an accelerated (or suppressed) tumor curve. This likely would not have addressed our interest in self-renewal but would have been an interesting tumorigenesis model to study. Third, T cells have such a rigorous selection process that any T cells with aberrant chromatin factor expression may have been selected against. We know by ISH that the injected factor was present in the thymus (Figure 3.9.G-J), but that does not guarantee that these cells made protein and survived long enough to disrupt thymic homeostasis.

The F1 transgenic screen performed in hematopoietic progenitor cells also did not yield any reproducible phenotypes from the thirty factors that were fully screened. Similarly, this list was enhanced for factors whose expression was up- or down-regulated in progenitor cells as well as chromatin factors that produced a hematopoietic phenotype upon knockdown in the embryo (H. Huang and K. Kathrein, personal communication). It was unexpected that none of these anticipated genes seemed to have an effect on either *globin* or *runx1/c-myb*

expression. We did see a wide variety of expression levels when performing expression analysis of the injected chromatin factor by ISH. Some factors seemed to express very strongly and others very weakly (Figure 3.7). Part of this could be due to ISH and probe variability; each probe was made from the open reading frame for each gene, so it is likely that some just worked better than others, depending on size and nucleotide sequence. Some factors may have expressed better due to genomic location of integration or interaction with other genes. Outcrossing the founders rather than incrosses helped increase the expression level variability, suggesting that differing integration locations between different fish caused some of the issues.

A stronger promoter may have been necessary to provide more consistently robust expression levels. In fact, another project in the lab took this advice, using the *draculin* promoter for a hematopoietic overexpression screen (J. Ganis, personal communication). The *draculin* promoter drives strongly in all blood lineages from early in development, and genes involved in globin switching were successfully screened using this method. Additionally, a cancer screen in the lab is looking at the role of chromatin-modifying factors in melanoma, using the *mitf* promoter to drive expression (E. van Rooijen, personal communication). The *mitf* promoter drives strongly and specifically in melanocytes, making this an ideal choice for a melanoma screen. Higher overexpression levels with a stronger promoter may have yielded the phenotypes we were looking for in our screen. The screen may have also produced weak modifiers of hematopoietic development, but these went undetected, either due to regular ISH variability or the inconsistent factor expression levels behind a weaker promoter.

Overexpression as a screening tool

Overexpression in a state of normal development may not be the best approach for screening factors. Knocking down chromatin factors in zebrafish had produced many interesting phenotypes for primitive RBC development and definitive HSC development (H. Huang and K. Kathrein, personal communication). By removing a specific factor that is necessary for a certain step in development, this is more likely to produce a phenotype in the embryo. The main reason this would not result in a disruption of hematopoiesis is compensatory genes. However, with overexpression, you may need a non-physiological level of overexpression to disrupt the balance of factors in the cell so much to create a phenotype. Many of these factors are ubiquitously expressed, so adding a little more would not disrupt the blood tissues dramatically. Many also act only in complexes, so without overexpressing their complex partners at the same time, the extra single factor in the cell would essentially be inactive despite its expression. These reasons imply that overexpression may not be the best screening tool to discover novel roles of known genes in development. Overexpressing screening in cancer seems to be more successful, likely because disrupted cells are being selected for during tumorigenesis.

Understanding the role of SUV39H1 in cell cycle and growth arrest

Cell cycle analysis

There are a number of tools available that can enhance our understanding of the dynamics of the SUV39H1 suppression of RMS initiation. Further cell cycle

analysis is warranted on the 5 and 7 dpf tumor forming larvae. We have preliminary data through FACS analysis that indicates the SUV39H1-overexpressing larvae may have a slightly higher proportion of cells in G1 phase, implicating a cell cycle arrest. Further analysis could be accomplished through the use of the FUCCI system, or the fluorescence ubiquitination cell cycle indicator [380]. This system would permit the real time following of cell divisions as the tumors develop. It has RFP fused to cdt1, which is present in G1 and ubiquitinated for destruction in S/G2/M, and GFP fused to geminin, which is ubiquitinated for destruction in G1 but present in S/G2/M. Depending on whether the cells are red or green, their cell cycle stage can be determined; they can also appear as yellow during the G1-S transition, as levels of cdt1 are decreasing and geminin are rising. This system would ideally require the development of transgenic lines expressing the two fusion proteins, and then microinjections of the *rag2*-hKRAS^{G12D}, *rag2*-hSUV39H1/mCherry constructs could be executed into the stable fish lines. It may also be possible in transient transgenics, though this would require the microinjections of six constructs. Using FUCCI would take advantage of the transparent fish for fluorescent cell cycle imaging. Additional cell cycle assessment could be performed through markers such as PCNA or MCM5, which can also be done in the zebrafish as fluorescent transgenics [381].

Since cyclin B1 was a gene of interest observed as downregulated in the SUV39H1-overexpressing 7 dpf larvae by microarray and was found to be bound by SUV39H1 in C2C12 cells, it may be interesting to determine its role in the suppression of these tumors. We could overexpress the cyclin B1 ORF with the *rag2*

promoter to restrict expression to the developing SUV39H1 tumors. This would allow us to determine if reinstating cyclin B1 expression would restore the SUV39H1 tumor curves to the control state. This is likely to occur however, since increasing expression of cyclin B1 would likely push the cells into mitosis. Cell cycle analysis of control tumors, SUV39H1 tumors, and SUV39H1/cyclin B1 tumors may present insights into how SUV39H1 is suppressing tumor initiation through cyclin B1.

Previous studies in the zebrafish have shown that cyclin B1 plays a central role regarding B-myb, a transcription factor that interacts with cell cycle regulators. A mutant for *bmyb* was revealed in a large-scale mutagenesis screen as having an increased number of mitotic cells, noted by increased phospho-H3 staining. The *bmyb* mutant zebrafish had decreased cell proliferation and defects in progression through the G2/M phase, which could be rescued by the overexpression of cyclin B1 mRNA [165]. A chemical genetic screen led to the discovery of persynthamide as a suppressor of the *bmyb* mutant; persynthamide caused a delay in S phase progression, leaving extra time for cyclin B1 mRNA to accumulate in the cells and to push the cells through mitosis [382]. These studies suggest that cyclin B1 levels can be manipulated *in vivo* to rescue cell cycle defects, and overexpression of cyclin B1 or treatment with persynthamide may be interesting experiments to try in our SUV39H1-overexpressing RMS model.

Senescence

To look at growth arrest further in this RMS model, we would need to examine markers of senescence. The gold standard for senescence currently

remains senescence-associated beta-Galactosidase (SA-beta-gal) [383]. In the zebrafish, senescence has been analyzed by SA-beta-gal staining in cryosections and on scales; this could be performed in tumor sections as well as potentially in the larval fish [181, 384]. Newer markers for senescence are constantly being discovered, and some of these could be tested, including p15, Dec1, DcR2, cathepsin D, and eEF1B2 [385, 386]. Interestingly, it has been proposed that in some tumors such as C cell adenocarcinomas, the H3K9me3 mark itself is a marker of senescence, which seems likely to be true in our SUV39H1-overexpressing RMS cells [264].

Our results add to the growing field of knowledge regarding how cellular senescence plays into tumorigenesis. It has been known for some time that oncogene expression can induce senescence and has more recently been shown that senescence is a mechanism to prevent tumor formation [186, 385, 387-389]. Our results with SUV39H1 demonstrate this effect, where overexpression of the enzyme in the presence of oncogenic KRAS likely directly suppresses cyclin B1 to cause growth arrest. As a consequence of growth arrest, fewer tumors form, leading to the suppressed phenotype we initially observed. This may have therapeutic implications, particularly since SUV39H1 associates with HDACs and DNMTs [94, 123]. In a complementary study to ours, loss of SUV39H1 led to a loss of senescence and therefore tumor development; this effect could be phenocopied by treatment with HDAC inhibitors or DNMT inhibitors [186]. This result suggests that by enhancing SUV39H1 or its HDAC and DNMT partners, tumor development would be halted. We demonstrated reduced tumor formation with SUV39H1 overexpression, and it may be interesting to overexpress HDACs or DNMTs in our model. In fact, the

only one tested in our screen, HDAC3, was a minor suppression of RMS formation, suggesting a similar mechanism is at play in our model. SUV39H1 is also known to interact with Rb, and Rb is uniquely required during senescence to repress growth-related genes [99, 100, 390]. This also implicates SUV39H1 in the regulation of senescence. In general, following up with the role of SUV39H1 in senescence could strengthen the conclusions from our RMS model.

Transgene expression

A question regarding overexpression of chromatin factors and tumor suppression is the expression of the oncogenic transgene; this is particularly valid in our model since SUV39H1 is a transcriptional repressor. However, hKRAS^{G12D} expression in the tumors does not correlate with tumor incidence among control, SUV39H1, and SUV39H1 mutant tumors within the tumors that form (Figure 2.A2). To demonstrate that SUV39H1 has not directly suppressed the *rag2*-hKRAS^{G12D} transgene in larvae as the mechanism for decreased tumor initiation, assessing how SUV39H1 impacts the integration of this transgene would be interesting. To study this, we will use a transgenic approach, comparing the effects of *rag2*-hSUV39H1 on fluorescent transgenes driven by different promoters to ensure that SUV39H1 does not specifically silence the *rag2* promoter. Additionally, our result that mRNA expression of GFP driven by the *rag2* promoter is the same in SUV39H1 and control larvae at 5 dpf demonstrates that SUV39H1 does not suppress the *rag2* promoter activity (Figure 2.5).

SUV39H1 and the muscle program

Even though we concluded that the impact of SUV39H1 on the muscle program was not solely responsible for the suppressive phenotype, it still may be interesting to follow up this part of the project. Our lab has KRAS^{G12D}-driven models of RMS initiated in various muscle cell types at different stages of differentiation. The tumor-initiating cell directly correlates to the differentiation state of the tumor that eventually develops; for example, driving the KRAS oncogene using promoters that express earlier in muscle differentiation, such as *rag2*- or *cadherin 15 (cdh15)*-KRAS^{G12D}, gives rise to undifferentiated RMS whereas tumors arising in cells expressing *myosin light polypeptide 2 (mylz2)*-KRAS^{G12D} are highly differentiated [257]. Repeating the experiments with SUV39H1 driven in RMS of various differentiation states may allow further assessment of its effects on the muscle program. For example, a tumor expressed from the *mylz2*-hKRAS^{G12D}, *mylz2*-hSUV39H1 transgenes may permit us to see the change from a differentiated control tumor to an undifferentiated SUV39H1 tumor. This observation would not have been possible in the *rag2* tumors, as they already start in an undifferentiated state. This result would indicate that SUV39H1 could impact the muscle program in the tumor. Assessing tumor rates driven by *cdh15*-hKRAS^{G12D}, *cdh15*-hSUV39H1 would be of particular appeal, since *cdh15* expresses early in the muscle lineage, like *rag2*. SUV39H1 would be expected to suppress this model of RMS with a similar mechanism as our current model, since these tumors should have the same muscle differentiation properties as *rag2* tumors.

Another fascinating experiment would be to perform chromatin immunoprecipitation followed by massively parallel DNA sequencing (ChIP-seq) for

a muscle master regulator like MyoD rather than for SUV39H1 or other HMTs themselves. In a recent study, it was demonstrated that different master regulators co-bind with signal transduction factors at lineage-specific genomic enhancers, depending on the cell lineage. For example, Wnt and BMP factors bind with erythroid factors at erythroid enhancers in an erythroleukemia context yet bind with myeloid regulators at myeloid enhancers in a monocytic environment [391]. This suggests that the master regulator is organizing the cellular state into the right context, with one of the likely mechanisms opening the chromatin at the desired genomic locations and ensuring others are closed. In fact, some chromatin complexes are known to interact with cellular regulators directly [392, 393]. Since MyoD is known to associate with repressive chromatin factors in precursors and chromatin activators in differentiating cells, it is likely that MyoD is acting in the same manner as the hematopoietic regulators. Performing ChIP-seq with MyoD would directly inform us of the most important genes involved in muscle or RMS development that MyoD is trying to open or close. In the context of the SUV39H1-overexpressing tumors, we could compare what MyoD binds in control and SUV39H1 tumors to determine where it cannot bind when ectopic SUV39H1 promotes the closure of chromatin. These data would directly elucidate the SUV39H1 target genes that MyoD is unable to control, leading to tumor suppression.

H3K9 methylation and cancer

An intriguing follow-up study on this project may be to fully examine the role of H3K9 methylation in RMS formation. In addition to SUV39H1 suppressing tumor

development, H3K9 HMT SETDB1 also suppressed RMS formation in zebrafish (data not shown). This is of particular interest since SUV39H1 and SETDB1 act in the same complex [95]. Though we did not examine them, it is likely the other HMTs in this complex, such as G9a and GLP, could also suppress RMS formation upon overexpression. Along with the data presented that SUV39H1 suppression relies on an active SET domain, having two H3K9 HMTs with a similar phenotype suggests that there is a specific set of genes that are being repressed when these enzymes are overexpressed to cause the decrease in tumor formation. Interestingly, when either of these genes is overexpressed in a zebrafish melanoma model, they accelerate tumor formation rather than suppress [181]. This implies that they are methylating a different set of target genes in skin as opposed to muscle. Trying to assess this difference could also prove interesting.

The most appealing experiment to determine the role of the H3K9 methyl mark in causing RMS suppression would be ascertaining what genes are silenced. In our model system, since the tumors form so early, it is possible to perform ChIP-seq in injected embryos to determine the target genes of the enzymes. This is feasible if the injected SUV39H1 and SETDB1 are Flag-tagged, allowing for ease of pull-down. This would require 1500-2000 embryos, making it technically feasible to perform by pooling a few rounds of injected embryos (E. Paik, personal communication). Additionally, other H3K9 HMTs could be examined, including G9a and GLP, which act in a complex together [95]. The fish could also be treated with histone methylation inhibitors, to determine if decreased methylation would promote tumor formation, though this could be in conflict with known LSD1 demethylase

overexpression in human RMS, though the enzymes may target a different set of genes [253, 254]. We could test the effects of LSD1 overexpression in our model as well.

A secondary screen to identify the critical target genes that result from ChIP-seq analysis may be necessary. It is possible that the tumor suppressive activity of SUV39H1 is dependent on several candidate genes. To solve this problem, putative target genes may need to be analyzed in combinatorial pools. Pooling of factors for screening has been successfully performed in the zebrafish (E. van Rooijen, personal communication). It is possible that the single gene approach limited our analysis involving Pax7 and myogenin, where neither gene alone was able to rescue the suppressed SUV39H1 tumor curve. In combination with reduced expression of a cell cycle or growth arrest factor, reduced levels of Pax7 or myogenin may still participate in the tumor suppression. These experiments would improve our understanding of how misregulation of H3K9 methylation contributes to RMS pathogenesis.

A potential mechanism for the SUV39H1 suppression of RMS formation involves Hox genes. Hox genes play an initial role in muscle development by specifying the somites [394]. Furthermore, Hox genes were found to be among the targets of SETDB1 overexpression in melanoma [181]. Therefore, Hox genes are a potential target of SUV39H1 overexpression in the muscle. In our system, it seems unlikely this would be impacting embryonic muscle development, as SUV39H1 driven by the *rag2* promoter should be expressed well after somite formation. However, the Hox genes are known to have differing roles in muscle regeneration,

so they potentially still could impact our RMS tumors [395]. In fact, repression of certain Hox genes may be required for quiescence, so further investigation of this mechanism in our tumors may be necessary [395, 396].

Chromatin-modifying factors in cancer

Increasingly, chromatin-modifying factors are being revealed as having key roles in tumor development, and the zebrafish is playing a major part in these discoveries. A study last year found that overexpression of SETDB1, another HMT, accelerates formation of $hBRAF^{V600E}$, $p53^{-/-}$ tumors in zebrafish [181]. This study represents an elegant experimental design, where a set of genes from a region known to be amplified in human melanomas could be examined in zebrafish to find the gene causing the phenotype and moved back to human cell lines to obtain the most relevant data. Another screen is ongoing in our lab utilizing the same model of melanoma to determine accelerator roles of other chromatin-modifying factors. Using the same set of ORFs used in this dissertation, SATB2 was revealed to be an accelerator of melanoma (E. van Rooijen, personal communication). Future screens utilizing our library of chromatin factors ORFs could discover novel roles of chromatin factors in other tumor types as well.

In addition, many different steps in embryonic and regenerative muscle development are regulated by chromatin-modifying factors. It is likely that many more perform important functions, but they currently remain undiscovered. Utilizing the capability of the zebrafish for high-throughput screens, a morpholino injection-based screen could be performed, knocking down every chromatin factor

in the zebrafish. The morphant embryos could be assessed for problems with muscle development, ranging from somite development to muscle regulatory factor expression to slow and fast fiber formation. The amount of information that could be learned from a project such as this would be staggering. A screen of this scale is feasible over several years with a small team of researchers (H. Huang and K. Kathrein, personal communication). These types of wide scale screens, whether in tumors or in embryos, are the optimal high-throughput ways to learn as much as possible about a collection of genes.

Based on this dissertation and other studies, it is becoming evident that H3K9 methylation plays a major role in cancer formation. We have shown that excess H3K9me3 suppresses ERMS initiation, whereas others have found it enhances proliferation in ARMS [251]. The mark was associated with growth arrest in murine lymphomas and has been referred to as a marker of senescence [186, 264, 265]. SETDB1 was found to be amplified in human melanomas and to accelerate zebrafish melanomas, likely mediated through aberrant H3K9 methylation [181]. Whether the presence of H3K9 methylation promotes or suppresses cancer formation seems to be context-dependent. This suggests that these enzymes are interacting with a wide variety of cell lineage regulators to have such varying cell-specific effects. This has been shown for the Polycomb Repressive Complexes (PRCs), as PRC1 interacts with Runx1 in megakaryocytes and thymocytes and PRC2 interacts with Gata1 in erythroid cells, recruiting these complex directly to genes of interest [392, 397]. Runx1 also interacts with the SWI/SNF complex to activate genes key for hematopoietic differentiation [393]. It is likely to assume HMTs are

acting in the same manner. Additionally, it can be appreciated how disrupting one of these interactions could promote or suppress tumorigenesis. If the chromatin-modifying enzyme cannot interact with its cell-specific regulator, it will silence or activate the wrong genomic regions. If there is too much of the enzyme, excess enzyme may interact with other complexes and act on the wrong genes. If there is too little, it may not silence or activate all the genes it should, leaving some to be unregulated. Each of these scenarios would impact differentiation or self-renewal of a cell lineage, and skewing these processes can lead to tumorigenesis. As these fields move forward, it is imperative to understand how chromatin modification, such as H3K9me3, interplay with the cell specific master regulators to truly comprehend the full biology at play.

Future screening approach in zebrafish with clinical relevance

Our studies demonstrated that the impact of chromatin-modifying factors on zebrafish RMS could be detected while the fish were still larvae. By using *rag2*-GFP as a fluorescent tracker of developing tumors, this approach could be adapted to a high-throughput manner for a primary drug screen. Ideally, a *rag2*-hKRAS^{G12D}; *rag2*-GFP stable line would be created for use in the screen to ensure microinjections are not the limiting step. Then, F1 embryos from this line could be treated with a library of drugs and assessed for fluorescence in the musculature at 5, 7, and 9 dpf (Figure 4.1). This rapid time scale is wide enough to show strong acceleration and suppression, as it is rare to see fish with fluorescence spots at 5 dpf

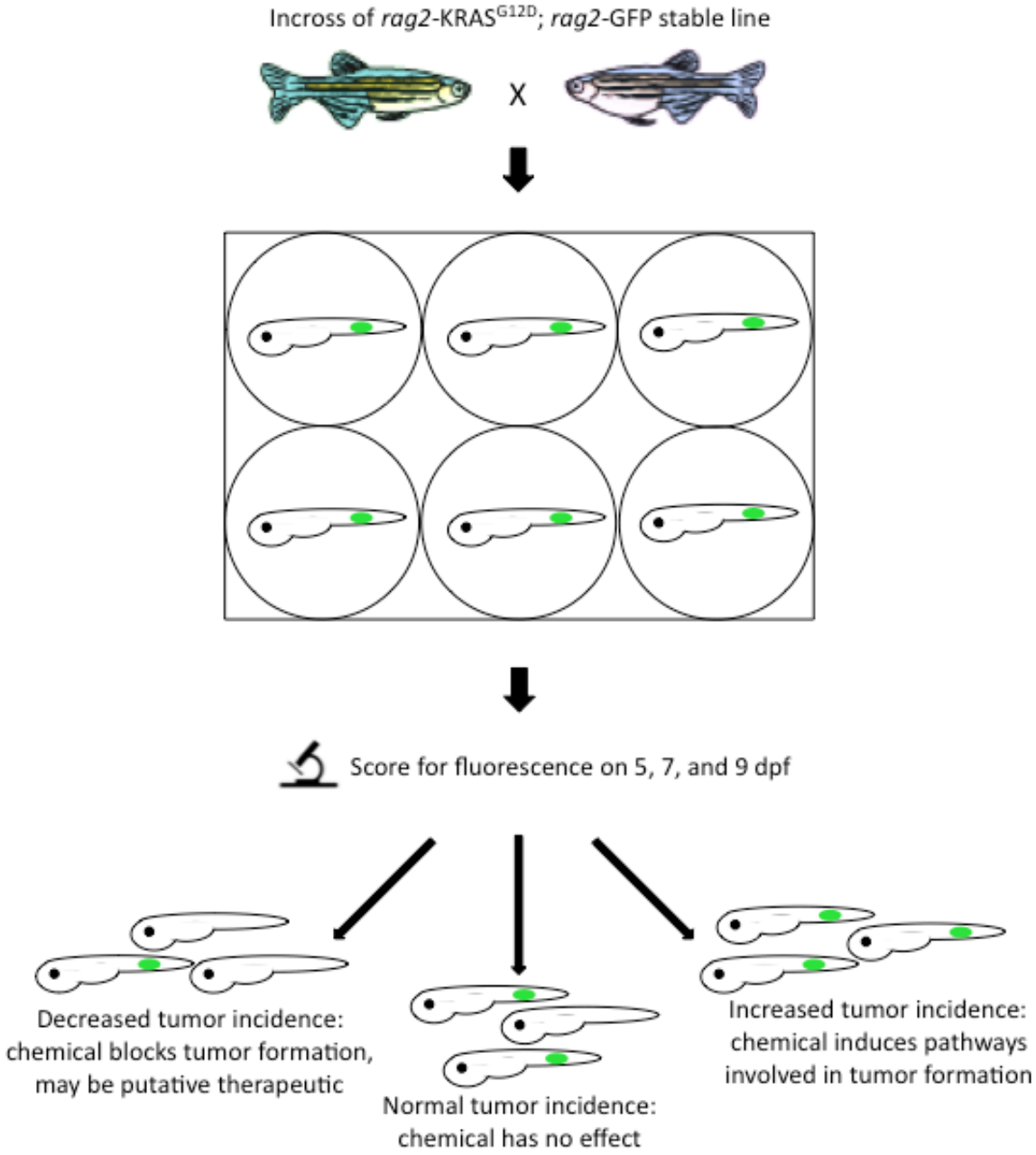


Figure 4.1: Schematic for a larval RMS screen in zebrafish to identify clinically relevant compounds. Following drug treatment, *rag2*-KRAS^{G12D}; *rag2*-GFP fish could be monitored for tumor formation by fluorescence at 5, 7, and 9 dpf. This screen would unveil chemicals that inhibit tumor formation, which represent potential therapeutics, as well as those that accelerate tumor formation, enhancing our understanding of tumorigenesis.

yet often over one-third of the control fish are positive by 9 dpf. To screen for drugs with clinical relevance, only the strongest modifiers are preferred to move towards human trials. Drugs that suppress tumor formation could be putative therapeutics. Drugs that accelerate tumor formation would reveal pathways interesting to tumor development; these drugs could also provide insight to potential therapeutics, as we would be looking for compounds with opposite effects. If automated fluorescent scoring could be developed, particularly since it is a straightforward binary question of fluorescent or not, then this screen could occur extremely rapidly. Automated fluorescent scoring has been developed utilizing microfluidics in *Caenorhabditis elegans*, so this technology may be applicable to the zebrafish system [398]. Even manually, researchers could still score a set of fish three days per week, leading to the completion of a few hundred drugs within a few months time.

This screen would truly encapsulate the best features of the zebrafish as a model organism. First, the screen takes place while the fish are still larvae. This allows us to take full advantage of the tools that the embryonic zebrafish has to offer. The screen would occur while the larvae are still optically clear and small enough to maintain in tissue culture dishes. Increased transparency could also be obtained by using the *casper* strain of fish, which lacks pigmented cells [154]. Second, despite being in a young zebrafish, this screen would occur in a setting of tumorigenesis. This approach allows for chemical inactivation of targeted molecules and pathways in a cancer environment, filling a void in zebrafish genetics. Current methods fall short, as the zebrafish lacks shRNA technology and the translation inhibiting effects of antisense morpholinos would not persist long enough for the screen. In contrast,

treating adult tumorigenic fish with chemicals is tedious, time-consuming, and requires a large amount of housing space (R. White, personal communication). Third, our assay enables a high-throughput cancer drug screen to occur in a whole organism rather than a cell line. This is preferable to ensure that any effects observed are occurring in the environment of a tumor and niche, rather than just isolated tumor cells, as they would be in a human cancer. Being able to use a vertebrate organism such as the zebrafish keeps novel drug discovery one step closer to getting to human patients. In conclusion, using fluorescence as a marker of developing RMS tumors could be converted to a novel, high-throughput assay for cancer drug discovery. This would be executed in larval zebrafish, where the embryonic advantages of zebrafish use could be combined with the tumorigenic environment to ascertain the most relevant drugs possible.

Conclusion

In summary, this dissertation utilizes the zebrafish as a model organism for cancer and hematopoietic development. We have shown SUV39H1, a histone methyltransferase, to be a suppressor of rhabdomyosarcoma initiation. Its mechanism cannot solely be explained by a defect in the muscle program and likely involves a cell cycle or growth arrest program through suppression of cyclin B1. We have also described a novel screening approach that assessed a number of chromatin-modifying factors for their roles in hematopoietic development. This strategy will be applicable in situations involving hard to reach tissues, toxic factors, and lethal mutants. The studies performed here will be the basis for future work

involving the role of histone methylation in cancer, muscle development, and hematopoiesis as well as cancer drug discovery.

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Appendix

Use of Zebrafish to Dissect Gene Programs Regulating Hematopoietic Stem Cells

Attributions

I was the primary contributor to this work.

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Use of zebrafish to dissect gene programs regulating hematopoietic stem cells

Colleen E. Albacker and Leonard I. Zon, M.D.*

Division of Hematology/Oncology

Harvard Medical School and Howard Hughes Medical Institute

Children's Hospital Boston

One Blackfan Circle

Boston, Massachusetts 02115

*To whom correspondence should be addressed.

E-mail: zon@enders.tch.harvard.edu

Phone: (617) 919-2069

Fax: (617) 730-0222

Running head: HSC regulation in zebrafish

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Abstract

Hematopoietic stem cells (HSCs) are responsible for creating each cellular component of the vertebrate blood system. However, HSCs must also self renew to maintain their own population so that the blood system always has the capacity to be reconstituted. This balance of HSCs producing more HSCs as well as differentiated blood cells is regulated by several extrinsic pathways. The *Cdx/Hox* pathway has been shown to have a role in regulating embryonic stem cells, with the zebrafish *caudal* genes *cdx4* and *cdx1a* acting upstream of the *Hox* clusters to aid in the formation of blood. The *Notch* pathway has been shown in mice and zebrafish to be a positive regulator of HSC self renewal; experiments in fish revealed that this pathway signals through the HSC transcription factor *runx1* to do so. Lastly, the action of prostaglandin E2 was found to positively regulate HSCs, and treatment with this compound leads to increased recovery kinetics of the hematopoietic system upon acute injury or transplantation. This knowledge is currently being applied in the clinic as a means to increase the success rates of umbilical cord blood transplantations in adult patients.

1. Introduction to Hematopoiesis and Zebrafish

All vertebrates require blood flowing through their circulatory systems that carries oxygen and other nutrients to cells of all tissues of the body and that provides defense against foreign pathogens. Hematopoiesis, or the differentiation of the mature blood lineages from immature progenitors, occurs throughout the lifetime of an organism to regularly replenish the different cellular components of the blood. It is ultimately supported by the hematopoietic stem cell (HSC). The HSC sits atop the entire hematopoietic hierarchy as it has the ability to differentiate into each member of every blood lineage; it also has the capacity to self renew, maintaining a population of undifferentiated, immature HSCs.

Vertebrate hematopoiesis occurs in the following two successive waves: primitive, to support the developing embryo, and definitive, to provide the organism with long term HSCs to last its entire lifetime. The primitive and definitive waves produce different cell types from different locations, with the primitive typically limited to nucleated erythrocytes containing embryonic globins and macrophages for non-specific defense. The definitive wave produces long term HSCs (LT-HSCs), which give rise to a multipotent progenitor; this produces a common lymphoid progenitor, which will become T lymphocytes, B lymphocytes, dendritic cells, and natural killer cells, and a common myeloid progenitor, which will become monocytes, neutrophils, eosinophils, basophils, thrombocytes, and erythrocytes.[1,2]

Danio rerio, commonly known as the zebrafish, is a member of the teleost family, bony fish with rayed fins. Technically easy to work with, the zebrafish is an excellent vertebrate model organism for studying hematopoiesis. Zebrafish eggs are

fertilized externally, development occurs entirely *ex vivo*, and embryos are optically clear. Additionally, zebrafish perform well as a genetic model organism; they are competent for large-scale genetic screens since they require less space than mammalian systems, females can produce one to two hundred eggs every week, and their generation time is short. The zebrafish genome is fully sequenced, and a series of large-scale mutagenesis screens have been completed, leading to the production of numerous genetic mutants that are available for study.[3,4]

Zebrafish development occurs rapidly over the course of a few days. Immediately after fertilization, the zygote remains in the one cell stage for forty-five minutes, facilitating genetic manipulations of the embryo as nucleic acid injections can be performed at this stage. Gastrulation commences around 5 hours post fertilization (hpf), as morphogenetic movements form the three primary germ layers. Somites and organs begin to form at 10 hpf. Shortly after 24 hpf, circulation and pigmentation commence; by 48 hpf the zebrafish begin hatching out of their clear chorions and by 72 hpf are swimming freely.[5]

2. Stages of Hematopoiesis

2.1. Primitive Hematopoiesis

In vertebrates, the first wave of hematopoiesis, the primitive wave, produces erythrocytes and myeloid cells for the developing embryo. In the zebrafish, the primitive erythropoiesis wave occurs within the intermediate cell mass (ICM); this hematopoietic site is functionally equivalent to the extraembryonic yolk sac blood islands in mammals and birds. The ICM is located in the trunk ventral to the

notochord. Following gastrulation, the developing primitive HSCs are marked by expression of the genes *stem cell leukemia (scl)* and *LIM domain only 2 (lmo2)*, first seen by the 2 somite stage (10 hpf). They exist in two bilateral stripes in the embryo until merging into the ICM at the 18 somite stage (18 hpf). Around the 6 somite stage (12 hpf), expression of *gata1*, an erythrocyte marker, commences in a subset of *scl*-positive cells in the ICM as the first primitive red blood cells are produced (Figure A.1A). About three hundred primitive erythrocytes will be produced by this hematopoietic wave. These cells are first seen in circulation by about 24 hpf and last until about 4 days post fertilization (dpf). The rest of the *scl*-positive cells, those that do not express *gata1*, will differentiate into vasculature, marked by expression of the *vascular endothelial growth factor receptor 2* (*vegfr2/flk1*).[1,6,7]

Primitive myelopoiesis occurs concomitantly with primitive erythropoiesis. However, whereas primitive erythropoiesis takes place in the ICM, primitive myelopoiesis occurs instead in the rostral blood islands (RBI) in the anterior mesoderm. The RBI are also marked by expression of *scl* and *lmo2*. Similar to how a proportion of cells in the ICM will express *gata1,* a subset of these cells in the RBI will additionally express *pu.1*, a myeloid-specific transcription factor. These cells will develop into primitive macrophages and granulocytes, which are functional and circulating in the bloodstream by 26 hpf.[1]

The appropriate proportion of erythroid to myeloid cells is maintained during the primitive wave by an equilibrium between expression of *gata1* and *pu.1* in the ICM. ICM cells have a bipotentiality, being able to form either cell lineage, so this regulation plays an important role in the embryo. Loss of *gata1* results in an

expansion of myeloid precursors at the expense of erythroid cells, with *pu.1* expression persisting longer than usual; conversely, loss of *pu.1* leads to ectopic *gata1* expression and an expansion of erythroid cells.[8,9] This reciprocal regulation suggests that each marker promotes development of one lineage while actively suppressing the other.

2.2. Definitive Hematopoiesis

The definitive wave of hematopoiesis provides an animal with LT-HSCs which, due to their ability to undergo self renewal, will support the generation of all blood lineages for the life of the organism. These differentiated lineages include not only erythroid and myeloid cells like the primitive wave, but also lymphocytes, thrombocytes, and a larger variety of myeloid cells. In mammals, the first anatomic site of definitive hematopoiesis is the aorta-gonad-mesonephros region (AGM; Figure A.2). The AGM equivalent in zebrafish is the ventral wall of the dorsal aorta. It is here at 31 hpf that expression of the first definitive HSC markers, the transcription factors *c-myb* and *runx1*, arises. *In situ* hybridizations at 36 hpf for these two genes reveal clusters of HSCs on the floor of the dorsal aorta (Figure A.1B). They then likely bud off from the epithelium to enter circulation and migrate to their next location.[1,6,7] Additionally, in mammals, a parallel population of definitive HSCs can be found in the placenta.[10]

In mammalian systems, HSCs are next found in the fetal liver; the equivalent intermediate larval site of hematopoietic expansion in the zebrafish is the caudal hematopoietic tissue (CHT; Figures A.1C, A.2). Lineage tracing of HSCs forming in

the vascular plexus below the floor of the dorsal aorta shows that the cells formed by 2 dpf will seed the CHT, later becoming myeloid cells and lymphoid precursors. The CHT develops in association with the caudal vein, consisting of endothelial stroma that harbors the hematopoietic cells.[11]

Finally, in mammals, HSCs seed the bone marrow, their permanent location. In zebrafish, the permanent niche for HSCs is the kidney marrow, in the reticular stromal cells of the kidney between the renal tubules and blood vessels (Figures A.1D, A.2). While most blood cell progenitors are also found in the kidney marrow, T lymphocyte progenitors, after being born in the kidney, can additionally be found in the thymus. HSCs formed at 2 dpf go through the CHT on their way to the kidney marrow and thymus. Those HSCs formed at 3 dpf will seed the kidney with fewer cells heading for the CHT and thymus; these are presumed to be the definitive HSCs.[11,12] By 5 dpf, nearly all HSCs can be found in the kidney marrow and will inhabit there for the remainder of the zebrafish's life. All mature blood cell lineages originate from the kidney, with only T lymphocytes progenitors then migrating to the bilateral thymi to be educated. Kidney marrow cells can be analyzed by flow cytometry; separation by forward scatter and side scatter, which are measures of cell size and granularity, respectively, reveals four distinct populations that correspond to lymphoid, myeloid, erythroid, and precursor cells (plots on Figure A.3).[13]

The definitive wave of erythropoiesis to replace the primitive red blood cells commences around 5 dpf. Erythrocyte differentiation is highly conserved between zebrafish and mammals, with the exception that mature erythrocytes in zebrafish

retain their nuclei and have an elliptical shape, as opposed to mammalian erythroid cells that have an enucleated discoid-shape. Myelopoiesis results in two granulocyte lineages, one resembling mammalian neutrophils and the other resembling a combination of mammalian eosinophils and basophils. They are found in the kidney by 7 dpf. The zebrafish immune system is supported by B cells that develop from the kidney by 19 dpf and by T cells that originate in the kidney and mature in the bilateral thymi beginning around 3 to 4 dpf. Thrombopoiesis in zebrafish yields small round nucleated thrombocytes that are equivalent to mammalian platelets, which appear beginning at 36 hpf.[1]

3. Hematopoietic Assays

Hematopoiesis can be studied *in vitro* through a series of colony-forming unit-culture (CFU-C) assays. Hematopoietic progenitors are grown in culture, consisting of a mixture of media, growth factors, and methylcellulose. When the progenitors proliferate and differentiate, a colony forms, which can be quantified and characterized.[14,15] Variations in the media conditions promote the growth of specific kinds of colonies with varying potentials.

In the murine system, the classic *in vivo* assay to determine the presence of HSCs is the colony-forming unit-spleen (CFU-S) assay. After lethal irradiation, progenitor cells from the bone marrow will form nodules of hematopoietic cells on the spleen. Those that appear relatively quickly, by eight days post-irradiation, likely arise from short-term HSCs or precursor cells; nodules forming later, by twelve or fourteen days, are from the long-term HSCs.[16]

HSCs can be sorted out in mammalian systems based on the antigens on their cell surface; recognition of these antigens by monoclonal antibodies facilitates the sorting process. In the human, the HSCs are marked by the presence of CD34 and the absence of Lineage marker. In the mouse, the long-term HSCs are enriched in the Lineage-negative, Sca-positive, c-kit-positive population.[17]

HSC presence can also be noted by performing an acute injury-recovery experiment, where the hematopoietic system is injured and assessed for recovery. In mice, the injury is traditionally 5-fluorouracil (5-FU) injection; then the recovery kinetics of the whole bone marrow are monitored. A similar assay has been developed for the zebrafish. The fish are sublethally irradiated, then analyzed over a time course by forward scatter-side scatter FACS analysis. In this way, the recovery of the different blood populations, erythroid, myeloid, lymphoid, and precursor, can be monitored over time (Figure A.3).[13,18]

Transplantation is another classic assay to definitively determine the presence of HSCs in a cell population. This can be executed in both mouse and zebrafish.[17,13] Transplanting donor marrow cells into an irradiated recipient allows for the assessment of long-term donor engraftment, which can only be fulfilled by long-term HSCs. If 5% of the recipient's blood is found to originate from the donor, either by cell surface antigens in the mouse or fluorescent markers in the zebrafish, the marrow cells are considered to have engrafted. The actual percentage of donor cells found in the recipient's blood is known as the percent chimerism. Competitive transplants are performed to test the desired cell population against a secondary population, serving as the challenger. The two cell populations are mixed, and co-

transplanted into the same animal; if one engrafts better than the other, this will be noted in the recipient. Serial transplants are performed to assess self renewal. A population containing HSCs is transplanted into a recipient; at a later time point, marrow cells from this first recipient are then transplanted into another animal, a secondary recipient. A population containing a greater number of HSCs or HSCs enhanced for self renewal will sustain more rounds of serial transplants; those with fewer HSCs or HSCs impaired in self renewal will undergo hematopoietic exhaustion, essentially running out of HSCs to repopulate the recipient's blood.

Alongside these traditional assays, recent work has combined pathway analysis, mutants, and transgenic animals to study hematopoietic stem cells. Understanding of the extrinsic pathways regulating HSCs has benefited greatly from this work. In the paragraphs that follow, three of these pathways will be discussed: Cdx/Hox, Notch, and Prostaglandins.

4. Regulatory Pathways for HSC Development

4.1. Cdx/Hox Pathway

Many aspects of embryonic patterning and development, including hematopoiesis, are determined by the homeobox transcription factors, which are encoded by the *Hox* genes. In vertebrates, there exist thirty-eight *Hox* genes clustered into four areas of the genome; these clusters are each oriented in the same transcriptional direction and are known as *Hoxa, Hoxb, Hoxc,* and *Hoxd*.[19] Within these clusters, there is a direct correlation between the order of genes along the chromosome and the gene's function according to the anterior-posterior (AP) axis of

the organism. This colinearity refers both to the location a gene is expressed along the AP axis as well as the time it is expressed in development. For example, the genes positioned at the 3' end of a *Hox* cluster tend to be expressed first and the most anterior; the more 5' genes in the cluster are expressed at later time points and spatially more posterior in the embryo. Any gene mutations or other perturbations in the *Hox* clusters typically lead to changes in cell fate and embryonic patterning. Loss of function in the most 3' region of a cluster will cause the embryo to become posteriorized, losing some anterior structures; conversely, gain of function in this genomic region would result in anteriorization of the embryo and loss of certain posterior structures.[19]

Vertebrates also have other homeobox genes that are not members of the main four *Hox* clusters. Among these genes include the *caudal* paralogues, *Cdx1, Cdx2,* and *Cdx4*. A proposed role for such genes, being that they are in a different genomic location from the *Hox* clusters, is that they affect AP patterning by directly regulating the *Hox* genes. Promoters of many of the clustered *Hox* genes contain binding sites with consensus sequences for *Cdx1, Cdx2*, and *Cdx4*.[20] Several studies of murine mutants for *Cdx1* and *Cdx2* have shown that these mutations led to shifts in the expression patterns and boundaries of several *Hox* genes, resulting in aberrant vertebral column and tail development.[21,22]

Being that the Cdx/Hox genes play key roles in development, it was not surprising that some of these genes would participate in hematopoietic development. A zebrafish mutant referred to as *kugelig* has a mutation in the *cdx4* gene. It has abnormal AP patterning, and several genes of the *Hoxa* and *Hoxb*

clusters display altered expression patterns. The *cdx4*-/- mutant presents with severe anemia by 1dpf that partially recovers by 5 dpf; pleiotrophic defects lead to the mutant's early death by 7 to 10 dpf. *Kugelig* also has reduced expression of hemoglobin, *scl*, and *gata1* with a complete lack of *runx1*. Overexpressing *hoxb7a* or *hoxa9a* in the *cdx4*-/- mutant rescues levels of *gata1* expression. However, overexpressing *scl* in the *cdx4*-/- mutant was unable to rescue these erythroid precursors, suggesting that *scl*'s role in determining hematopoietic cell fate is *cdx4*-dependent and further downstream. Overexpression analysis of *cdx4* was also performed, which showed a posteriorization of the zebrafish embryo and ectopic expression of *scl* and *gata1*.[23] These observations showed that *cdx4* is upstream of *Hox* gene expression.

A follow-up study was performed involving one of the other *caudal* genes, *cdx1a*, since it was noted that the *cdx4*-/- mutant was not completely bloodless, likely due to redundancy. Knocking down *cdx1a* by injection of an antisense morpholino oligonucleotide led to an increase in expression of the gene, implying that it normally negatively regulates itself. Injection of this same *cdx1a* morpholino into the *cdx4*-/- mutant zebrafish embryos yielded loss of posterior structures and development of fewer somites; it appears that the two genes normally act simultaneously to form the posterior tissues of the animal. *Cdx1a*-morpholino;*cdx4*+/- embryos had fewer *scl*-positive cells in the ICM as well as a reduction in *gata1*-positive cells; these phenotypes were more severe in *cdx1a*-morpholino;*cdx4*-/- embryos, suggesting a dose-dependent effect of *cdx4*. The *cdx1a*-morpholino;*cdx4*-/- embryos also suffered from a complete failure to specify

the ICM blood precursors; these also lacked in forming definitive HSCs, as noted by a reduction in *runx1a* expression. However, these effects could be rescued by co-injection of *hoxa9a* mRNA.[24] The *cdx* pathway plays a major role in primitive hematopoiesis in the ICM, as this region was particularly sensitive to the gene dosage of *cdx4*; the *cdx-hox* pathway is also required to form definitive HSCs in the AGM. These studies confirmed the idea that the *caudal* genes act upstream of and regulate the *Hox* clusters, playing an important role in blood formation.

Other studies of *Cdx4* have confirmed the pathway's role among HSCs. In the mouse, *Cdx4* is expressed in adult stem and progenitor population yet is significantly reduced in differentiated blood cells.[25] Induction of *Cdx4* in embryoid bodies promoted formation of CD41-positive and c-kit-positive presumptive HSCs in a colony-forming assay. Additionally, an increase in blood markers was observed upon *Cdx4* induction, including *lmo2*, *scl*, *gata1*, embryonic and adult globins, *c-myb*, and *runx1* expression. Similar to *cdx* regulation in zebrafish, *Hox* genes were found to be the downstream mediators of *cdx4 in vitro*.[26,27] These observations show that the *Cdx* genes play a role in regulating blood stem and progenitor stems through the *Hox* genes.

4.2. Notch Signaling

The Notch pathway is an evolutionarily conserved signaling pathway that is known for playing important roles in development, and it has been shown to indeed participate in the formation of HSCs. The Notch proteins themselves are type 1 single pass transmembrane glycoprotein receptors that activate downstream

transcriptional targets upon binding to their transmembrane ligands, Jagged and Delta. Receptor-ligand interactions occur when a cell expressing the ligand is juxtaposed to a cell expressing the Notch receptor. This interaction results in an external cleavage of Notch, caused by an ADAM metalloprotease, that splits the receptor into the Notch extracellular domain and an activated membrane-bound form of Notch. A second cleavage then occurs within the activated transmembrane domain by presenilin-dependent gamma-secretase; this releases the Notch intracellular domain (NICD), which translocates to the nucleus and participates as a member of various transcriptional activation complexes.[28]

Examination of live Notch expression in HSCs was facilitated by the creation of a transgenic Notch reporter mouse. The transgene contained an upstream Notch response element as well as four binding sites for CBF-1, Notch target gene, driving enhanced green fluorescent protein (GFP). In the transgenic mice, HSCs, designated by the marker c-kit, were also GFP-positive, showing *in vivo* that Notch signaling is active in HSCs. Analysis of more mature blood cells revealed significantly less GFP expression, suggesting that Notch activation decreases with differentiation. This was confirmed *in vitro* by comparing the amount of CFU-S12 colonies formed by HSC-enriched GFP-positive and GFP-negative cells; those expressing GFP, and thus having active Notch signaling, tended to form more CFU-S12 colonies and thus had more HSC function.[29]

Results of overexpression studies of Notch are consistent with these conclusions. Mice containing an expanded population of osteoblasts in the murine bone marrow, which serve as a stem cell niche, possessed increased numbers of

Notch-expressing HSCs. The osteoblast niche presents the appropriate Notch ligands to HSCs, leading to an increase in NICD within HSCs and a concomitant increase in HSC number, presumably reflecting an increase in HSC self renewal.[30] If primary murine bone marrow cells enriched for stem cells are retrovirally transduced with the NICD, differentiation is inhibited, the cells appear primitive, and more colonies form in the CFU-C *in vitro* assay, implying there are more HSCs present than in controls. Upon competitive transplantation of the NICD-transduced bone marrow cells into irradiated mice, there are more primitive cells and fewer mature cells present, as the Notch gain of function appears to impede exit from the undifferentiated state of the HSCs. Secondary recipients in serial transplants do not experience the hematopoietic exhaustion that control cells undergo, possibly due to enhanced self renewal creating a larger HSC pool.[31] Forced expression of NICD in irradiated zebrafish adults enhanced recovery of multi-lineage precursor cells, and thus all the blood lineages; HSCs were likely increased as well, as shown by increased *runx1* and *lmo2* expression.[32] Similarly, retroviral transduction of Hairy-Enhancer of Split (HES-1), a Notch target gene encoding a basic helix-loop-helix transcription factor, cDNA into fetal liver and bone marrow HSCs maintained the cells as immature, undergoing self renewal. Upon transplantation, the HES-1 transduced HSCs also yielded a higher proportion of immature cells in the recipient mouse as well as a higher donor chimerism.[33]

Loss of function studies have also helped elucidate the role of Notch in definitive HSC induction. A zebrafish known as *mindbomb*, harboring a mutation in an E3 ligase required for Delta ligand trafficking, lacks expression of *runx1* and *c-*

myb in the AGM; this suggests that Notch signaling is required for the definitive wave of hematopoiesis.[32] To assess the effect of Notch1 on AGM HSC formation, AGM cells from day E9.5 embryonic Notch1-/- mice were removed and cultured *in vitro*, then assayed for functional implications. The cells were found to have impaired ability to produce functional HSCs in a colony forming assay or to reconstitute an irradiated mouse. These effects could not be rescued by the addition of signaling factors belonging to other pathways in the environment, suggesting that HSCs have a cell autonomous requirement for Notch.[34]

The zebrafish has proved particularly useful in the elucidation of a genetic pathway involved in definitive HSC formation. Crossing two fish, one carrying the yeast transactivator Gal4 driven by the zebrafish heat-shock promoter (*hsp70:gal4*) and the other with the Gal4-responsive *upstream activating sequence* (*uas*) driving the zebrafish NICD (*uas:NICD*), led to a fish harboring heat shock inducible NICD. A pulse of heat shock at 14 hpf led to NICD induction, which caused ectopic *runx1* and *c-myb* expression at 36 hpf. This included an expansion of these definitive HSC markers into the aortic roof and vein. Similar results were found by overexpressing *runx1* by injection of *runx1* mRNA into single cell stage embryos. Conversely, knocking down *runx1* by morpholino injection led to a lack of definitive HSC formation; both this and the *mindbomb* mutant could not be rescued by NICD induction by heat shock or overexpression of *runx1* mRNA. This implied that *runx1* and Notch acted together in a pathway to induce HSC formation. This pathway was teased out by knocking down either *mindbomb* or *runx1* in the heat shock inducible NICD fish. Forced NICD expression showed Notch gain of function effects when

mindbomb was knocked down, thus showing that Notch is downstream of *mindbomb*; however, with *runx1* knocked down and NICD induced, the fish still had a lack of HSCs, thus displaying that *runx1* is downstream of Notch. These zebrafish experiments enabled the finding that Notch is necessary to establish HSC fate, sufficient to expand HSCs *in vivo*, and signals through the transcriptional factor *runx1* to perform these effects.[32]

4.3. Prostaglandin Signaling

Prostaglandins (PG) are a type of eicosanoid that have a variety of physiological effects at low concentrations. They are derived from twenty-carbon unsaturated fatty acids, the most common being arachidonic acid. Arachidonic acid is stored in the cell membrane and is turned into PGH_2 by cyclooxygenases (COX). COX1 is expressed in most tissues constitutively whereas COX2 expression is regulated within specific tissues; aspirin inhibits both COX enzymes. The PGH_2 intermediate then gives rise to a variety of PGs through PG synthases; the most common PG is PGE_2. The PGs bind to G-protein coupled receptors to mediate their actions.[35]

A few studies have shown the role of PGE_2 in promoting blood cell proliferation. Adding PGE_2 to a murine bone marrow suspension stimulated cell proliferation at concentrations comparable to those found in human serum. A colony forming assay was performed and revealed a higher proportion of colonies were in S phase than the controls. These results implied that PGE_2 prompts quiescent HSCs to move into the cell cycle.[36] A similar effect was shown in the

zebrafish thymi. Embryos treated with PGE_2 contained increased expression of the lymphocyte-specific *recombination activation gene* (*rag1*) in the thymi whereas those treated with COX inhibitors or antagonist of the PGE_2 receptor lacked *rag1* expression. Additionally, PGE_2 treatment could rescue the COX2 inhibitor phenotype. These effects of PGE_2 were shown to be due to increased cell proliferation as shown by the presence of the proliferating cell nuclear marker (PCNA) that was missing in controls.[37]

Recently, a major study on PGE_2 and HSC development was completed in the zebrafish. It was shown that treatment of zebrafish embryos with PGE_2 increased AGM expression of *runx1* and *c-myb*, while exposure to COX1 and COX2 inhibitors gave a decrease in HSC number. Knockdown of COX1/2 by morpholino injection similarly decreased *runx1* and *c-myb* expression; this effect was rescued by addition of dimethylPGE_2 ($dmPGE_2$), a long-acting form of PGE_2, showing the loss of AGM HSCs was a result of the loss of PG signaling. COX1 is expressed in the endothelial cells of the AGM region, yet both COX1 and COX2 are expressed in CD41-positive cells, likely the HSCs themselves. This implies that PGE_2 could be regulating both the HSC and the niche. In adult zebrafish, exposure to $dmPGE_2$ enhanced the recovery kinetics of the kidney marrow following irradiation, leading to faster repopulation of the precursor cells.[38]

Follow-up studies in mammalian systems confirmed that the regulation of HSCs by PGE_2 was evolutionarily conserved. Addition of $dmPGE_2$ to murine embryonic stem cells increased the amount of hematopoietic colonies formed in a dose-dependent manner. Similar to zebrafish AGM analysis, treatment with an

inhibitor of COX1/2 prevented colony growth, and this was rescued by $dmPGE_2$ treatment. Murine bone marrow was exposed *ex vivo* to $dmPGE_2$ and then transplanted into an irradiated recipient. This led to an increase in the number of CFU-S8 and CFU-S12 colonies formed, indicating an increase in HSC and precursor populations, respectively; complementary results were obtained upon treatment with COX inhibitors. Similar results were obtained when these experiments were performed on isolated HSCs. $dmPGE_2$-treated bone marrow showed an increase in the frequency of repopulating cells in a limiting dilution competitive transplant assay. Since no difference in homing to the bone marrow was observed, these results indicate an increase in HSC number.[38] COX2-/- mice have normal hematopoiesis and HSC formation due to maternal and sibling contribution of the enzyme. However, upon 5FU injury treatment, these mice recover slowly, have significantly lower numbers of cells in all blood lineages by day 12 post-injury and fail to repopulate the bone marrow with hematopoietic cells.[39] These findings conclude that PGE_2, produced by COX2 in particular, plays a key role in the creation of HSCs.

5. Clinical Applications

Umbilical cord blood transplantation is a common therapy used when an immunological match donor cannot be found for a patient. However, cord blood contains few HSCs and typically takes longer to engraft than bone marrow transplantations. It has been successful for the pediatric population, but expansion of HSCs is necessary to be more useful for adult patients.[40] Understanding the

extrinsic pathways that impact HSC development can lead to the development of an *ex vivo* treatments that increase HSC frequency in cord blood samples in the clinic.

Researchers have made several attempts to take knowledge of the above pathways to expand HSC populations in cord blood. Similar to overexpression of the *Hox* genes in zebrafish and mouse studies, by treating cord blood *ex vivo* with a peptide containing *Hox* sequences, the amount of HSCs and multipotent progenitors doubled; additionally, the cord blood reconstituted the blood system when transplanted into irradiated mice more efficiently and with faster kinetics.[41] Since Notch signaling seems to be required for HSC formation, adding Notch ligands at appropriate concentrations to cord blood has been shown to increase the number of immature precursor cells.[42] Whereas lower concentrations of the ligands result in better repopulation of irradiated mice in comparison to no *ex vivo* treatment, higher concentrations appear to cause too much apoptosis and hence a decreased population.[43] Similar trials are underway for prostaglandin E2, since overexpression has also been shown to increase HSC numbers and functionality in zebrafish and mice.[44] This is just one example of how work in model organisms, especially the zebrafish, can be translated into therapies for human disease.

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Figures

Figure A.1: *In situ* hybridizations of blood markers during the various stages of zebrafish hematopoiesis.

(A) *Gata1*, an erythrocyte marker, at the 5 somite stage (12 hpf). The 2 bilateral stripes form the ICM and will merge together by 18 somites (18 hpf) (X. Bai)

(B) *C-myb* and *runx1*, markers of definitive stem cells, at 36 hpf in the ventral wall of the dorsal aorta (T. North)

(C) *Scl*, an HSC and progenitor marker, is seen expressed in the CHT at 4 dpf (T. Bowman)

(D) *Scl* at 6.5 dpf; additional expression near the head is the kidney marrow (T. Bowman)

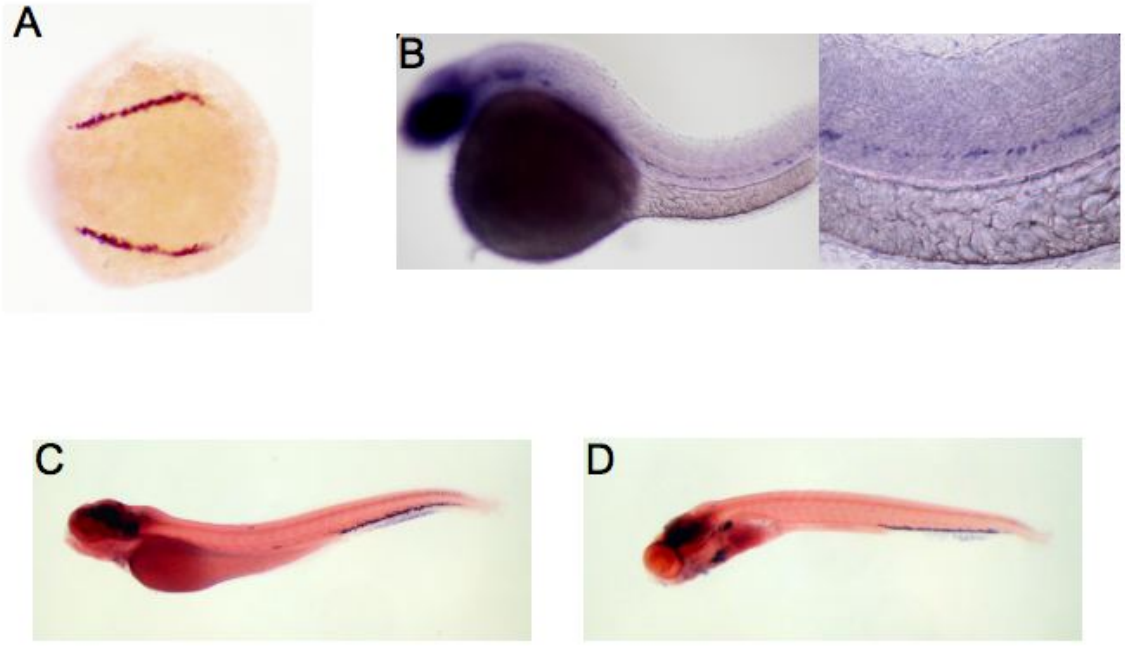

Figure A.2: Approximate temporal and anatomical locations of hematopoietic activity, based on current knowledge (adapted from reference 7)

(A) Mouse

(B) Zebrafish

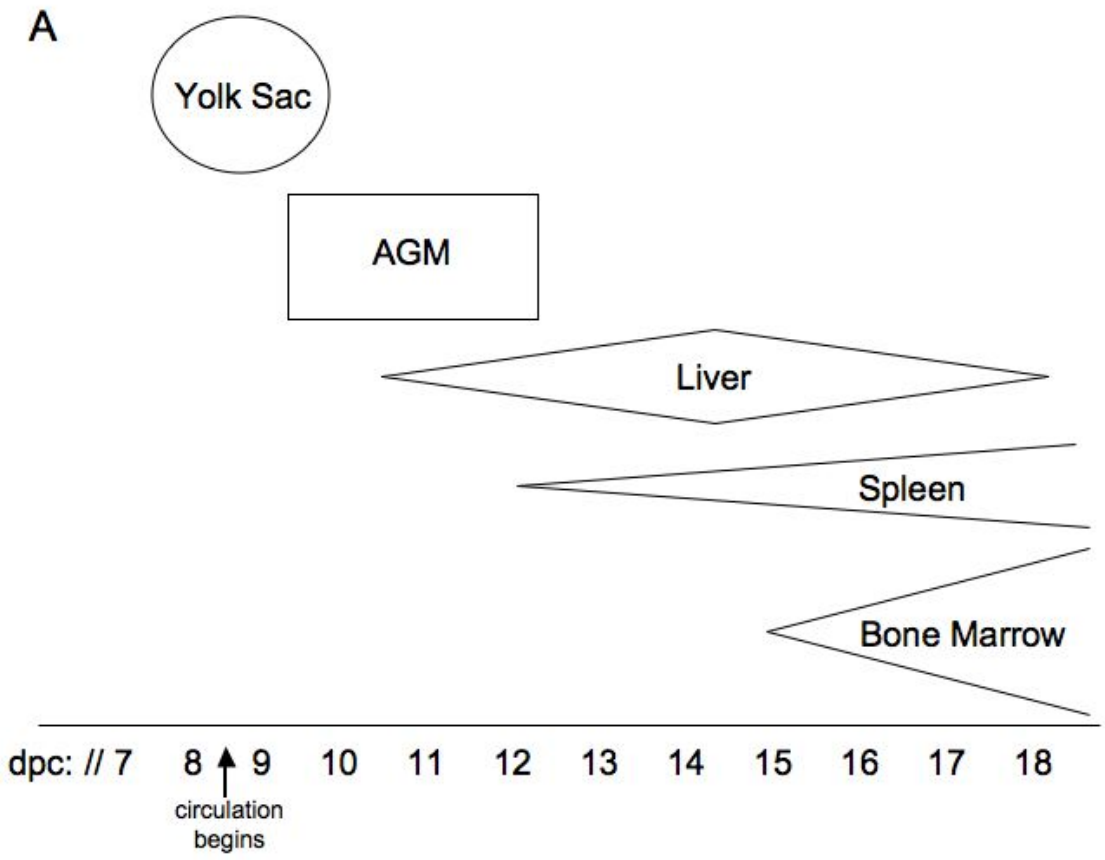

Figure A.2 (Continued)

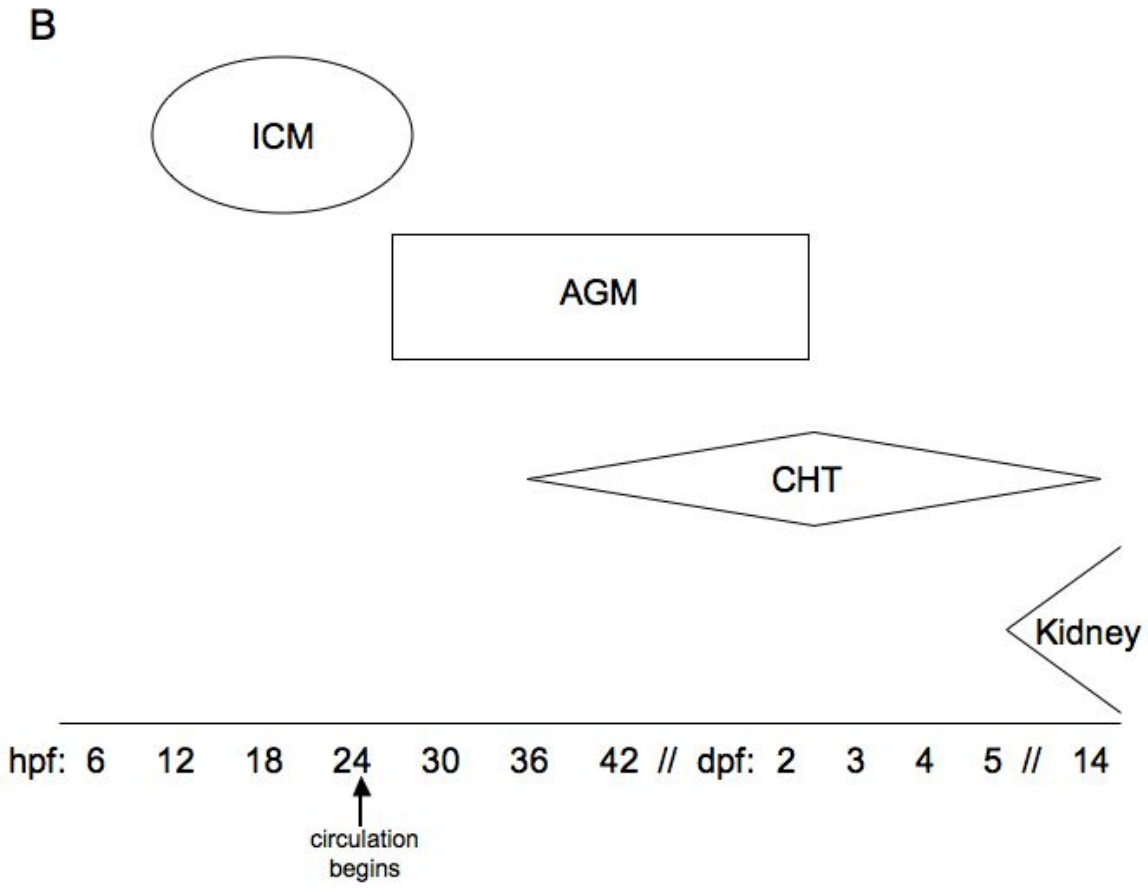

Figure A.3: Schematic of the acute injury recovery assay. By treating zebrafish with a sublethal dose of irradiation, the blood system undergoes injury and recovers over time. By day 7 post-irradiation, the HSCs will begin to produce cells that will differentiate into the blood system. Common lymphoid and myeloid progenitors recover by day 10 with more mature erythroid, lymphoid, and myeloid cells recovering by day 14. These kinetics are observed via the forward scatter-side scatter FACS plots. Within a few weeks, levels of all populations are back to and can even surpass pre-irradiation levels (T. Bowman).