Using zebrafish to elucidate the epigenetic mechanisms controlling neurogenesis and brain tumorigenesis

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Using zebrafish to elucidate the epigenetic mechanisms controlling neurogenesis and brain tumorigenesis

by

Laura Elizabeth Schultz

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in partial fulfillment of the requirements for the degree of

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2018

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DEDICATION

I would like to dedicate this dissertation to Nick, my mom and dad, and Logan, who all supported me though this journey.
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Retinoblastoma-Binding Protein 4 (RBBP4) is a chromatin adaptor protein that associates with numerous activating and repressive chromatin regulatory complexes. RBBP4 is overexpressed in human gliomas, and has been shown to promote survival of GBM cells and p300-dependent expression of DNA repair genes (Kitange et al., 2016). We previously established a zebrafish RB-defective neural progenitor-like brain tumor model (Solin et al., 2015) and recently found Rbbp4 is highly upregulated in the RB-brain tumors. Here we demonstrate Rbbp4 is essential for zebrafish brain development and show it has distinct requirements in neural stem and progenitor cells. CRISPR/Cas9 targeting was used to isolate a 4 base-pair frameshift mutation in the zebrafish homolog rbbp4. Homozygous mutant rbbp4^{Δ4/Δ4} are lethal at approximately 5-7 days post fertilization and show a dramatic reduction in the size of the brain and retina. Overexpression of rbbp4 cDNA in a Tg(Tol2<ubiquitin:rbbp4>) transgenic line rescues the rbbp4 mutant phenotype, demonstrating disruption of neurogenesis in rbbp4^{Δ4/Δ4} homozygotes is due to lack of functional Rbbp4. Beginning at 2 days post fertilization, γ-H2AX and activated caspase labeling was detected in post-mitotic regions in the larval midbrain and retina, suggesting RBBP4 is required to prevent DNA-damage and apoptosis in neural progenitors or newborn neurons. γ-H2AX and caspase were not detected in the stem cell niches at the brain ventricle or retinal ciliary marginal zone, however, stem cell morphology was abnormally large. Pulse chase BrdU labeling experiments confirmed a delay in stem cell proliferation and a lack of survival of post-mitotic neural progenitors. TP53 morpholino knockdown in rbbp4^{Δ4/Δ4} homozygotes transiently suppressed neural progenitor apoptosis, indicating the absence of Rbbp4...
activates TP53-dependent programmed cell death. Together these results suggest distinct requirements for Rbbp4 in neural stem cell proliferation and neural progenitor survival. Overall, our results indicate RBBP4 may drive RB-defective brain tumor growth by promoting proliferation and preventing DNA damage induced apoptosis of tumor cells.
CHAPTER 1. INTRODUCTION

General Introduction

In the influential paper by cancer researchers Douglas Hanahan and Robert Weinberg, the hallmarks of cancer are defined as the ability to sustain proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis, activation of invasion and metastasis, and enabling genome instability and mutation (Hanahan and Weinberg, 2011). In addition to these core hallmarks, the view of cancer as a misappropriation of normal developmental processes is also an important notion in the attempt to understand cancer (Aiello and Stanger, 2016). Development is a unique period when cells are highly dynamic and undergoing broad changes including proliferation, cell cycle exit, migration, and differentiation. These processes are normally tightly controlled by signaling molecules and genetic regulation at the transcriptional and epigenetic level. However, in the progression of tumorigenesis these processes become re-activated and dysregulated to promote the development of a tumor which can itself be seen as a complex organ consisting of different cell types with different functions.

The classic approach to studying cancer has been the use of mouse models containing genetic mutations in a few key genes. The problem with this approach is that often times the mouse models display different phenotypes than what is observed in humans. This underlines the limitations in relying solely on one model to understand the mechanisms of tumorigenesis. The zebrafish is coming to the forefront as a viable and useful model organism in which to study cancer (Mione and Trede, 2010). Zebrafish are highly fecund and produce large clutches of transparent embryos that can be closely
followed during development. Additionally, a fully sequenced genome and numerous genetic resources including cutting edge CRISPR/Cas9 technologies make zebrafish an ideal alternate model in which study cancer. Therefore, the approach of this dissertation is a two-prong effort to understand the mechanisms specifically involved in the initiation of tumorigenesis in the zebrafish model. The use of both germline and somatic mutagenesis has allowed for the study of undifferentiated neural tumors in the adult zebrafish. This has provided a model for understanding how signaling molecules like Leptin and the tumor suppressor Rb1 contribute to phenotypically similar human pediatric brain tumors. Additionally, this work has been expanded on by examining the role of a candidate oncogene, Rbbp4, in the normal neural developmental process. By understanding how the inactivation of Rbbp4 affects development we can gain insight into how dysregulation of this gene contributes to the initiation of tumorigenesis.

**Literature Review**

**Leptin**

Leptin was discovered in the 1990’s as the cause of the obesity phenotype seen in *ob/ob* mice (Zhang et al., 1994). It was determined to be a master regulator of appetite and energy homeostasis, being secreted from adipose tissue and having its signal transduced through its receptor in the hypothalamus (Friedman and Halaas, 1998). The functions of Leptin are mediated through interaction with its receptor which activates several pathways including the JAK/STAT, PI3K/AKT, and MAPK pathways, all of which are implicated in cell growth and proliferation (Banks et al., 2000) (Donato et al., 2010) (Sweeney, 2002). It has received a great deal of attention for its role in metabolic disorders such as obesity, however, additional studies have elucidated roles for leptin in
processes such as angiogenesis (Sierra-Honigmann et al., 1998b), lipid and glucose metabolism (Bryson et al., 1999), wound healing (Tadokoro et al., 2015), and immunity and inflammation (Fernández-Riejos et al., 2010). Furthermore, Leptin expression has been detected in tissues other than adipose tissue such as placenta (Cameo et al., 2003), stomach (Bado et al., 1998), skeletal muscle (Wang et al., 1998) and several areas of the brain including cortex, cerebellum, and pituitary (Morash et al., 1999).

Given that Leptin’s involvement in numerous key physiological processes and in the regulation of cell growth and proliferation, it is not surprising that Leptin has recently been implicated in progression of many cancers. For instance, up-regulation of Leptin expression has been found in breast (Ishikawa et al., 2004) (Andò et al., 2014), colon (Uddin et al., 2014), and prostate (Habib et al., 2015) cancers. Many of the studies examining Leptin’s role in cancer have found that is often associated with increased aggressiveness and worsened prognosis. These studies have reported that Leptin’s role in cancer progression is through promotion of invasiveness (Attoub et al., 2000), epithelial-to-mesenchymal transition (Andò et al., 2014), proliferation (Konopleva et al., 1999), cell survival and suppression of apoptosis (Somasundar et al., 2004), and finally increased angiogenesis in concert with VEGF (Ambrosini et al., 2002). Much of the current research on Leptin focuses on the relationship between obesity and cancer and the role Leptin plays in this connection. Many studies hypothesize that Leptin levels seen in cancer, particularly in an obese subject, are controlled by de-regulated signals such as insulin, glucocorticoids, or feedback loops of the sympathetic nervous system (Somasundar et al., 2004).
However, there is another mechanism by which Leptin levels are altered which may also play a role in the changes seen in Leptin in cancer. Specifically, Leptin has been shown to be a hypoxia-responsive gene, containing a HRE (hypoxia responsive element) in its promoter sequence which allows its expression to be altered by HIF-1α in response to decreased oxygen levels (Ambrosini et al., 2002). Research has shown that Leptin responds to hypoxia in several different tissues, including smooth muscle cells (Chiu et al., 2015) and alveolar epithelial cells (Chen et al., 2015). Additionally, increased Leptin expression has been seen in several glioblastoma cell lines (Morash et al., 2000) (Ferla et al., 2011) (Yeh et al., 2009), and its expression has been correlated with increased malignancy in brain tumors (Riolfi et al., 2010). This is of particular interest because malignant gliomas are known to contain areas of hypoxia within the central core of the tumor mass. These hypoxic areas show features typical of hypoxic response such as secretion of cytokines and growth factors that promote angiogenesis (Evans et al., 2004), maintenance of cancer stem cell populations (Irshad et al., 2015), and changes in metabolism favoring glycolysis over oxidative phosphorylation (Kucharzewska et al., 2015). Therefore, it is possible that in transformed glial cells within the hypoxic core, decreased levels of oxygen drive expression of Leptin, which in concert with other factors such as VEGF, promote changes in growth, metabolism, and apoptosis allowing the tumor to not only survive in hypoxic conditions, but also giving it a growth and metastatic advantage. Specifically, one study has shown an increase in Leptin in response to radiation in colon cancer cells (Suman et al., 2015). It can be surmised that Leptin confers apoptotic resistance to radiation, allowing for selection of increasingly resistant cells with treatment.
Retinoblastoma 1

A search of RB1 (Retinoblastoma 1) can find it referred to as the master cell cycle regulator, gatekeeper of the cell cycle, or major tumor suppressor. It is understandable given its history why RB1 is usually so narrowly categorized. It was initially discovered that mutations in RB1 served as the genetic basis of familial and sporadic retinoblastoma (Knudson, 1984). In these cases, when RB1 function is lost either though an inherited mutant allele or sporadic mutation, patients develop bilateral tumors of the retina before adulthood. Furthermore, patients with germline RB1 mutations show an increased risk for developing small cell lung cancer and osteosarcoma and later in life (Weinberg, 1995) (Wistuba et al., 2001) (Marees et al., 2008). It was through his initial studies of RB1 and retinoblastoma that Alfred Knudson developed his “two hit” theory of how mutations in tumor suppressor genes could cause predispositions to cancer and the idea of the tumor suppressor was born (Knudson, 1971) (Comings, 1973).

Initial molecular studies identified a straightforward and overarching role for RB1 in regulation of the cell cycle (Weinberg, 1995) (Goodrich et al., 1991). It was discovered that hypophosphorylated pRB binds to and inhibits E2F family members and prevents the expression of genes necessary for progression of the cell cycle (Classon and Harlow, 2002) (Hiebert et al., 1992). Upon mitogenic signals, cyclin dependent kinases phosphorylate pRB which then releases E2Fs and allows for transcription of cell cycle genes and the progression of G1 to S phase (Mittnacht, 1998). Continuing through the cell cycle, upon mitosis pRB is re-activated (de-phosphorylated) by PP1 (Kolupaeva and Janssens, 2013). Numerous studies have shown that loss of pRB leads to excessive proliferation (Lipinski et al., 2001) (Sidle et al., 1996) as well as inappropriate re-entry of
both stem and differentiated quiescent cells into the cell cycle (Mayhew et al., 2005) (Sage et al., 2003) (Burkhart and Sage, 2008).

While it is tempting to focus on the paradigm of phosphorylation of pRB as a master switch for G1 to S phase, a close examination of the structure of pRB give the first insights that there is more to the story than simple repression of E2F transcription factors. Human pRB contains three main domains: an amino terminal domain (RBN) consisting of cyclin folds (Hassler et al., 2007), a central pocket domain consisting of an A and B subdomain (Lee et al., 1998), and an intrinsically disordered carboxy-terminal domain (Rubin et al., 2005). The pocket domain is the most important interaction domain for pRB and gives the first clues to the function of pRB beyond E2F inactivation. The pocket domain contains two binding regions; the E2F transactivation domain which binds E2F factors (Lee et al., 2002) (Xiao et al., 2003) as well as an L-X-C-X-E binding cleft which has been shown to mediate interaction between pRB and chromatin modifying enzymes and viral proteins (Kim et al., 2001) (Morris and Dyson, 2001). Importantly, pRB can interact with multiple binding partners simultaneously through these different domains making it function more as an adaptor rather than a simple inhibitor (Dick and Rubin, 2013) (Goodrich, 2006). In fact, pRB has been shown to have over 150 different interacting partners (Chinnam and Goodrich, 2011). Thus pRB can interfere with E2F both through binding and inhibiting formation of transcription initiation complexes, but also through association of various chromatin modifying complexes at particular promoter sites to alter chromatin structure.

As mentioned above, studies have shown that pRB can interact physically with several different chromatin modifying proteins through its L-X-C-X-E binding cleft
Experiments have shown that this interaction is not necessary for pRB to regulate transition from G1-S phase (Isaac et al., 2006), suggesting roles for these interactions in modulating RB function beyond cell cycle control. It has been long known that pRB and the histone deacetylases HDAC1 and HDAC2 form a complex (Brehm et al., 1998) (Luo et al., 1998) (Magnaghi-Jaulin et al., 1998). As deacetylation is associated with closed/transcriptionally inactive chromatin it is thought that pRB serves to bring HDAC1/2 to specific (usually E2F associated) locations on the genome and promote gene silencing (Brehm and Kouzarides, 1999). The functionality of this interaction has been supported by studies that have shown treatment with the HDAC inhibitor trichostatin A does lead to inhibition of pRB repression at some promoters (Luo et al., 1998). Additional studies in mouse embryo fibroblasts (MEFs) show that loss of pRB leads to a more open, accessible chromatin structure again supporting the idea that pRB in association with chromatin remodelers plays a role in transcriptional repression (Trouche et al., 1996). Furthermore, the DNA methyltransferase DNMT1 has also been found, in complex with RB/E2F1/HDAC1, to promote DNA methylation and silencing (Robertson et al., 2000). Additionally, pRB is known to interact with the histone methyltransferase SUV39H1 which methylates lysine 9 of histone H3 (Nielsen et al., 2001) (Vaute et al., 2002) and Suv4-20h1 and Suv4-20h2 which trimethylate H4K20 (Gonzalo et al., 2005). The methylation of histones promotes spreading of heterochromatin and gene silencing (Spencer and Davie, 1999). Recent studies have also shown that pRB can form a complex with histone methyltransferase enhancer of zeste homolog 2 (EZH2), a component of the Polycomb Repressive Complex, to promote H3K27me3 and thus silencing of repetitive elements in the genome such as transposons,
LINEs, and SINEs (Ishak et al., 2016). In addition to repressors, pRB has also been shown to interact with HBRM and BRG1 which are part of the ATP-dependent remodeling complex SWI/SNF (Dunaief et al., 1994) (Strober et al., 1996) (Trouche et al., 1997). This complex uses helicase and ATPase to unwind DNA and make it more accessible thereby activating gene expression (Cairns, 1998). Thus, pRB can participate in the activation of gene expression through chromatin remodeling which may be relevant in its role promoting differentiation. As will be discussed below, many of these interactions mediate the diverse array of roles pRB plays in various different cellular processes beyond just control of the cell cycle.

In addition to its canonical role in regulation of the cell cycle, pRB is also involved in regulation of many other processes that suppress tumorigenesis including senescence, genome stability, and differentiation. Senescence is the process whereby a cell permanently withdraws from the cell cycle due to stress and is an important barrier to cellular immortalization and transformation (Prieur and Peeper, 2008). Studies have shown that pRB plays a crucial role in the initiation and maintenance of senescence (Chicas et al., 2010) (Dannenberg et al., 2000) (Sage et al., 2000) (Ben-Porath and Weinberg, 2005). One of the ways it most likely does this by aiding in the formation of senescence associated heterochromatin foci and overall reorganization of the chromatin into a less permissive state (Talluri et al., 2010).

Maintenance of genome stability as well as the ability to detect and repair DNA damage is another important process in the prevention of cellular transformation (Knudsen et al., 2006). Loss of pRB leads to aneuploidy and other chromosomal defects in mouse ES cells or human primary fibroblasts (Zheng et al., 2002) (Amato et al., 2009).
pRB contributes to genome stability through the repression of the E2F target gene MAD2 which is a mitotic checkpoint gene that has been shown to cause chromosomal instability when overexpressed (Schvartzman et al., 2011) (Kabeche and Compton, 2012). Additionally, experiments have shown that triple knockouts of all three pocket proteins leads to decreased methylation, tangled chromosomes, and aneuploidy (Gonzalo et al., 2005). pRB has also been shown to be a binding partner of Condensin II (Longworth et al., 2008) and upon pRB loss defects in condensin loading and subsequently chromosome condensation are observed (Manning et al., 2010) (Coschi et al., 2010) (van Harn et al., 2010). Mutations in the LXCXE which inhibit pRB interactions with chromatin remodelers also leads to chromosomal defects (Isaac et al., 2006), further indicating that pRB mediates its effect on genome stability primarily through its interaction with chromatin remodelers.

pRB is also well known to influence cellular differentiation, which is the process whereby precursor or progenitor cells exit the cell cycle and undergo global alterations in gene expression to activate genes that promote the function and morphology of the mature cell type (Korenjak and Brehm, 2005) (McClellan and Slack, 2007) (Nguyen and McCance, 2005). Several studies have demonstrated that pRB interacts with different transcription factors to promote cellular differentiation. For instance, pRB interacts with MyoD to promote muscle differentiation (Gu et al., 1993), GATA1 to promote erythrocyte differentiation (Kadri et al., 2009), CBFA1/RunX2 to promote bone differentiation (Thomas et al., 2001), and C/EBP to promote adipocyte differentiation (Chen et al., 1996). Furthermore in plants, loss of pRB homolog actually causes failures in differentiation rather than proliferation (Borghi et al., 2010). In mice it has been
shown that pRB loss leads to defect in differentiation as well (Zhang et al., 2004a) (Papadimou et al., 2005). Clearly, pRB doesn’t just regulate the cell cycle; instead it is an important master regulator that integrates signals about genome integrity, differentiation status, and response to stress and helps the cells respond appropriately to these conditions.

While many of the observed functions of pRB play an obvious role in tumor suppression, pRB does have a paradoxical role in apoptosis that would suggest an oncogenic effect (Indovina et al., 2015). Many studies have reported an increase in apoptosis, particularly in differentiating rather than cycling cells, upon the loss of pRB (Chau and Wang, 2003) (Khidr and Chen, 2006) (Wang et al., 2010a). Studies have also found that the loss of pRB leads to increased apoptosis and sensitivity to anti-cancer agents and UV radiation (Ishii et al., 1997) (Zagorski et al., 2007) (Sharma et al., 2007) (Biasoli et al., 2013) (Chau et al., 2006) (Agerbaek et al., 2003). This controversial activity seems to be based primarily on a specific pattern of phosphorylation of pRB and its interaction with E2F family member E2F1. E2F1 has been shown to bind to pro-apoptotic gene promoters and induce their expression in response to DNA damage (DeGregori et al., 1997) (Trimarchi and Lees, 2002). Furthermore, E2F1 displays a unique interaction with pRB through binding to the RBC c-terminal domain rather than the pocket domain (Dick and Dyson, 2003) (Julian et al., 2008) (Seifried et al., 2008). Models have been proposed whereby during normal progression through the cell cycle, phosphorylated pRB can still bind E2F1 and block expression of genes involved in apoptosis while at the same time permitting progression through cell cycle (Wells et al., 2003) (Cecchini and Dick, 2011) (Calbó et al., 2002). However, upon DNA damage pRB
and E2F1 undergo a series of post-translational modifications by ATM and CHEK1/2 allowing for the formation of pRB/E2F1/p300/CBP activating complexes at promoters of apoptotic genes (Korah et al., 2012) (Avni et al., 2003) (Munro et al., 2012). However, additional research suggests that pRB also has a pro-apoptotic interaction with E2F1 by inducing its acetylation and activation (Ianari et al., 2009) (Carnevale et al., 2012). Additionally, some studies suggest that active, hypo-phosphorylated pRB can actually promote apoptosis in some circumstances (Day et al., 1997) (Wagner and Roemer, 2005) (Bowen et al., 2002). Overall, the observation of whether or not loss of pRB contributes to increased apoptosis is context dependent and may also be dependent on the type of mutation. For instance, increases in apoptosis are often observed in mutants with null mutations in RB1 rather than mutants with defects in phosphorylation or regulation of pRB (Delston and Harbour, 2006). This could explain the puzzling observation that many mutations in RB1 occurring in tumorigenesis are in the regulation of its phosphorylation rather than direct null mutant as the tumor cells are selecting for mutations that maintain suppression of apoptosis (Chau and Wang, 2003). Clearly, there is still much to be elucidated about the role pRB plays in apoptosis which is a critical barrier to the formation of transformed cells.

Given all of the roles pRB plays in normal cellular processes, what does this indicate in terms of its role in promoting or preventing tumor formation? One observation is that the effect of loss of pRB on tumor formation is highly context dependent. For instance, as mentioned above, germline loss of RB1 only leads to cancers in certain tissues with regularity even though pRB or pathway regulating it is mutated/dysregulated in almost all cancers (Sherr, 1996) (Knudsen and Knudsen, 2008) (Hanahan and
Weinberg, 2000). In the case of retinoblastoma, osteosarcoma, and small cell lung cancer, RB1 clearly plays a crucial role in homeostasis in those cell types and loss of RB1 appears to be an initiating event (Knudson, 1984). However, most other cancers mutation in RB1 is a late event in the progression of transformation (Burkhart and Sage, 2008). This may be due to the fact that it does contain paradoxical oncogenic effects which may be beneficial to early tumors and only when other pathways have been deactivated, like p53, are RB1 mutations helpful (Sherr and McCormick, 2002). Whether it plays a role in promoting de-differentiation of more mature cells or promoting excess proliferation in stem and progenitor cells that already contain the potential to divide or both remains to be determined.

There have been several null alleles of RB1 generated in different species with the intent of studying both the mechanistic role of pRB in normal cellular processes as well as its role in tumorigenesis. In early mouse models containing an Rb1 null allele, homozygotes were embryonic lethal at approximately 15 days post conception and displayed abnormal proliferation, defects in erythropoiesis, and apoptosis in the nervous system, lens, and skeletal muscle (Clarke et al., 1992) (Jacks et al., 1992) (Lee et al., 1992) (Chau and Wang, 2003). However, while heterozygous and chimeric mice did develop pituitary tumors, they did not develop retinoblastoma as is the case in humans. Furthermore, several follow up studies have suggested placental defects as a possible contributing factor to the observed embryonic apoptosis (Wu et al., 2003) (de Bruin et al., 2003). Due to the confounding placental effects and the lack of observed retinoblastoma, in the intervening years many conditional and tissue specific Rb1 alleles have been generated in mice. In particular, when Rb1, pocket protein p107, and tumor suppressor
p53 are ablated in mice models the animals do develop retinoblastoma (Zhang et al., 2004b).

The zebrafish rb1 gene shows 67% homology to the human and mouse RB1/Rb1. The first rb1 null alleles generated in zebrafish were named space cadet (Gyda et al., 2012). In fish homozygous for these alleles, retinal ganglion cell (RGC) precursors displayed delayed cell cycle exit and subsequent defects in post mitotic RGC axon guidance, retinotectal connectivity, and phototactic defects. Unlike Rb1 null mouse models, homozygous rb1 null zebrafish embryos did not display embryonic apoptosis, which is possibly due to the fact that zebrafish larvae develop externally with maternal RNA possibly rescuing early gene loss phenotypes. However, homozygous embryos did fail to survive past approximately 10 days post fertilization (dpf) due to the failure to develop swim bladders which zebrafish need to swim and feed properly. Thus, the rb1-homozygotes are larval lethal and could not be tracked for the development of juvenile retinoblastoma. Recently, our group showed that injection of the genome editing nuclease TALEN RNA targeting rb1 leads to somatic inactivation of rb1. F0 animals survive to adulthood and 11-33% develop undifferentiated, primitive neuroectodermal-like tumors starting at approximately 3 months of age. Another recent study has also used TALENs to generate germline rb1 null alleles in zebrafish (Zhang et al., 2018). This study focused on lymphocytes and found that loss of rb1 led to early lymphocyte apoptosis due to elevated caspase-3. Similar to results mentioned above, these results suggest rb1 plays a role in inhibiting E2F1-driven apoptosis. While there has been a great effort recently in using zebrafish to model cancer (Shive, 2013) (White et al., 2013), there is still much to be learned from studying loss of rb1 in zebrafish beyond just retinal or
lymphocyte phenotypes. Detailed explanations of how loss of RB1 affects proliferation and function of neural progenitors leading to both developmental defects and tumorigenesis remain to be fully elucidated.

**Primitive neuroectodermal tumors**

As mentioned above, somatic inactivation of *rb1* in zebrafish results in undifferentiated tumors similar to human primitive neuroectodermal tumors of the central nervous system (CNS-PNET). This class of brain tumors is usually present in children, though can present in adults, and makes up approximately 3-5% of all pediatric brain tumors (Pizer et al., 2006). These tumors are defined by small, poorly differentiated or undifferentiated embryonal cells that can differentiate into both neural and glial subtypes (Louis et al., 2007). CNS-PNETs are aggressive tumors and the 5-year survival rate is only 48-73% (Jakacki et al., 2015). A major obstacle in understanding and treating CNS-PNETs is the difficulty in defining and categorizing tumor types. Early studies by Picard et al. used gene expression, copy number, and immunohistochemical data to define three main CNS-PNET subtypes (Picard et al., 2012). These include the primitive-neural subtype which displays high LIN28 expression and is generally highly aggressive; the oligoneural subtype displays high OLIG2 expression and is seen in older children; and the mesenchymal subtype shows low OLIG2 and LIN28 expression, increased metastases, and is present in all ages. However, a more recent analysis combining data from gene expression, genome-wide sequencing, copy number, histopathology, and DNA methylation analyses categorized CNS-PNETS into four subtypes (Sturm et al., 2016b). In addition to our recent work, other studies in zebrafish have shown that activation of the RAS/MAPK pathway in oligoneural precursor cells leads to oligoneural subtype-like
brain tumors expressing Olig2 and Sox10 (Modzelewska et al., 2016). Clearly, additional work is needed to understanding the molecular mechanisms driving transformation of early progenitor and precursor cells in the brain resulting in the formation of CNS-PNETs.

**Retinoblastoma binding protein 4**

RBBP4 (Retinoblastoma Binding Protein 4) and its homolog RBBP7 (Retinoblastoma Binding Protein 7) are WD40 repeat proteins that share a 90% identical amino acid sequence. While these two proteins do exist in certain complexes together, they appear to have distinct functions. For example, RBBP7 is exclusively associated with the HAT1 (histone acetyltransferase) (Verreault et al., 1998), while RBBP4 is exclusively associated with the CAF-1 (chromatin associated factor 1) complex (Verreault et al., 1996). Much of the research to date on these homologous proteins has focused on RBBP4 which is highly conserved from plants, nematodes, yeast, drosophila, and zebrafish among others, and in fact it shows 100% amino acid sequence homology between mouse and human (Qian and Lee, 1995).

RBBP4 contains an n-terminal alpha helix, seven WD40 repeats forming a seven blade β-propeller structure, and a one turn helix c-terminus (Song et al., 2008). While the protein itself does not contain enzymatic activity, there are two known binding sites where it binds to a diverse array of interactors (Gururaja et al., 2002). First is the binding pocket formed at the side of the propeller structure formed by the n-terminal alpha helix and binding loop emerging from seventh blade (Song et al., 2008). This is most notable for binding the n-terminal helix 1 of histone H4. This binding pocket has also been shown to bind the SUZ12 subunit of PRC2 (polycomb repressive complex 2) (Nowak et al.,
2011b) and the MTA1 subunit of the NuRD (nucleosome remodeling deacetylase complex) (Lejon et al., 2011). Additionally, there is a channel on the top of the b-propeller structure that has been shown to bind un-methylated histone H3 (Song et al., 2008) and the transcriptional regulator FOG-1 (Lejon et al., 2011). And while RBBP4 can bind H3 or H4 individually, it can also bind H3/H4 dimers (Murzina et al., 2008) (Zhang et al., 2013). The presence of multiple binding sites as well as its affinity for histones H3 and H4 has given RBBP4 the designation as an adaptor protein that serves to tether or locate specific chromatin modifying complexes to the chromatin.

While the specific nature of all of the binding interactions may not be known, a large body of research has shown that RBBP4 interacts with a wide array of different partners (Wolffe et al., 2000). As its name suggests, RBBP4 was first identified when it co-immunoprecipitated with RB1 in HeLa cell lysates (Qian and Lee, 1995). RBBP4 likely interacts with the c-terminus of un- or hypo-phosphorylated RB1 (Qian et al., 1993) (Qian and Lee, 1995). It has been further shown that RBBP4 complexes with RB1/HDAC1/E2F1 and appears to help mediate RB1-based suppression of E2F promoters (Nicolas et al., 2000) (Nicolas et al., 2001). However, contrary evidence has shown that the formation of this complex is dependent on the LXCXE binding domain of RB1, suggesting that the interaction between RB1 and RBBP4 is indirect and mediated by HDAC1 (Nicolas et al., 2000). Further evidence has also suggested that de-regulated E2F activity, as in the case of tumor cells with RB1 deficiency, actually promotes the increased expression of RBBP4 via GC repeat sites in the RBBP4 promoter region (Kitamura et al., 2015).
The association of RBBP4 with HDACs (histone deacetylases) is well documented and it has been found as part of several repressive histone deacetylating complexes including the Sin3 complex (Vermaak et al., 1999) and the NuRD complex (Xue et al., 1998) (Zhang et al., 1999) (Millard et al., 2016). The Sin3 complex contains HDAC1, HDAC2, SAP30, SAP18, and both RBBP4 and RBBP7 (Alland et al., 1997). This complex is recruited to specific regulatory sites by DNA-binding proteins and co-repressors such as Mad-Max to mediate gene silencing (Ayer et al., 1995). RBBP4 is also a component of NuRD which is a major co-repressor complex. The NuRD complex contains CHD3 and CHD4 which are ATP-dependent helicase chromatin remodelers, HDAC1 and 2, MBD2 which binds CpG methyl domains, MTA1, 2, and 3 which target the complex to different genomic locations, and finally RBBP4 and RBBP7 (Allen et al., 2013). The multi-combinatorial subunits of the NuRD complex allow it to perform various functions in different cellular contexts. It is especially critical in embryonic development and in particular in promoting proper cellular differentiation (Ahringer, 2000) (Yoshida et al., 2008) (Denslow and Wade, 2007) and it has also been implicated in tumorigenesis (Lai and Wade, 2011). One possible function of the NuRD complex may be to use ATP-dependent chromatin remodeling to allow RBBP4 access to helix 1 of H4 which may be inaccessible when assemble into the nucleosome octamer.

In addition to HDAC-containing repressor complexes, RBBP4 is also a component of the methylating repressive complex PRC2 (Schmitges et al., 2011) (Kuzmichev et al., 2002). PRC2 is comprised of four subunits including EED (Embryonic Ectoderm Development), SUZ12 (Suppressor of Zeste 12), RBBP4, and finally the catalytic subunit EZH1 or EZH2 (Enhancer of Zeste Homolog 1 or 2)
EZH1 or 2 is the catalytic lysine methyltransferase subunit responsible for trimethylation of lysine 27 in histone 3 (H3K27me3) (Margueron and Reinberg, 2011). PRC2 plays a key role in early epigenetic regulation during embryonic development and differentiation (Pasini et al., 2004) (O'Carroll et al., 2001) and is also dysregulated in cancer (Conway et al., 2015). RBBP4 has been shown to bind the SUZ12 subunit in drosophila via the binding pocket at the side of the beta propeller and it thought to facilitate high affinity binding of the PRC2 complex to the nucleosome (Nowak et al., 2011b). In addition to PRC2, studies have also shown RBBP4 and RBBP7 to be found in a complex with HDAC1/2 and the K9H3 methyltransferase SUV39H1 and together are thought to facilitate the formation of heterochromatin (Vaute et al., 2002).

RBBP4 is also an integral component of the CAF-1 complex (Verreault et al., 1996). CAF-1 consists of three subunits including p150, p60, and RBBP4 and functions to assemble nucleosomes onto newly synthesized DNA (Kaufman et al., 1995). RBBP4 has also been shown in yeast to be required for proper loading of the kinetochore-specific histone CENP-A to the centromere (Hayashi et al., 2004). Besides just repressive complexes, RBBP4 is also a member of the activating acetylating complex CBP/p300 (Zhang et al., 2000). CBP (CREB binding protein) and its homolog p300 are histone acetyltransferases which activate gene transcription either through direct interaction or post-translational modification of the basal transcriptional machinery or other transcriptional activators (Shiama, 1997). Experiments have shown that RBBP4 interacts with CBP/p300 and in fact enhances its activity (Zhang et al., 2000). Finally, RBBP4 is a member of the DREAM (dimerization partner, RB-like, E2F and multi-vulval class B)
complex which is an RB-independent complex that controls cell cycle gene expression during quiescence (Litovchick et al., 2007) (Sadasivam et al., 2012).

To date much of the work done analyzing the functional role of RBBP4 has been performed *in vitro* or in invertebrate models. However, several clear roles for RBBP4 have become apparent in chromatin assembly and stability, development, aging, and cancer. Experiments in chicken cells have demonstrated that loss of RBBP4 leads to decreased DNA synthesis resulting in S-phase delays, chromatin instability, mitotic defects and abnormal chromosomal segregation, and apoptosis (Satrimafitrah et al., 2016). These defects are most likely caused by loss of functionality of the CAF-1 complex when RBBP4 is depleted leading to defects in packaging of newly synthesized DNA into chromatin. Additionally, in yeast, loss of the RBBP4 homolog Mis16 results in misloading of CENP-A and subsequent chromosomal segregation defects and aneuploidy (Hayashi et al., 2004). In both cases, the loss of RBBP4 is also results in an increase in acetylation of histones highlighting the additional role of RBBP4 in proper targeting of HDACs. Further supporting this idea, in mouse oocytes knockdown of RBBP4 led to defects in meiosis, chromosomal alignment, multipolar spindles, aneuploidy, and histone H3 and H4 hyperacetylation (Balboula et al., 2015). The authors posit that the hyperacetylation resulting from the loss of RBBP4 prevents the spindle assembly checkpoint protein AURKC from properly localizing and thus the formation of meiotic defects.

To date studies examining the role of RBBP4 in development have been limited to cell culture and invertebrate models such as nematodes and planarians. In *C. elegans*, the RBBP4 homolog LIN-53 is expressed throughout the nuclei of most if not all cells
during embryogenesis and loss of LIN-53 leads to embryonic lethality (Lu and Horvitz, 1998). It was shown that LIN-53, in coordination with the RB1 homolog LIN-35, antagonizes the Ras/RTK pathway and prevents vulval cell fate specification. In human pluripotent stem cells, higher expression of RBBP4 was associated with a more undifferentiated state and knockdown of RBBP4 led to an increase in differentiation and neurogenesis-specific genes and thus in these cells likely functions to maintain pluripotency (O’Connor et al., 2011). It was proposed by the authors that RBBP4 acts to sequester RB1 and prevent it from inducing genes necessary for differentiation programs. Contrary to the results presented in human pluripotent stem cells, in planarians the loss of RBBP4 leads to apoptosis due to a failure of neoblast cells to commit to a cell fate and differentiate suggesting a role for RBBP4 in promoting differentiation rather than pluripotency (Bonuccelli et al., 2010). Finally, in Arabidopsis reduction in the RBBP4 homolog MSI1 led to ectopic expression of floral homeotic genes, inability of primordia to differentiate properly, and loss of heterochromatin structure (Hennig et al., 2003). The authors surmise that in this case MSI1 is required to maintain epigenetic silencing of developmental patterns of gene expression from one cell division to the next.

Interestingly, several links have been made between RBBP4 and aging-related phenotypes. For instance, 48% of patients with the accelerated aging condition known as Progeria show reduced levels of RBBP4 (Pegoraro et al., 2009). Furthermore, RBBP4 fails to bind with the mutated form of LaminA, or Progerin, which causes the disorder. This reduction of RBBP4 was also seen in normal aged cells vs. normal young cells. To confirm the role of RBBP4 in this disease and the physiological process of aging, researchers demonstrated that silencing of RBBP4 and RBBP7 produced phenotypes
similar to those seen in cells from progeria patients including defects in heterochromatin formation, loss of CENP-A, re-expression of SatIII, and increases in DNA damage. Based on the fact that the NuRD complex components MTA2 and HDAC1 were also decreased in cells from Progeria patients, the authors concluded that these defects are mediated by mis-regulation of the NuRD complex. Experiments in mice have confirmed that the amount of RBBP4 is reduced in the brain of older animals, and furthermore the knockdown of RBBP4 in young animals led to defects in memory (Pavlopoulos et al., 2013). Finally, experiments in vitro have shown an association between loss of RBBP4 and induction of cellular senescence (Tsujii et al., 2015). In this study the authors show novel binding between RBBP4 and Importin alpha and suggest that RBBP4 plays a role in regulating importin-mediated nuclear import to prevent replicative senescence.

Given the number of major regulatory complexes and pathways RBBP4 is associated with, it is no surprise that it has been implicated as being dysregulated in many different cancers. Several large-scale expression studies have found altered RBBP4 expression in cancers including multiple myeloma (Gao et al., 2017), mesothelioma (Vavougios et al., 2015), medulloblastoma (Bunt et al., 2013), childhood germ cell tumors (Haskins et al., 2012), oropharyngeal tumors (Lohanichbutr et al., 2009) (Bai et al., 2015), salivary gland carcinomas (Leivo et al., 2005), and hepatocellular carcinoma (Song et al., 2004). Studies are now beginning to examine the specific role of RBBP4 in the progression of these malignancies. In malignant melanoma, a W382X truncation is thought to cause improper cytoplasmic localization of RBBP4 contributing to tumorigenesis (Pauty et al., 2017). In thyroid tumors, increased levels of RBBP4 were observed as compared to normal tissue, and tumor cell proliferation was diminished upon
RBBP4 inhibition (Pacifico et al., 2007). In this study it was determined that RBBP4 gene transcription is controlled in part by the oncogenic NF-kB transcription factor. Several other studies have actually found that RBBP4 expression is decreased in tumor tissues. For example, in cervical cancer a decrease in expression in RBBP4 is found in malignant tissue and this decrease is associated with increased proliferation, decreased senescence, and decreased RB1 and p53 expression (Kong et al., 2007). Additionally, in liver tumors a decrease in RBBP4 expression is associated with increased tumor aggressiveness and an increase in E2F1-responsive oncogenes including Oct4, Myc and Sox2 (Li et al., 2015a). In this study the authors determined that reduced RBBP4 expression was due to suppression by the microRNA miR-429. Several additional studies have also looked at the role of RBBP4 in response to different therapeutic cancer treatments and have generated conflicting results. Increased expression of RBBP4 was associated with increased sensitivity to radiation in breast cancer cells (Torres-Roca et al., 2005) (Scuto et al., 2007) and photodynamic therapy in cervical cancer cells (Wu et al., 2017). While in the case of breast cancer cells RBBP4 is thought to mediate this effect through antagonizing the Ras pathway (Scuto et al., 2007), in cervical cancer cells it was shown that RBBP4 is associated with increased expression of tumor suppressor genes such as TP53, RB1, and Caspase-3 (Wu et al., 2017). However, contrary to these results, studies in glioblastoma cells have found that decreased RBBP4 is associated with increased susceptibility to temozolomide treatment and that RBBP4 is likely mediating resistance to drug sensitivity through interaction with CBP/p300 to activate expression of DNA repair genes (Kitange et al., 2016). Overall, these results suggest that the
mechanism and nature of RBBP4 function in a tumorigenic paradigm is dependent on the tumor and treatment type.
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CHAPTER 2. VEGFA AND LEPTIN EXPRESSION ASSOCIATED WITH ECTOPIC PROLIFERATION AND RETINAL DYSPLASIA IN ZEBRAFISH OPTIC PATHWAY TUMORS

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Abstract

In the central nervous system (CNS) injury induces cellular reprogramming and progenitor proliferation, but the molecular mechanisms that limit regeneration and prevent tumorigenesis are not completely understood. We previously described a zebrafish optic pathway tumor model in which transgenic Tg(flk1:RFP)is18/+ adults develop non-malignant retinal tumors. Key pathways driving injury-induced glial reprogramming and regeneration contributed to tumor formation. Here we examine a time course of proliferation and present new analyses of the Tg(flk1:RFP)is18/+ dysplastic retina and tumor transcriptomes. Retinal dysplasia was first detected in 3-month old adults but was not limited to a specific stem cell or progenitor niche. Pathway analyses suggested a decrease in cellular respiration and increased expression of components of Hif1-α, VEGF, mTOR, NFκβ, and multiple interleukin pathways is associated with early retinal dysplasia. Hif-α targets VEGFA (vegfab) and Leptin (lepb) were both highly upregulated in dysplastic retina, however each showed distinct
expression patterns in neurons and glia, respectively. Phospho-S6 immunolabeling indicated mTOR signaling is activated in multiple cell populations in wild type retina and in the dysplastic retina and advanced tumor. Our results suggest multiple pathways may contribute to the continuous proliferation of retinal progenitors and tumor growth in this optic pathway tumor model. Further investigation of these signaling pathways may yield insight into potential mechanisms to control the proliferative response during regeneration in the nervous system.

**Introduction**

Elucidating the molecular mechanisms controlling cellular reprogramming and regeneration is important for improved treatment of nervous system injury and disease. Proper regulation of regenerative programs is necessary to limit proliferation and prevent cellular transformation and neoplasia. In injured tissue dying cells and damaged vasculature lead to inflammation and a hypoxic environment (Corcoran and O'Connor, 2013; Rock and Kono, 2008). The pro-inflammatory cytokine and hypoxia inducible factor Hif-α pathways are critical for mediating the cellular response to cell death and hypoxia (Kono et al., 2014; Semenza, 2012). How activation of hypoxia and inflammatory pathways contribute to proliferation and regeneration in the nervous system is still not completely understood.

Hypoxia has been shown to activate regeneration gene programs in injured peripheral sensory neurons by Hif1-α induced expression of its direct target, the vascular endothelial growth factor VEGF (Cho et al., 2015). VEGF receptor tyrosine kinase signaling is essential to embryonic blood vessel development, adult angiogenesis, tumor vascularization, and disease related vascular retinopathies (Koch et al., 2011). In addition,
multiple non-vascular functions have been described for VEGF in the developing and adult CNS (Lange et al., 2016; Mackenzie and Ruhrberg, 2012). VEGF is expressed by glial progenitors and astrocytes in response to hypoxia in the neonatal subventricular zone and stimulates proliferation of glial progenitors (Bain et al., 2013). In the subgranular zone/neurogenic niche of the adult mouse hippocampus, secretion of VEGFA and VEGFB by neural stem and progenitor cells is thought to influence the microenvironment and maintain the stem cell pool (Kirby et al., 2015). Treatment of embryonic cortical neural precursors in vitro with VEGF induces expression of E2F family members and cell cycle regulators cyclin D1, cyclin E and cdc25, supporting the above observations that VEGF may have a direct role in promoting progenitor proliferation in vivo (Zhu et al., 2003). In the retina, VEGF has been shown to act directly on chick retinal progenitors to promote proliferation (Hashimoto et al., 2006), however, whether VEGF mediates the proliferative response to hypoxia in the retina has not previously been shown.

A second transcriptional target of Hif-α, the cytokine Leptin, is expressed in response to hypoxia (Ambrosini et al., 2002), and has been shown to stimulate proliferation of neural progenitors (Perez-Gonzalez et al., 2011) and regulate angiogenesis (Bouloumie et al., 1998; Cao et al., 2001; Sierra-Honigmann et al., 1998a) and wound healing (Murad et al., 2003). The presence of Leptin in the eye vitreous is associated with inflammation and vision loss in patients with diabetic retinopathy (Gariano et al., 2000). Experimentally, Leptin expression is increased as part of the inflammatory response in a guinea pig model of uveitis (Kukner et al., 2006) and induces accumulation of reactive oxygen species in human endothelial cells (Bouloumie et al.,
This suggests Leptin and VEGF together might integrate hypoxia and inflammatory signaling pathways, which are both implicated in solid tumor initiation and cancer progression (Candido and Hagemann, 2013; Coussens and Werb, 2002; Semenza, 2013). Most recently it has been shown that extended exposure of normal bronchial epithelial cells to hypoxia and proinflammatory cytokines TNF-α and IL1-β induces cancer-like phenotypic changes, providing a link between hypoxia, inflammation and cellular transformation in vitro (Baird et al., 2016). How sustained signaling due to a combination of hypoxia and chronic inflammation contributes to transformation in solid tumor initiation in vivo remains an open question. Moreover, the connection between hypoxic induction of Hif-α targets, such as VEGF and Leptin, to neoplastic transformation and tumor induction is less clear than the well-documented role of these factors in vascularization supporting tumor growth (Connolly et al., 1989).

Zebrafish has been used extensively as a model system for studying activation of latent progenitor populations and the molecular pathways controlling injury induced regeneration in the vertebrate nervous system (Alunni and Bally-Cuif, 2016; Kizil et al., 2012). Two populations of progenitor cells contribute to the growth of the zebrafish retina; latent progenitors derived from Müller glia in the inner nuclear layer, and neural progenitors from neuroepithelial stem cells present in the ciliary marginal zone at the retina periphery (Alunni and Bally-Cuif, 2016). Chemical, mechanical, genetic and light-induced injury of the zebrafish retina has revealed multiple growth factor and cytokine signaling pathways stimulate latent progenitor proliferation and Müller glia dedifferentiation and reprogramming, both of which contribute to regeneration (Goldman, 2014; Gorsuch and Hyde, 2014; Lenkowski and Raymond, 2014). Together
these studies reveal the importance of coordinated activation of multiple pathways, including Heparin-Binding EGF-like Growth Factor, Wnt, Leptin, Interleukin-6, and Jak/Stat signaling, in progenitor proliferation and subsequent fate specification and differentiation (Goldman, 2014; Zhao et al., 2014). The initial response to injury in the zebrafish retina involves transient expression of the proinflammatory cytokine TNF-α, which prevents gliosis and stimulates induction of inner nuclear layer progenitor proliferation and regeneration (Nelson et al., 2013). The limited regenerative response indicates control mechanisms are enacted to prevent unregulated growth and dysplasia. Hif-α and mTOR signaling have been reported to work together in regulating ciliary marginal zone progenitor proliferation in Xenopus retina after nutrient starvation (Khaliullina et al., 2016; Love et al., 2014), and mTOR has been shown to be required for Müller glia-derived progenitor proliferation in injured chick retina (Zelinka et al., 2016). A role for Hif-α or mTOR signaling in zebrafish retinal development and regeneration has not previously been reported.

We previously reported the characterization of a zebrafish optic pathway tumor model in which transgenic Tg(flk1:RFP)i518/+ adults develop non-malignant retinal tumors at ~80% penetrance (Solin et al., 2014). The molecular basis for tumor induction is not known, however our analyses showed the retinal tumors may originate in part from Müller glia derived progenitors, and activated signaling pathways in the tumor transcriptome are similar to injury induced regeneration pathways driving Müller glia reprogramming and progenitor proliferation. In contrast to injury induced regeneration models, our retinal tumor model is distinct in that TNF-α expression is not upregulated and proliferation is not transient. Once initiated, proliferation is sustained and contributes
continuously to the growth of dysplastic tissue and the development of non-malignant glial-like tumors. These observations indicate other molecular pathways can stimulate retinal progenitor proliferation and override the normal controls that limit regeneration.

To identify pathways that might be associated with proliferation and the responsive retinal cell populations, we performed additional immunocytochemical and differential gene expression analyses on adult Tg(flk1:RFP)is18/+ retina. Early proliferation and dysplasia was not restricted to the ciliary marginal zone or to Müller glia-derived progenitors in the inner nuclear layer, the normal stem cell/progenitor niches of the teleost retina (Alunni and Bally-Cuif, 2016; Than-Trong and Bally-Cuif, 2015). Ingenuity Pathway Analysis of the early dysplastic retina identified Hif-α signaling targets VEGF and Leptin, components of inflammation pathways NFκβ, IL-1, IL-6, and IL-8, and pathways required for Müller glia-derived progenitor proliferation in injured retina including HBEGF (Zhao et al., 2014) (Wan et al., 2014) and mTOR (Zelinka et al., 2016). Novel pathways that act in tissue repair and regeneration (Baker and Tuan, 2013), but not previously implicated in retinal regeneration, were represented, such as GADD45 growth arrest and DNA damage response, endothelin-1 signaling, and caveolin-1-mediated endocytosis. Our results suggest multiple signaling pathways might trigger progenitor proliferation and contribute to tumor growth. The Tg(flk1:RFP)is18 tumor model may provide insight into the molecular mechanisms that prevent neoplastic transformation during regeneration in the nervous system.

**Results**

**Analysis of cellular proliferation and dysplasia in Tg(flk1:RFP)is18/+ adult retina**

To examine morphological changes and dysplasia in the Tg(flk1:RFP)is18 retina,
we performed histopathology on serial sectioned head tissue from cohorts of age matched wild type sibling and Tg(flk1:RFP)is18/+ zebrafish. Wild type and transgenic siblings were sacrificed at 4 weeks (n=10 wild type, n=10 Tg(flk1:RFP)is18/+), 3 months/11 weeks (n=8 wild type, n=9 Tg(flk1:RFP)is18/+), and 4 months (n=5 wild type, n=15 Tg(flk1:RFP)is18/) of age. In all individuals 6um sections extending from the anterior to posterior limits of the eyes were examined for the frequency and location of ectopic cell proliferation and abnormal retinal architecture.

Comparison of 10 four-week-old Tg(flk1:RFP)is18/+ with 10 age-matched wild type siblings did not reveal any obvious morphological abnormalities or ectopic proliferation in the retinas, indicating that during juvenile stages in Tg(flk1:RFP)is18/+ heterozygotes, the growth of the retina and the rate of progenitor proliferation and production of neurons and Müller glia proceeds normally. At three months of age, in comparison to wild type retina (Figure 2.1 a, e), 6/9 Tg(flk1:RFP)is18/+ fish had regions of ectopic proliferation in the retina in at least one eye that contained clusters of proliferating cells in the inner nuclear layer, inner plexiform layer, or retinal ganglion cell layer (Figure 2.1 b, f). Cells with processes stretched across the inner plexiform layer were frequent (Figure 2.1 f arrows). In contrast, the ciliary marginal zone appeared normal compared to wild type. A similar frequency of retinal abnormalities was observed in 4 month old Tg(flk1:RFP)is18/+ fish, with 10 out of 15 individuals presenting with ectopic proliferation in one or both retina. However, phenotypes ranged from retina with...
Figure 2.1 Detection of aberrant cell migration and ectopic proliferation in young adult Tg(flk1:RFP)is18 dysplastic retina. a, e Histological staining of retina section from 3-month old adult wild type zebrafish showing nuclear layers and organized laminar structure. b, f Representative image of section through retina of 3-month-old Tg(flk1:RFP)is18 adult reveals aberrant migration of cells across the inner nuclear layer (arrows, f) (n=9). c-h Representative images of retinal sections from 4-month-old Tg(flk1:RFP)is18 adults (n=15). c, g Retina from 4-month-old Tg(flk1:RFP)is18 adult with dysplasia reveals disruption of inner, outer and ganglion cell layers with numerous mitotic Figures (arrows, g). d, h 4-month-old Tg(flk1:RFP)is18 adult with advanced retinal tumor filling the vitreous space. Tumor tissue is composed of fibrous material interspersed with numerous mitotic Figures, cells showing heterogeneous nuclear morphology and forming occasional rosettes (arrows, h), and blood vessels (arrowheads, h). pe, pigmented epithelium; onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bars a, b 200 μm; c, d 500 μm; e, f, g, h 50 μm.

a normal appearance, to retina containing multiple regions of proliferation and disorganization with gaps in the inner nuclear layer or large lesions distending the nuclear layers (Figure 2.1 c, g), and retina completely replaced by neoplastic tumors that filled the vitreal cavity (Figure 2.1 d, h), which was not observed in the 3 month old retinas. Regions of proliferation and disorganization were expanded across the retina layers and
included the outer nuclear layer (Figure 2.1 c, g). Mitotic figures, which are rarely detected in mature regions of wild type adult retina, were numerous in these regions (Figure 2.1 g arrows). Larger tumors contained rosettes with mitotic figures (Figure 2.1 h arrows) and blood vessels (Figure 2.1 h arrowheads) embedded in a fibrous matrix, similar to the advanced retinal tumors previously reported in older adult Tg(flk1:RFP)is18/+ fish (Solin et al., 2014).

To examine proliferation and progenitor cells in early dysplastic retina, serial sections from three wild type and three Tg(flk1:RFP)is18/+ four-month-old adults were histologically stained with H&E and adjacent sections labeled with PCNA and Sox2 antibodies (Figure 2.2). In wild type (Figure 2.2 a-e) a small number of PCNA and Sox2-labeled cells were present at the ciliary marginal zone (Figure 2.2 c arrow) and in the region just adjacent to the ciliary marginal zone (Figure 2.2 c bracket). Few PCNA-positive cells were detected in the outer nuclear layer at the periphery (Figure 2.2 c arrowhead) or central regions of the retina (Figure 2.2 e arrowheads). The Sox2 expressing cells detected in the inner nuclear layer and ganglion cell layer (Figure 2.2 c, e) have previously been described as Müller glia, amacrine, and displaced amacrine cells (Jusuf and Harris, 2009). In contrast to wild type, 3/3 four-month-old Tg(flk1:RFP)is18/+ adults had regions of ectopic proliferation in multiple serial sections (Figure 2.2, Figure 2.3). Groups of cells in the inner plexiform layer and gaps in the inner nuclear layer could be detected at multiple locations in a single retina (Figure 2.2 f). The ciliary marginal zone (Figure 2.2 g, h arrow), and adjacent progenitor region (Figure 2.2 g, h bracket), appeared expanded in the Tg(flk1:RFP)is18/+ retina
Figure 2.2  Ectopic proliferation and Sox2 expressing cells in young adult
Tg(flk1:RFP)is18 dysplastic retina. a-e Wild type 4-month-old adult (n=3)
shows PCNA-positive cells are restricted to stem/progenitor cells at the
ciliary marginal zone (cmz, arrow, c) and rod precursor cells in the
photoreceptor outer nuclear layer (arrowheads, e). Sox2-expressing cells
overlap with PCNA-positive stem cells at the ciliary marginal zone (bracket,
c) and are present in amacrine/displaced amacrine cells in the inner nuclear
and ganglion cell layers (c, e). f-j Retina from 4-month-old Tg(flk1:RFP)is18
adult (n=3) shows numerous regions of proliferation distributed throughout
the neural retina (f). The ciliary marginal zone (g, h) and ventral ciliary
circumferential artery (asterisk, g) are expanded. Small masses of PCNA and
Sox2 positive cells (arrowheads g, h) are present in the normally single cell
layered non-pigmented epithelium extending from the ciliary marginal zone.
(Figure 2.2 continued) (small arrow, g, h – compare to small arrow in wild type b). Ectopic regions of proliferation in the neural retina contain disorganized cells expressing PCNA and Sox2 (arrowheads, j). cmz, ciliary marginal zone; gel, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; pe, pigmented epithelium. Scale bars a, f 200 μm; all other panels 50 μm.

compared to wild type. The diameter of the ventral ciliary circumferential arteries was increased (Figure 2.2 g, h asterisk), and the non-pigmented epithelium that lines the vitreal face of the iris was expanded (Figure 2.2 g, h small arrow) and contained small masses with PCNA and Sox2 expressing cells (Figure 2.2 g, h arrowheads). In the internal retina, PCNA was detected in cells within each nuclear layer and in disorganized masses in the inner plexiform layer (Figure 2.2 i, j arrowheads). Sox2 expressing cells were present scattered throughout the masses and inner nuclear layer and ganglion cell layer. Quantification of Sox2 and PCNA positive cells at the ciliary marginal zone and regions of dysplasia in the inner retina showed the increase in $Tg(flk1:RFP)is18/+$ compared to age-matched wild type siblings was significant (Figure 2.3).

In summary, the histological analysis of tumor onset indicates that in the $Tg(flk1:RFP)is18/+$ retina, neurogenesis proceeds normally through juvenile stages of development. Abnormal proliferation begins in early adulthood and occurs in the inner nuclear layer and ganglion cell layers of the retina (Figure 2.1). The non-pigmented epithelium that extends away from the ciliary marginal zone along the vitreal surface of the iris is also affected (Figure 2.2 g). In contrast, the ciliary marginal zone appears expanded but relatively normal overall, even in fish with advanced tumors (Figure 2.1 d, Figure 2.2 g). The data suggest multiple cell populations in the $Tg(flk1:RFP)is18/+$ retina are stimulated to proliferate, and may include cells in the pigmented epithelium or
Figure 2.3 The ciliary marginal zone and dysplastic regions of Tg(flk1:RFP)is18/+ retina in 4 month old adults contain increased numbers of PCNA and Sox2 positive cells. Total number of PCNA and Sox2 labeled cells were counted at the ciliary marginal zone and in a 300μm long section of the internal retina in three wild type and three age matched 4 month old Tg(flk1:RFP)is18/+ siblings containing a region of dysplasia identified by H&E staining. a Ciliary marginal zone PCNA positive cells 19 ± 4 vs. 7 ± 1, \( p = 0.0082 \). b Ciliary marginal zone Sox2 positive cells 19 ± 4 vs. 7 ± 1, \( p = 0.0168 \). c Internal retina PCNA positive cells: 11 ± 2 vs. <1, \( p = 0.0001 \). d Internal retina Sox2 positive cells: 47 ± 5 vs. 28 ± 3, \( p = 0.007 \). n=9 retinal sections, three each from three individuals of each genotype. Error bars represent standard error of the mean. cmz, ciliary marginal zone; ir, internal retina.

ganglion cell layer, in addition to the known progenitor populations in the inner nuclear layer and at the ciliary marginal zone (Than-Trong and Bally-Cuif, 2015).

Transcriptomics reveals elevated expression of VEGF, leptin, and mTOR signaling pathways correlates with Tg(flk1:RFP)is18/+ retinal dysplasia

We previously reported transcriptome analysis of age matched adult wild type
retina, \textit{Tg(flk1:RFP)is18/+} dysplastic retina (formerly designated Pretumor) and \textit{Tg(flk1:RFP)is18/+} retinal tumor tissue was consistent with activation of signal transduction pathways known to be required for Müller glia reprogramming during injury induced regeneration (Solin et al., 2014). GO Term analysis of the RNASeq dataset using genes with an FPKM of $\geq 1$ and a significant change in expression level between samples ($p\leq0.01$) revealed translation, vascular development and blood vessel development processes were upregulated in \textit{Tg(flk1:RFP)is18/+} dysplastic retina (Figure 2.4 a; Supplementary Table S2.1-S2.4). In retinal tumor the analysis revealed a significant decrease in phototransduction and ion transport and an increase in cellular processes necessary for proliferation, including DNA replication, cell division, and ribonucleoprotein complex biogenesis (Figure 2.4 b, Supplementary Table S2.1-S2.4).

We used IPA analysis to further examine the altered molecular pathways that might initiate transformation and proliferation of progenitor cells leading to tumor formation. To increase the stringency of the analysis the original RNASeq data was first filtered to remove genes with a read count of less than 10 in wild type retina, dysplastic, or tumor tissues. Next, genes with less than three-fold change in expression level between wild type retina and dysplastic retina, or between wild type retina and tumor tissue, were removed, leaving a set of 2587 genes. Human homologs of the 2587 gene set were recovered at BioMart (Smedley et al., 2015). Individual zebrafish genes of interest absent from the BioMart conversion (ie., ENSDARG00000045548\_lepb,
Figure 2.4  *Tg(flk1:RFP)is18/* dysplastic retina associated GoTerm and Ingenuity Pathway Analysis canonical pathways. a GoTerm analysis of DGE of dysplastic retinal tissue suggests decreased oxidative metabolism and increased vasculogenesis pathways. b GoTerm analysis of DGE of retinal tumor tissue is consistent with loss of phototransduction and increased proliferation. c IPA canonical pathways to which the *Tg(flk1:RFP)is18/* dysplastic retina transcriptome is associated. d IPA regulator pathway analysis of VEGF targets involved in cytokine signaling, transcriptional response and extracellular matrix remodeling underlying invasion of tumor cells.

ENSDARG00000076972_si:dkey-208k22.3_plvap) were identified using BLAST, leaving 2570 genes for IPA analysis (Supplementary Table S5). Pathways with the most significant overlap in the dysplastic tissue were EIF2 Signaling (p=4.65E-26), Phototransduction Pathway (p=4.65E-15), mTOR signaling (p=2.28E-08) and Cell Cycle Control of Chromosomal Replication (1.46E-07). General categories of disease, disorders
and molecular and cellular function were consistent with injury and tumorigenesis and included cancer, organismal injury and abnormalities, and neurological disease, and predicted an increase in cell movement, growth, and proliferation and a decrease in cell death and apoptosis. Specific canonical signaling pathways represented in the dysplastic retina included caveolin-mediated endocytosis, EIF2 translation, Hif1-α, endothelin-1, VEGF, mTOR, and gadd45B (Figure 2.4 c; Supplementary Table S2.5). NFκβ and multiple interleukin pathways (IL1, IL6, IL8) were present (Supplementary Table S2.5). A glioma invasiveness pathway was activated (Figure 2.4 c; Supplementary Table S2.5) as expected given the involvement of glial reprogramming pathways and the glioma-like cellular and molecular features of the tumors. Together the data suggest activation of multiple signaling pathways is associated with dysplasia in the Tg(flk1:RFP)is18/+ retina.

**VEGFA and Leptin are induced in distinct cell populations in the early dysplastic Tg(flk1:RFP)is18/+ retina**

A number of genes with the greatest change in expression between wild type and Tg(flk1:RFP)is18/+ dysplastic retina are direct targets of hypoxia inducible factor Hif1-α (Benita et al., 2009; Greenald et al., 2015; Ortiz-Barahona et al., 2010; Wang et al., 2005) or components of the Hif1-α (egln1a, egln3, lepb, vegfab, stc2a), vascular endothelial growth factor VEGF (hmox1, ptgs2b) or JAK/Stat signaling pathways (socs3a) (Supplementary Table S5). Other hypoxia-inducible targets whose expression was significantly increased included cxcr4b, cxcl12, ddit4 and gadd45ba. Hypoxia regulates the level of Hif1-α through protein stabilization (reviewed in (Corcoran and O'Connor, 2013)). The level of hif1aa and hilab gene expression was not significantly changed
between wild type, \textit{Tg(flk1:RFP)is18/+} dysplastic retina and tumor tissue. Together these observations suggest a hypoxic environment is one possible trigger associated with ectopic proliferation in the \textit{Tg(flk1:RFP)is18/+} retina. Because both VEGF and Leptin are direct targets of Hif1-\(\alpha\) and have been shown to promote neural progenitor proliferation (Forsythe et al., 1996; Shweiki et al., 1992) we examined these two signaling molecules in more detail.

In dysplastic \textit{Tg(flk1:RFP)is18/+} retina tissue the level of \textit{vegfaa} increased 2.9 fold and \textit{vegfab} increased 11-fold. IPA analysis showed increased expression or activation of many components in the VEGF regulatory pathway that promotes tumor cell invasion (Figure 2.4 d), including multiple growth factors (\textit{TGF\(\beta\), hbegf}), cytokines (\textit{endothelin-1, cxcl12}), matrix metalloproteases and peptidases (\textit{mmp2, mmp9, plau}), and enzymes involved in prostaglandin synthesis (\textit{cox2, ptgs2}). In the retina, VEGF promotes proliferation of retinal neural progenitors through activation of E2F via the MEK-ERK signal pathway (Hashimoto et al., 2006; Nishiguchi et al., 2007). The E2F family of transcription factors has been shown to activate expression of gene networks regulating cell cycle progression, DNA damage and mitotic checkpoints, chromosome maintenance and chromatin assembly (Ren et al., 2002). Many direct targets of E2F were significantly increased in the dysplastic retina transcriptome, including cyclin dependent kinase \textit{cdk1} and \textit{cdk2}, minichromosome maintenance deficit family members \textit{mcm3, mcm5, mcm6}, and mitotic kinases aurora kinase \textit{aurkb} and polo-like kinase \textit{plk1}. Together, the data is consistent with VEGF’s role as a mitogen that induces cell migration and stimulates cytokine signaling and angiogenesis.

A second target of Hif1-\(\alpha\) signaling, the cytokine Leptin (Grosfeld et al., 2002),
was dramatically increased in the dysplastic retina transcriptome (~500 fold) and remained elevated in tumor tissue. IPA analysis did not indicate a significant overlap of genes in the canonical pathways of which Leptin is a component including Leptin signaling in obesity, insulin or Jak/Stat. However, Leptin can mediate activation of the Jak/Stat pathway through induction of Socs3, one of the genes most highly altered in the dysplastic retina transcriptome. Leptin has also been shown to synergize with interleukin IL-6 in Müller glia reprogramming in injury-induced regeneration (Zhao et al., 2014).

Together, this indicates that like VEGF, Leptin may play an important role in mediating the response to metabolic changes in the Tg(flk1:RFP)is18/+ retina, either contributing to dysplasia and tumorigenesis or as a result of increased proliferation.

To examine which cell types in the retina express vegfaa, vegfab and lepb, in situ hybridization was performed on retinal sections from wild type and Tg(flk1:RFP)is18/+ adults (n= 3-5 biological replicates). The panel of in situ hybridization results shown in Figure 5 is composed of images from the same Tg(flk1:RFP)is18/+ retina, in order to illustrate expression for multiple genes in a retina with dysplasia as well as an advanced tumor. As shown in Figure 2.5, dysplastic retina was defined by a thickening and distortion of the neural retina layers compared to wild type, whereas tumor represents a mass that completely distorts the retinal layers and extends into the vitreous. In wild type retina the vegfaa signal was weak in all three nuclear layers (Figure 2.5 a, b) and was only slightly elevated in dysplastic retina and tumor (Figure 2.5 c box, d asterisk). vegfab, the VEGF receptor flk1, lepb, and the Leptin receptor lepr were not detected in
Figure 2.5  Induction of vegfab and lepb expression in distinct cell populations in Tg(flk1:RFP)is18 dysplastic retina and retinal tumor. *in situ* hybridization of retina cryosections from +/+ (a, b, e, f, i, j, m, n, q, r, u, v) and Tg(flk1:RFP)is18 (c, d, g, h, k, l, o, p, s, t, w, x) adult siblings. Weak expression of vegfaa is detected in the three nuclear layers of the retina (a, b) and in early dysplastic (c box, d) and retinal tumor tissue (c asterisk).
Expression of vegfab (e, f), the VEGF receptor flk1 (i, j), lepb (m, n), or the Leptin receptor lepr (q, r) are not detected in wild type +/+ retina. g, h In early dysplastic retina vegfab is detected throughout the inner and outer nuclear layers (g small bracket, h). In the tumor containing region (large bracket) vegfab is detected in a central region of the lesion (asterisk). k, l The VEGF receptor flk1 was present in small groups of cells scattered throughout the tissue that may overlap with microvessels. o, p lepb is highly expressed in a subset of cells in the inner nuclear layer and cells in the ganglion cell layer in dysplastic retina, and present in many cells evenly distributed in the early tumor (bracket). s, t The lepr receptor expression is faint and diffuse in dysplastic retina and tumor tissue. u, v In wild type retina the glial marker apoeb is strongly expressed in inner nuclear layer Müller glia and astrocytes in the ganglion cell layer/nerve fiber layer. w, x apoeb expression is highly elevated in dysplastic retina and is detected in projections crossing the inner plexiform layer. Numerous cells throughout the tumor tissue labeled intensely with apoeb. gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; pe, pigmented epithelium. All scale bars, 100 μm, except panels g, k, o, s, t scale bars, 500 μm.

any layer of the wild type neural retina (Figure 2.5 e, f, I, j, m, n, q, r), as expected given the low number of reads in the wild type retina transcriptome. The glial marker apoeb was readily detected in the inner nuclear layer and ganglion cell layer (Figure 2.5 u, v), presumably expressed in Müller glia and nerve fiber layer astrocytes, respectively. In regions of the Tg(flk1:RFP)is18/+ retina with dysplasia a high level of vegfab labeling was present and appeared to be expressed in the inner and outer nuclear layers but was absent from the ganglion cell layer (Figure 2.5 g small bracket, h). The retina also contained a region with a tumorous lesion that distended the nuclear layers (Figure 2.5 g large bracket). vegfab was detected in a central region in the lesion (Figure 2.5 g asterisk). The VEGF receptor flk1 showed a low level of expression in cells scattered throughout the dysplastic region and larger lesion in the retina (Figure 2.5 k, l). In contrast to vegfab, in dysplastic tissue lepb expression appeared to localize specifically to Müller glia in the inner nuclear layer and in cells in the nerve fiber layer, presumably
astrocytes (Figure 2.5 o, p). In addition, high levels of lepb were detected in clusters throughout the tumorous lesion (Figure 2.5 o, bracket), whereas little vegfab was present in a similar section (Figure 2.5 g large bracket). The lepr receptor showed diffuse expression throughout both regions in the dysplastic retina (Figure 2.5 s, t).

Apolipoprotein ApoE is expressed by activated Müller glia and astrocytes in the injured retina and promotes neurite outgrowth of various neuronal cell types (Lorber et al., 2009). lepb expression was similar to apoeb, which showed a substantial increase throughout the inner nuclear layer and ganglion cell layer and widespread expression in the retinal lesion, but appeared absent from neuronal cells (Figure 2.5 w, x), further supporting the restricted expression of lepb in glia within the Tg(flk1:RFP)is18 dysplastic retina. Together these results indicate that in the Tg(flk1:RFP)is18 retina, neurons and glia respond by expression of distinct growth factors and cytokines. vegfab expression is highly induced in neurons, and possibly Müller glia, in the inner nuclear layer. In contrast, lebp expression overlapped with apoeb and appeared restricted to the Müller glia and astrocytes in the nerve fiber layer.

A third canonical pathway identified by IPA analysis of the Tg(flk1:RFP)is18/+ transcriptome was mTOR signaling (Figure 2.4 c), which regulates cell growth and metabolism (Laplante and Sabatini, 2009). Activation of mTOR in response to hypoxia or growth factor signaling stimulates translation through phosphorylation of eukaryotic initiation factor 4E-binding protein (4EBP) and ribosomal protein S6-kinase, which stimulates phosphorylation of ribosomal protein S6 (Figure 2.6). mTOR can also act upstream of HIF1-α to activate VEGF signaling (Figure 2.6) indicating positive feedback between the hypoxia and mTOR pathways. Components of mTOR signaling
Figure 2.6 Tg(flk1:RFP)is18/+ dysplastic retina genes associated with IPA canonical pathway mTOR signaling. Upstream components phospholipase D (PLD), DNA damage induced transcript (DDIT), RAS and phosphoinositol 3-kinase (PI3K) predicted to activate mTOR signaling are upregulated. Downstream effectors of mTOR signaling, such as translation initiation factors eIF4E and eIF3 and ribosomal proteins including RPS6 are also upregulated. Mixed red and green color in VEGF symbol reflects ~11-fold vegfab upregulation and vegfb three-fold downregulation.

located upstream and downstream of the mTORC1 complex that were upregulated in the dysplastic retina transcriptome included phospholipase D (PLD), DNA damage inducible transcript (DDIT4), RAS, phosphoinositol 3-kinase (PI3K), eukaryotic translation
initiation factors eIF4E and eIF3, ribosomal protein S6 (RPS6), and many ribosomal proteins (Figure 2.6).

To examine mTOR signaling in the Tg(flk1:RFP)is18/+ retina tissues were labeled with an antibody specific for phosphorylated ribosomal protein S6 (phospho-S6) (n=7 wild type and n=8 Tg(flk1:RFP)is18/+ biological replicates). In wild type adult zebrafish retina phospho-S6 labeling was detected in numerous cell types. In the inner nuclear layer of the mature retina phospho-S6 labeled putative horizontal cells (Figure 2.7a, b small arrowheads) and amacrine cells and/or Müller glia (Figure 2.7a, b large arrowheads). Phospho-S6 labeling was also detected in the retinal ganglion cell layer, present in retinal ganglion cells and/or displaced amacrine cells (Figure 2.7a, arrows) (Diekmann et al., 2015). Regions of the Tg(flk1:RFP)is18/+ retina with dysplasia (Figure 2.7c, d bracket, e, f) or advanced tumor (Figure 2.7g) showed phospho-S6 labeled cells distributed throughout the lesions. However, the percentage of phospho-S6 positive cells in Tg(flk1:RFP)is18/+ tumor tissue was not significantly different than in wild type retina (Figure 2.7h, 11.4% ± 0.9 vs. 13.9% ± 1.7, p=0.2291, n=9 sections, three sections each from three individuals of each genotype). In the Tg(flk1:RFP)is18/+ retina phospho-S6 was also detected in the nerve fiber layer on the vitreal side of the retina (Figure 2.7d, e arrows). This labeling may represent active mTOR signaling in retinal ganglion cells, displaced amacrine cells, astrocytes in the nerve fiber layer, or blood vessels of the capillary plexus that extends across the vitreal surface of the retina. Together the data indicate mTOR signaling may contribute to the sustained growth of tumors in Tg(flk1:RFP)is18/+ retina.
Figure 2.7  Activated mTOR signaling marker phosphorylated-S6 in adult zebrafish wild type retina, Tg(flk1:RFP)is18/+ dysplastic retina and retinal tumor. a, b phospho-S6 (green) is detected in putative horizontal cells (small arrowheads), amacrine and/or Müller glia (large arrowheads) and retinal ganglion cells and/or displaced amacrine cells (small arrows) in the mature region of the retina. Nuclei are labeled with DAPI. Asterisk marks a blood vessel. c-g In Tg(flk1:RFP)is18/+ dysplastic retina phospho-S6 labeling is present in a
subset of cells throughout regions of the retina with increased cell number and disorganized retinal layers (c-f). Intense labeling of phospho-S6 was present in the ganglion cell and nerve fiber layers (c, d arrows). Large lesions contained phospho-S6 positive cells distributed throughout the tumor mass (d bracket, g). h Percentage of phospho-S6 cells/total cells in wild type retina and Tg(flk1:RFP)is18/+ tumor. +/- 11.4% ± 0.9 vs. Tg(flk1:RFP)is18/+ 13.9% ± 1.7, p=0.2291. n=9 sections, three sections each from three individuals of each genotype. gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; pe, pigmented epithelium. Scale bars, a, g, f 50 μm; b, c 20 μm; d, e 100 μm; h 25 μm.

Discussion

In this study we examined the onset of ectopic proliferation in the retina of adult Tg(flk1:RFP)is18/+ zebrafish predisposed to non-malignant retinal tumors and analyzed activation of signaling pathways that correlate with retinal dysplasia and tumor growth. A major finding from our time course analysis of proliferation is that multiple cell populations proliferate in the early dysplastic Tg(flk1:RFP)is18/+ retina, including cells in the pigmented epithelium, ganglion cell layer, and inner nuclear layer, as well as at the ciliary marginal zone. We also observed aberrant cell migration across the inner nuclear layer in the very early dysplastic retina, which could contribute to dysplasia and tumor growth. These cells may originate from angiogenic sprouting of blood vessels in the nerve fiber layer in response to release of VEGF by inner nuclear layer neurons. Together, our data indicate cells outside of the zebrafish retina ciliary marginal zone, in addition to Müller glia-derived progenitors, have the potential to proliferate.

A second major finding of our study is that expression of VEGFA and Leptin is significantly increased in early dysplastic retina, before formation of large lesions or tumors. Within the dysplastic retina neural and glial cell populations appear to respond differently, with vegfab expression upregulated in neurons, and lepb expression
overlapping with the glial marker apoeb. How this contributes to dysplasia in the retina, and separating and evaluating the distinct roles VEGF plays in neurogenesis directly vs. indirectly through enhanced angiogenesis, will require additional investigation. Leptin has previously been reported to function in Müller glia reprogramming (Zhao et al., 2014), and our results showing increased expression of lepb in Tg(flk1:RFP)is18/+ retinal glia support a role for leptin signaling in glial-derived progenitor proliferation. Recent studies have identified a tissue regeneration enhancer element in the zebrafish lepb promoter that mediates high levels of leptin expression during fin and heart regeneration (Kang et al., 2016), suggesting the elevated expression of lepb in Tg(flk1:RFP)is18/+ retina may simply be due to enhancer activation. It is well documented that Hif1-α activates the VEGF and Leptin promoters (Cho et al., 2015; Grosfeld et al., 2002), consistent with elevation of the Hif1-α pathway in the dysplastic retina transcriptome. However, it is equally likely that additional upstream triggers may activate VEGF and Leptin expression in the Tg(flk1:RFP)is18/+ retina, leading to proliferation and dysplasia.

Our study also provides evidence that in wild type adult retina mTOR signaling is active in numerous cells in the inner nuclear layer and retinal ganglion cell layer, which has not previously been reported in zebrafish. mTOR activation was detected in putative horizontal cells, amacrine cells and retinal ganglion cells but was absent from the ciliary marginal zone. In Tg(flk1:RFP)is18/+ retina phospho-S6 positive cells were present in dysplastic regions with high cellularity and large tumor lesions. Compared to VEGF and Leptin signaling, mTOR activation may not be a factor in initiating inappropriate proliferation in the early dysplastic retina, but could contribute to the sustained growth of
retinal tumors. mTOR has been shown to be required for proliferation of Müller glia progenitors in response to injury in chick retina (Zelinka et al., 2016). While multiple signaling pathways most likely drive proliferation and tumor growth in the Tg(flk1:RFP)is18/+ model, our data is consistent with our previous analyses that suggest mTOR signaling could influence activation of Müller glia regeneration pathways in the Tg(flk1:RFP)is18/+ retina.

In summary, differential gene expression and cytological analyses of retinal dysplasia in adult Tg(flk1:RFP)is18/+ zebrafish predisposed to optic pathway tumors reveals ectopic proliferation is associated with elevated expression of markers in the VEGF, Leptin and mTOR pathways. The causative molecular mechanism inducing retinal dysplasia and tumor formation in the Tg(flk1:RFP)is18 line is not known. Our previous molecular characterization of the Tg(flk1:RFP)is18 line revealed a 500-copy number <flk1:RFP> transgene inserted into a lncRNA. Isolation of a 147kb deletion allele that removed the majority of the lncRNA gene did not cause tumor formation in heterozygous or homozygous adults (Solin et al., 2014). It is possible that the nature of this particular high copy number transgene is linked to deregulation of multiple signaling pathways, and this underlies proliferation and dysplasia. One possibility is that hundreds of copies of the flk1 promoter alters global gene expression, or a subset of vascular genes including VEGF. Elevated levels of VEGF can cause vascular permeability and leakage (Lange et al., 2016), which would disrupt exchange of nutrients and metabolic waste. Alternatively, high levels of membrane targeted RFP expressed from the transgene in vascular endothelial cells may compromise vascular integrity, leading to defective retina function and metabolism. One of the most highly overexpressed genes in the
Tg(flk1:RFP)is18 dysplastic retina transcriptome is homologous to Plasmalemma Vesicle Associated Protein (plvap), a marker of vascular permeability. This supports the idea that defective vasculature and metabolic recycling could contribute to persistent stimulation of neural progenitor proliferation, leading to retinal dysplasia and non-malignant tumor formation. Determining whether similar mechanisms regulate stem and progenitor cells throughout the nervous system will be important for a better understanding of CNS injury and repair.

**Materials and Methods**

**Zebrafish**

The Tg(flk1:RFP)is18 transgenic zebrafish line predisposed to optic pathway tumors was previously described (Solin et al., 2014). Zebrafish were housed in an Aquatic Habitat system (Aquatic Ecosystems, Inc., Apopka, FL) and maintained on a 14-hr light/dark cycle at 27°C. Transgenic fish predisposed to tumor formation were raised side by side with non-transgenic siblings. Heterozygous and homozygous transgenic fish and sibling fish were monitored daily during routine feeding for viability and morbidity, and monitored bi-weekly for gross presentation of ocular tumors. Juvenile and adult fish were anesthetized and euthanized in MS-222 Tricaine Methanesulfonate according to experimental protocols approved by the Iowa State University Institutional Animal Care and Use Committee (Log # 11-06-6252-I) in compliance with the American Veterinary Medical Association and the National Institutes of Health guidelines for the humane use of laboratory animals in research. Adult fish were anesthetized and euthanized in MS-222 Tricaine Methanesulfonate prior to sacrifice and tissue dissection for RNA isolation, histopathology and immunolabeling.
Histopathology and Immunocytochemistry

One month old intact juvenile fish or whole heads dissected from adult zebrafish were fixed in Davidson’s fixative (2:3:1:3 Formalin:Ethanol:Glacial Acetic Acid:Water) for 16 hr at 4°C, decalcified in Cal-Ex (Fisher) for 2 days at 4°C, and processed and embedded in paraffin blocks at the Clinical Histopathology Laboratory at Iowa State University. Paraffin blocks were serial sectioned at 6 µm on a Shandon Finesse 325 microtome. Slides were stained with Hematoxylin 7211 Richard-Allan Scientific (Fisher) and 3% Eosin Y (Argos Organics), and mounted in Permount (Fisher). To aid antigen retrieval for PCNA labeling, slides with sectioned tissue were pretreated with 10 mM Sodium Citrate. Mouse monoclonal anti-PCNA (Sigma P8825) was used at 1:1000; rabbit anti-Sox2 polyclonal (Sigma-Aldrich/Millipore AB5603) was used at 1:200; rabbit anti-phospho-S6 ribosomal protein (Ser235/236) (Cell Signaling 2211) was used at 1:300; Alexa-488 and Alexa-594 conjugated secondary antibodies (Invitrogen) were used at a dilution of 1:500. Antibody-labeled slides were stained with DAPI and mounted in Fluorogel (EMS). Hematoxylin and Eosin stained slides were imaged on a Zeiss Axioskop II using a Nikon Rebel camera. Immunofluorescence was imaged on a Zeiss LSM700 laser scanning confocal microscope. Images of histological staining and antibody labeling from the same fish were captured from tissue sections separated by ~100 µm.

Quantification and Statistics

For analysis of proliferation in early dysplastic regions of the retina, sections of retinal tissue adjacent to a section stained with H&E (Figure 2) were labeled with PCNA and Sox2 antibodies. The total number of PCNA and Sox2 labeled cells was counted in
three retinal sections each from three wild type and three Tg(flk1:RFP)is18/+ age matched siblings (18 sections total). The total number of positively labeled cells was counted separately in two regions of the retina: at the ciliary marginal zone and in a 300 μm length of the inner retina. To quantify phospho-S6 labeling, three individual wild type and three individual Tg(flk1:RFP)is18/+ retinal tumors were examined. From each individual, three serial sections were imaged at 63X for a total of 18 images. The total number of DAPI positive cells and phospho-S6 positive cells were counted in a 100μm X 100μm region of wild type retina and Tg(flk1:RFP)is18/+ tumor tissue in each section and used to calculate the percentage of phospho-S6 positive cells. Statistical comparison of quantification in wild type and Tg(flk1:RFP)is18/+ was performed using student’s t-test in GraphPad Prism software.

**in situ Hybridization**

cDNA was amplified by RT-PCR out of total RNA isolated from wild type 5 dpf embryos and was cloned into the pCR4-TOPO vector (Invitrogen). Primers for amplification were as follows: *apo*e forward 5’ TAGCGGCCGCGAATTCGCCC 3’, *apo*e reverse 5’ TAGTCCTGCAGGTTTAAACGA 3’; *lep*b forward 5’ TGCTTGTTAATATCATCCCTGGT 3’, *lep*b reverse 5’ GAGAATGAATGTCTCAGCCACA 3’; *lepr* forward 5’ CGCTGTAAAGACGTGAACGA 3’, *lepr* reverse 5’ TCTGCCTGAAGTCCATTCCT 3’; *flk1* forward 5’ AAGTGGCTAAAGGCATGGAGTTC 3’, *flk1* reverse 5’ GACACTCCATCTCCGAGTCAAGG 3’; *vegfab* forward 5’ CGCGTGCTCCAGTTATTTATTGTG 3’, *vegfab* reverse 5’ CACCTCTTGGTTTGTCACTCTG 3’; *vegfaa* forward 5’
TGATACAGTTATTCTCGCGGCTC 3’, *vegfaa* reverse 5’

TTTGCAGGAGCATTTACAGGTGAG 3’. Digoxigenin-labeled probes for *in situ* hybridization were synthesized using a DIG RNA labeling mix (Roche #11277073910) and T3 RNA polymerase (Roche #11031163001) and stored in 50% formamide at −20°C. Adult zebrafish tissues were dissected, fixed in 4% paraformaldehyde, and embedded in OCT medium. *in situ* hybridization on 12–16 µm cryosections of head and eye tissue was performed as described (Trimarchi et al., 2007). Tissues were photographed on a Zeiss Axioskop II microscope using a Nikon Rebel camera.

**RNA-Seq and Transcriptomic analyses**

The transcriptome of retinal and tumor tissue from age-matched wild type sibling and *Tg(flk1:RFP)is18/+* 6-month-old adults was reported previously (Solin et al., 2014) and the data deposited at the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2886. Briefly, total RNA from dissected retinas or tumor tissue from age matched 6-month-old sibling fish was isolated using an RNeasy RNA Isolation Kit (Qiagen). Three dissected wild type sibling retina, heterozygous *Tg(flk1:RFP)is18/+* dysplastic retina, and heterozygous *Tg(flk1:RFP)is18/+* retinal tumor tissue were pooled and used for total RNA isolation. *Tg(flk1:RFP)is18/+* retinas were classified as dysplastic or tumor based on the gross appearance of the tissue. Dissected *Tg(flk1:RFP)is18/+* retinas that were thicker than wild type but did not have obvious lesions were used for the dysplastic retina sample. Dissected *Tg(flk1:RFP)is18/+* eyes that contained large masses filling the vitreous were used for the retinal tumor tissue sample. A single RNA-Seq library for 100bp paired end sequencing was prepared for each sample and the three libraries sequenced at the Genome Sequencing and Analysis
Core Resource, Duke Institute for Genome Sciences and Policy, Duke University. Wild type, dysplastic retina, and retinal tumor RNA-Seq libraries contained 436,511,378, 444,838,528, and 422,324,454 reads, respectively. As previously described (Solin et al., 2014), sequences were mapped to the zebrafish reference genome v9 using GSNAP, counted with HTSeq-count, upper quartile normalization applied, and the Fisher’s exact test used to determine differential gene expression. Q-value estimation of false discovery rate was performed in R using the open source software qvalue (http://bioconductor.org/biocLite.R). In the present study genes with at least 10 read counts were used for downstream analyses. Gene ontology GO Term (Boyle et al., 2004) analyses were done using the Princeton GO Term Finder website (http://go.princeton.edu/cgi-bin/GOTermFinder). Cluster frequency was calculated as the percentage of genes from the data set significantly associated with a particular term, while genome frequency (or background frequency) was calculated as the percentage of all genes in the Danio rerio genome associated with a particular term. p-value cutoffs were set at 0.01. p-values were Bonferroni corrected with an estimated false discovery rate of less than 0.01.

Transcriptome data were analyzed with QIAGEN’s Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) using genes having a read count of at least 10 in all samples and a three-fold change in expression between wild type retina and Tg(flk1:RFP)is18/+ dysplastic retina or retinal tumor samples. Ensembl gene IDs for human homologs were extracted using Biomart (Smedley et al., 2015) at (http://useast.ensembl.org/biomart/martview/da30878652120cac4e352ddac342a16e).
Availability of supporting data

Raw transcriptome dataset supporting the conclusions of this article is available in the ArrayExpress repository (E-MTAB-2886 https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-2886/). An excel file containing normalized read counts for all genes was previously published in Solin et al., 2014, Supplemental Table S7 (Solin et al., 2014).

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## Supplemental Tables

### Supplementary Table S2.1  Princeton Go Term Analysis and Annotated Genes in each Term Category Downregulated in Tg(flk1:RFP)is18

<table>
<thead>
<tr>
<th>Gene Ontology term</th>
<th>Cluster frequency</th>
<th>Gene term frequency</th>
<th>Correction D P value</th>
<th>FDR</th>
<th>P-value</th>
<th>Genes annotated in the term</th>
</tr>
</thead>
<tbody>
<tr>
<td>cellular repulsion</td>
<td>32 of 6358 genes, 5.0%</td>
<td>43 of 22445 genes, 0.2%</td>
<td>0.0007</td>
<td>0.0000</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>energy derivation by oxidation of organic compounds</td>
<td>39 of 6358 genes, 0.5%</td>
<td>61 of 22445 genes, 0.0%</td>
<td>4.0055</td>
<td>0.0000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ATP metabolic process</td>
<td>43 of 6358 genes, 0.7%</td>
<td>71 of 22445 genes, 0.3%</td>
<td>0.0125</td>
<td>0.0000</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>gene molecule de novo phosphate metabolic process</td>
<td>50 of 6358 genes, 0.8%</td>
<td>93 of 22445 genes, 0.0%</td>
<td>0.0003</td>
<td>0.0000</td>
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<tr>
<td>gene molecule de novo phosphate metabolic process</td>
<td>50 of 6358 genes, 0.8%</td>
<td>93 of 22445 genes, 0.0%</td>
<td>0.0004</td>
<td>0.0000</td>
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<tr>
<td>Golgi vacuole transport</td>
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<tr>
<td>generation of precursor metabolites and energy</td>
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<td>electron transport chain</td>
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<tr>
<td>gene molecule de novo biosynthetic process</td>
<td>38 of 6358 genes, 0.6%</td>
<td>63 of 22445 genes, 0.0%</td>
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Supplementary Table S2.2  Princeton Go Term Analysis and Annotated Genes in each Term Category Upregulated in \( Tg(flk1:RFP)is18 \) dysplastic retina.

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<tr>
<th>Gene Ontology term</th>
<th>Cluster frequency</th>
<th>Genome frequency</th>
<th>Corrected P-value</th>
<th>FDR</th>
<th>FALSE Positives</th>
<th>Genes annotated to the term</th>
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<td>translation</td>
<td>162 of 6701 genes, 2.4%</td>
<td>311 of 22409 genes, 1.4%</td>
<td>4.50E-13</td>
<td>0.00%</td>
<td>0</td>
<td>eif3i, tceu1, pl12, pdc511, eef11b2, efl1, rps15a, RPS11, mns6, eif3e, epp, denr, eif4ebp2, eef1d, pl10, farsa, sars, pl19, eif3b, RPS6, pl22, pl118, pl27, pl17, rps15, tmt6, sars, sars, rps4, iars, pl23a, larsa, rps26, eif4a3, eef1g, tca3, pl23, eif3c, pl37, eif3d, rps16, eef2a1, eif2b4, eif2x2, mnp20, pl5a, rpsa, pl14, eif5a, eif6, rpl9, plp1, eif4a1a, eif5s6p, rpl5b, rpl13a, eef1a1a, RPS20, rps3a, cars, rpl36a, rpl3, eif2d, gra, dars, mif4gdb, RPL38, lasb, eif3s10, eif4ebp1, eif2b2, eif11axa, rpl36, TNRC6A, plp2, pl18a, RPS17, rps27.1, nagoh, caprin2, rps29, mettl7, WARS, rps23, farsb, yars, rps8a, eif2ak1, rbn8a, mif4gda, rpl9, EEF3F, eif3ha, eif5a, mpl1, rpl30, rpl10a, rps26l, eif5a2, eef2b, rps7, eif3ea, rps21, EIF3K, uba52, tars, eif2ak3, p114, rps14, rps5, ticcr, hars, fana, rpl26, rps2, eif4eb, impact, pl26, rars, rps50, rpl8, eef2k, rps3, RPL31, p115, rps9, rps27a, ngda, rpl2211, kars, gars, eef1a1l1, rps5a, eif3g, mars, eif2d, rps27.2, eif1axb, rpl3, rps28, eif2b1, rps18, eil, rpl32, eif4bb, pl21, gfm2, EIF1B, eif2b3, br1a1, rps24, rps14, rps19, qki2, plp21, eif4ebp3, rpl6, pl11, rps3</td>
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<tr>
<td>ribonucleo-protein complex biogenesis</td>
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<tr>
<td>regulation of translational initiation</td>
<td>20 of 6701 genes, 0.3%</td>
<td>23 of 22409 genes, 0.1%</td>
<td>5.62E-05</td>
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</tr>
<tr>
<td>ribosome biogenesis</td>
<td>45 of 6701 genes, 0.7%</td>
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<td>9.28E-05</td>
<td>0.00%</td>
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## Supplementary Table S2.2 continued

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<th>vascular development</th>
<th>136 of 6701 genes, 2.0%</th>
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<th>0.00609</th>
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<td>vhl, figf, LAMA1, colc12, fzd6, meis1, tns1f19, NTN4, jnmj6d, frzb, rasgrp4, ephb4a, bnpir2a, femt2, alox5ap, erg, notch1b, msna, etv2, ANGPTL1, sars, vegfaa, appb, smad5, foxc1a, jak2a, mus1, fzd8a, lpar1, acvri1, foxo1b, lgals2a, foxm1, flt4, hmgcrb, d1a, ELMO1, rsf213a, sox7, amotl2a, wdr43, wnt2bb, flt1, pak2a, calcria, dll4, np2b, birc5a, ptp51, amot, sox18, ung, tbx6, itagv, lpar6a, tie1, cxc12b, gnb2ll, ctnmb1, rab13, lama4, SHC1, snk5, pdgfb, egfl7, sat1b, vegf, piprb, fzd9b, TAL1, dle, igf7p7, mb, kdrl, rps29, twsg1b, PLXND1, sno, hech, flt1a, pphb, lmx1a, tpc1, dcp1, PDGFB, paxla, pldla, eif3ka, ecer, per2, FOXJ2, utpi5, pggt1b, foxc1b, cdk5, fltax1la, unc5b, lmo2, notch3, fzd2, spry2, loxl2b, smarcall1, twsg1a, slpr1, sfpr1, ctsz, inagl1, flt1b, runx1, kdr, pak2b, angpt1, phlb2a, iga2b, ephx2, vap, fzd7b, np2a, notch2, CFI, sfpr2, rbpjb, fln2b, mcam, robos4, f5b, fnm13, ell, tp53, piprja, lef1, itgb6, gata2a, ilk, plekh1</td>
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<table>
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<tr>
<th>positive regulation of cell death</th>
<th>25 of 6701 genes, 0.4%</th>
<th>37 of 22409 genes, 0.2%</th>
<th>0.00666</th>
<th>0.00%</th>
<th>0</th>
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<tbody>
<tr>
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<td>casp9, fas, bnip4, caspa, phlda3, tradd, bbc3, bnip3lb, zgc:73226, dusp6, bmf2, tnsf1a, baxa, pmaip1, fadd, rola, casp3a, bida, tp53, caspb, rest, crib, CASP8, bnp3la, baxb</td>
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<table>
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<tr>
<th>positive regulation of apoptotic process</th>
<th>25 of 6701 genes, 0.4%</th>
<th>37 of 22409 genes, 0.2%</th>
<th>0.00666</th>
<th>0.00%</th>
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<tbody>
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<tr>
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<th>37 of 22409 genes, 0.2%</th>
<th>0.00666</th>
<th>0.00%</th>
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<tr>
<th>regulation of apoptotic process</th>
<th>85 of 6701 genes, 1.3%</th>
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<th>0.00759</th>
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<td>phlda3, m2f, tnsf10l, akt2, traf4a, rola, ldmm5e, mcl1b, sno, mpxb, sicch211-218c6.8, bc1210, bc1l2, tp73, rad21a, casp9, park7, FOXO2, zgc:158434, bbc3, bnip3lb, ddt, dusp6, foxc1b, bmf2, tnsf1a, PPM1K, cand9, foxc1a, pmaip1, fadd, casp3a, dusp1, caspb, apex1, rest, esco2, foxo1b, pyca, mcl1a, bnip4, sidkey-10c21.1, pmpr5s, tradd, baxa, spast, dusp2, egr1, HEATR1, traf3, nod2, perp, mcm5, nes, bida, apafl, crib, pak2a, snx7, pak2b, wt1b, bcor, bnp3la, birc5a, baxb, dig3a, fas, egr2a, caspa, GRINA, epars1b, zgc:73226, casp812, bic2c, bc1l21, EGER3, gbp5, ctnmb1, tp53, lefl, zgc:174906, plbg1, boka, CASP8, andvb</td>
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</table>
### Supplementary Table S2.2 continued

<table>
<thead>
<tr>
<th>Regulation of programmed cell death</th>
<th>86 of 6701 genes, 1.3%</th>
<th>188 of 22409 genes, 0.8%</th>
<th>0.00829</th>
<th>0.000%</th>
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</thead>
<tbody>
<tr>
<td>phlda3, m2f, tfusfl10, akt2, traf4a, rela, kdm5f, mecl1b, smo, mxsb, sich211-218c6.8, bc1210, bcl2.2, tp73, rad21a, casp9, park7, FOXJ2, zgc:158343, bcc3, bnp31b, ddt, dusp6, fox1b, bm2f, tfusfla, cct1, PPM1K, card9, foxcl1a, pnaip1, fadd, casp3a, dusp1, caspb, apex1, rest, escocl2, foxolb, pycard, mecl1a, bnp4, sidkey-10c21.1, pmpr33, tradd, baxa, spast, dusp2, egr1, HEATR1, traf3, nod2, perp, mcem5, nes, bclb, apafl, scrib, pak2a, sno7, pak2b, w1b, bc0r, bnp3la, binc5a, baxb, dlgl5a, fas, egr2a, caspa, GRINA, eps11b, zgc:73226, casp812, birc2, bc1211, EGR3, gbp3, ctnnb1, tp53, lefl1, zgc:174906, plrg1, boka, CASP8, aurkb</td>
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<table>
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<td>vhl, figf, LAMA1, TAL1, colc12, fd6, igfbp7, meis1, dle, mb, tfusfl9, NTN4, tps20, kdr, jund6, twsg1b, PLXND1, ephb4a, femr2, smo, alox5ap, hexb, flila, pph1, trpc1, loxl2a, erg, PDGFB, dzip1, notch1b, pld1a, msna, etv2, esocr, per2, FOXJ2, utpi5, ANGPTL1, sars, pgt1b, foxclb, vegfaa, ceh5, smad5, fbxv11a, foxcl1a, jak2a, unc5b, ilmo2, notch3, nus1, spry2, bpar, loxl2b, smacq11, acvr1l, foxolb, twsg1a, ghba2a, ctsz, foxn1, tinaq11, flt4, hmgcob, dlia, ELM01, mfs23a, flilb, runt1, son7, amot2a, wdr43, wnb28b, fl1l, pak2a, calcrla, kdr, pak2b, dli4, nrp2b, angi1, birc5a, pts1a, amot, igba2b, pchb2a, epdx2, vap, sso18, gng2, flx6, igfb, nrp2a, lspar6a, rbjpb, tie1, CFI, cxd12b, fmn2b, rob04, mncamb, ell, fmn13, ctnlb1, gnh2l1, tp53, lefl1, ptrprj, tgb8, rblb3, lam4, SHC1, sno5, gata2a, pgfibf, egfl7, sat1b, vegf, ilk, plekhh1, ptrpe</td>
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<table>
<thead>
<tr>
<th>Regulation of cell death</th>
<th>87 of 6701 genes, 1.3%</th>
<th>191 of 22409 genes, 0.9%</th>
<th>0.00902</th>
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### Supplementary Table S2.3 Princeton Go Term Analysis and Annotated Genes in each Term Category Downregulated in Tg(flk1:RFP)is18 tumor tissue.

<table>
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<tr>
<th>Gene Ontology</th>
<th>Cluster frequency</th>
<th>Genome</th>
<th>Term Category</th>
<th>Downregulated in Tg(flk1:RFP)is18 tumor tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>sensory perception of light stimulus</td>
<td>64/7295 genes, 0.8%</td>
<td>71 of 22400 genes, 0.3%</td>
<td>2.00E-04</td>
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<tr>
<td>visual perception</td>
<td>44/7295 genes, 0.6%</td>
<td>70 of 22400 genes, 0.3%</td>
<td>4.00E-04</td>
<td>0.00%</td>
</tr>
<tr>
<td>cellular respiration</td>
<td>29/7295 genes, 0.4%</td>
<td>42 of 22400 genes, 0.3%</td>
<td>3.11E-03</td>
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<tr>
<td>ion transport</td>
<td>278/7295 genes, 3.8%</td>
<td>680 of 22400 genes, 3.0%</td>
<td>3.67E-03</td>
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</table>

<table>
<thead>
<tr>
<th>Gene Ontology</th>
<th>Cluster frequency</th>
<th>Genome</th>
<th>Term Category</th>
<th>Downregulated in Tg(flk1:RFP)is18 tumor tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>monovalent inorganic ion transport</td>
<td>118/7295 genes, 1.6%</td>
<td>256 of 22400 genes, 1.1%</td>
<td>0.00772</td>
<td>0.00%</td>
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Supplementary Table S2.4  Princeton Go Term Analysis and Annotated Genes in each Term Category Upregulated in Tg(ﬂk1:RFP)is18 tumor tissue.

<table>
<thead>
<tr>
<th>Term Category</th>
<th>Gene Ontology Term</th>
<th>Number of Genes</th>
<th>Upregulated Genes</th>
<th>Corrected P-value</th>
<th>FDR</th>
<th>Genes annotated to the term</th>
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<tbody>
<tr>
<td>Ribonucleo-</td>
<td>Protein complex</td>
<td>82 of 8294 genes, 1.0%</td>
<td>107 of 22409 genes, 0.5%</td>
<td>1.99E-13</td>
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<td>eif3b, exosc6, gnl2, gnl3, rpsa8a, ga/phb4, eif3b, tes2, std1, ppp1, dcd13, wdr6, wdr65, daa11, EIF3F, eif2am, eif2ha, mdad1, rsv1, wdr12, raf241, utp15, supe, RPS6, eif6a, rps, mphosphp10, rpp21, psl1, eif5aa, col1, EIF3K, psyp2h, tbl3, noc48, dint11, rps6, psypf11, nup7, ticer, dke1, MRT04, eif3fj, icnu, eif3c, dicer1, cld2, HEA1R1, nup10, eif3d, bop1, pes, tor1, rpsa, eif9, kv1, npl30, eif6up, eif9, shag1, q1, ybe1, xbp2, tbl, nq2p3, pmp7, sar3, WBP11, rps28, rps18, snf1, xbp2p1s, gpl1, gnl3, xbp2p, eif3s10, eif5ja, bnr1, lsg1, RPS17</td>
</tr>
<tr>
<td>Translation</td>
<td>184 of 8294 genes, 2.2%</td>
<td>311 of 22409 genes, 1.4%</td>
<td>3.86E-12</td>
<td>0.00</td>
<td>0.00</td>
<td>eif3i, teac1, mps15, rpl12, pdc11b, eif1b2, rps15a, eif1, RPS11, mps6, eif3e, MPR32, case1, eprs, eif4e2p2b, dem, efr1b1, rpl10, sars, larsa, rpl19, RPS6, eif3a, rpl22, rpl18, rpl27, rpl17, rpl15, trn5, eif32e2x, nars, qars, aar, rps4, rpl23a, iars, larsa, rps4, eif3a, eif5b, mps24, eef1g, con10, rpl23, eif3c, rpl37, eif3d, rpl16, eor2a, eif2b4, eif3a2, eif4e2p3, mpr20, eif4a2p, rpsa, mtrf1, rpl4, eif3a, eif6, sepsc, rpl39, rpl9, rpl20, eif36p, eif4a1a, rpl3b, eif35, rpl13a, RPS20, rpsa3, teac1, efr1, rpl3a, eif3x, gra, efr1da, dars, tufrn, RPL38, larsb, tarbp2, rpl36, eif3b2, eif3a10, eif6eb1p, eif3ja, rpl2p, rpl18a, rps21, RPS17, EIF2A, mbrod, capsin2, rps29, EIFIAD, WARS, rps23, farsb, yars, rpsda, rqud1, eif2ak1, rofsh, gsh, mup24q, eif3f, ncb2p, eif3ha, eif3un, rplp1, rpl30, rpl10a, rps26a, eif32b, eef2b, rpl7, rps21, eif5ea, EIF3K, uba52, dars2, tars, efr3e1c, vhrs, eif2ak3, rpl14, rps15, rps14, NRF8, mrrf, ticer, mpr37, hars, fauna, rpl28, rps2, eif1eb, impact, rars, rps26, rps30, eif1f, mps10, rpl13, RPL31, rpl15, rps9, rps27a, ngdn, mpr113, kars, rpl2211, efr3a11, gars, rpl35a, eif5a, mrs, notog2, rps27, eif2f, rpl13, rpl35, rps28, eif3ea, eif3b1, rps18, rpl32, eif4b, rpl21, EIF5B, eif2b3, rps24, rps19, con6t7, mps18a, rpl34, rpl22, eif4eb3l, qks2, rpl6, rpl11, rps3</td>
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<tr>
<td>Mitotic cell</td>
<td>cycle</td>
<td>101 of 8294 genes, 1.2%</td>
<td>157 of 22409 genes, 0.7%</td>
<td>9.48E-09</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>Mitotic cell</td>
<td>cycle</td>
<td>87 of 8294 genes, 1.0%</td>
<td>131 of 22409 genes, 0.6%</td>
<td>2.30E-08</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>Regulation</td>
<td>of</td>
<td>translational initiation</td>
<td>23 of 8294 genes, 0.3%</td>
<td>23 of 22409 genes, 0.1%</td>
<td>3.52E-07</td>
<td>0.00</td>
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</table>
Supplementary Table S2.4 Continued

| ribosome biogenesis | 55 of 8294 genes, 0.7% | 74 of 22409 genes, 0.3% | 2.09E-07 | 0.000 0 | dicer1, MRT04, exosc6, gnl2, HEATR1, c1d1, glnl1, nopl10, pes, bop1, rps8a, giupb4, tbr1, tbr2, sdafl1, rpsa, dacl3, wdr36, wdr55, noll11, eif6, krr1, rplp0, mad11, rsi112, rsi24d1, rtp15, gaur1, ybev, nhg2, fln, nopl2, RPS66, eif7a, WBP1, rps7, rps27a, mhpohp10, rps21, ppsp2b, rps18a, naf1, thi3, dmtr11, nhp21b, noc4l, rps2a, fusi, gnl3, bms11, lag1, ry36, npii, RPS17 |
| cell division | 122 of 8294 genes, 1.5% | 208 of 22409 genes, 0.9% | 5.02E-07 | 0.000 0 | mastl, ccb2b, prelb, prela, pgfhasa, ndc80, gnl2, cdc73, snx33, cdc8a, dixl8, cdc26, tpr2, mad111, pafal1b1b, smc2, fam83d, cdeo40, actr4, ercc4, zwilch, pafalha1a, ANAPC4, anaph, bora, helix, snap25a2, katan1, we1, nrd2a1, CCNA2, mana, mcmdb, nekl8, conf, cdeo124, zgc65674a, ncapd1, ced11, vegfasa, skca3, msc18a, TXNL4B, mcp13, SETDB2, musap1, kif23, dtn2, notch1, cdeo2, cep63, sam6, khb21, zgc56231, cdeo51, pgfhasb, ncapg, chrh, tbs4b, ced1, ueb212, snap10, RNFSF, vegFb6b, tik, tbd4b, cdeo16, tex15, smc3, cdeo45, ffox5b, mad211, mceptr1a, CDC37, fgg3, wec2, esp, KIF20a, top2b, haus6, sehl1, settha8a, apo2a, tex4a, dsc1, ect2, spe2c, peleo, cdeo14b, arf8aa, h2afs2, rec2, ueb21, ced1k, mnd1, nns1, uap37, ccb1b, skiv2d2, sak1, pns1, tipin, cod20, cod2da, cehb3, cenpa, gln211, p53, RBBP8, swap70b, ncapd2, fdi1, ocd27, gnl3, zw10, ercco8, vegf6b, mst3, aurkb, top2a |
| mitotic nuclear division | 62 of 8294 genes, 0.7% | 88 of 22409 genes, 0.4% | 5.60E-07 | 0.000 0 | mastl, scl2, mad211, ffox5, nde80, gnl2, wec2, snx33, haus6, cdc8a, seh11, cdc26, tpr2, mad111, set78a, pafal1b1b, smc2, actr4, fam83d, cdeo44a, dsc1, zwilch, pafalha1a, ANAPC4, anaph, bora, spe2c, arf8aa, katan1, rec2, we1, rad21a, ube2i, uap37, memdb, nekl8, conp, ncapd3, sk3a, tipin, skca3, snx18a, TXNL4B, SETDB2, musap1, cenpa, dtn2, cep63. RBBP8, Khb21, swap70b, ncapd2, fdi1, chrh, ncapg, gnl3, zw10, ube212, ercco8, set7ap10, mfs2, aurkb |
| translational initiation | 46 of 8294 genes, 0.6% | 60 of 22409 genes, 0.3% | 1.30E-06 | 0.000 0 | tcc1r, eif2a, EIF2A, eif3b, eif3b, eif3c, EIF1AD, eif2d, eif2e2, eif2g2, eif2eg3, eif4a1, eif4a2, eif4eb2, eif4e1, eif4f3, dnr2, eif3g, eif3h, eif3i, eif3l, eif3k, eif3l, eif3m, eif3n, eif3o, eif3p, EIF3K, eif2b1, eif2c2, gsa, cfl4, EIF1B, ECF4L1, trns6, eif2kb6, eif2kb6, eif3s10, eif3a, eif3gb11 |
| cell cycle process | 125 of 8294 genes, 1.5% | 218 of 22409 genes, 1.0% | 2.26E-06 | 0.000 0 | mastl, prelb1, fn115, prela1, nde80, gnl2, snx33, cep72, cdeo8a, cdeo26, tpt2, mad111, pafalha1b1b, smc2, fam83d, actr8, ercco4, zwilch, pafalha1a, ANAPC4, anaph, cdkln1a, bora, helix, ip73p, snap25a2, katan1, napl1a, fdm13b1b, wec2, rad21a, memdb, cdeo245, nhg2, conp, zgc56764, ncapd3, cond1, skca3, pkld4, snx18a, TXNL4B, stil, mtp13, SETDB2, musap1, chek1, bre, kif23, dtm2, pkcb1, cep63, fdi1, snx33, khb21, zgc56231, stk35, vps24, cenpa, chrh, sem2, vang21, ube2a, snap10, rkc, tcc1r, tex15, uap33, ncapg, cdkln1a, fmn11, ffox5, mad211, CKDN1C, fam175a, haus3, insm1a, sem2, cemph, wec2, KIF20A, top2b, haus6, int3, seh11, ppp2a, atm, set7a8a, pold1, mndm2, tex4a4, dsc1, ecc2, haus1, spe2c, arf8aa, rec2, ube2i, uap37, HUA54, skak, pns1, tipin, mcts2a, idm8b, haus5, cenb1, cenpa, cmpoa, p53, RBBP8, nd1, swap70b, ncapd2, fdi1, war11, gnl3, zw10, ercco8, mfs2, rhoab, aurkb, top2a |
| ribonucleo-protein complex assembly | 32 of 8294 genes, 0.4% | 39 of 22409 genes, 0.2% | 3.13E-05 | 0.000 0 | tcc1r, eif3b, eif3j, icnh1, dicen1, eif3e, eif3d6, eif6b, rps8a, EISF3F, eif6, eif6a, eif6m, eif6g, eif6s3, shq1, surp, eif6a3a, pm7, sart3, eif6a1, pab1, col, EIF3K, pps2b, eif3o10, tarb2, eif3a, nip7, ppr62, RPS17 |
| nuclear division | 77 of 8294 genes, 0.9% | 125 of 22409 genes, 0.6% | 6.02E-05 | 0.000 0 | mastl, nde80, gnl2, snx33, cdeo8a, cdeo26, tpt2, mad111, pafalha1b1b, smc2, fam83d, actr8, ercco4, zwilch, pafalha1a, ANAPC4, anaph, bora, helix, katan1, we1, rad21a, memdb, nekl8, conp, ncapd3, sk3a, snx33, TXNL4B, stil, mtp13, SETDB2, musap1, dtn2, cep63, sam6, khb21, ncapg, chrh, ueb2i, uap37, rkc, tex15, cdeo124, mad211, set7a8a, apo2a, tex4a4, dsc1, spe2c, arf8aa, h2afs2, rec2, ube2i, mns1, mnd1, uap37, pns1, sk1, tipin, cenpa, cmpoa, RBBP8, ncapd2, swap70b, fdi1, gnl3, zw10, ercco8, mfs2, aurkb, top2a |
Supplementary Table S2.4 Continued

<table>
<thead>
<tr>
<th>Process</th>
<th>Genes Affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle</td>
<td>206 of 8294 genes, 2.5%</td>
</tr>
<tr>
<td>DNA replication</td>
<td>61 of 8294 genes, 0.7%</td>
</tr>
<tr>
<td>RNA metabolic process</td>
<td>36 of 8294 genes, 0.4%</td>
</tr>
<tr>
<td>Organelle fission</td>
<td>81 of 8294 genes, 1.0%</td>
</tr>
<tr>
<td>RNA processing</td>
<td>34 of 8294 genes, 0.4%</td>
</tr>
<tr>
<td>Cellular response to DNA damage stimulus</td>
<td>114 of 8294 genes, 1.4%</td>
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</table>

Notes: MASTL, prel1b, nde80, cep72, cdc26, mad11, rps15a, pafah1b1b, fam58d, ercc4, hin54, cne2, cdkn1b1a, snp25a, katan1a, hm:nh20351, npabla1, cdkn1blb, weel1, CCNA2, mecs, mcmbp, rb4, smarcba1a, cdc124, zgc:86764, sk3d, plk4e, str18a, TXN4LB, int37, tripl3, sepol8a, nsupa1, cnu, chk1cl1, pdepi, cpep3, bl3, esoc2, chfr, sesna2, gadd45g, cens1, ubc322, anapc10, gadd45ab, p132a, tkl, sep5a, tdo29, usp22, smc3, foxn1, cdc45, fltx05, mre11a, insunla, fam58a, CDC37, ecmph, rb1, mcum5, int3s, seh11, prkeda, ppp24i, gadd45a, gadd45gip1, ec22, arf88a, rec2, mndl, usp37, tsp33p2, bl11, antx2a, havs5, ccs2a, sepol5b, ecmph, rpl36a, swap70b, chaf1b, wnt11, cdc7, zw10, p35e, muf2, zgc:53587, rhob, bapl, top2a, plk1, cehn2, flm115, muf2, gna2, pprea1, cdc7, smx33, rps29, eceas8, rps2, smc2, rps4a, actf6, cdo40, zwoch, pl7, ANAPC4, pafah1b1a, cdc42l, bora, gadd45l, hdl3, ypl1, rad21a, cdc25, cks2, cnef, nck8, nacpd3, ccd11, still, SETDB2, rps7, aspm, bre, serpla, src, try23, det2, cdc42, chaf1a, mbl22, sas6, khl21, zgc:50321, sid:koey-283B15.3, cdc5, tftp1a, cdc5p, dfl, nacpg, smacal1, vang2, atoh7, conk, tier, cdc16, ten15, usp33, cdm14a, mad21, fam75a, CDRK1C, havs5, gadd45fob, sesn1, wce2, spad, KIF20A, top2b, hau1, atm, meft1, set8a, bop1, psol2, pnd1, ndex1, dtx4a, disce1, hau1, sgc24, pelo, cdc14b, h2af5, ube24, cdk1, mms1, bop1, bop2, ccdn1, has4, skat1, psm1, tipl, ripl13, rps5, kmn8, cdc20, ccb3, rps18, tampon, tsp5, RBBP8, vep, rad1, nacpd42, fsl1, gulf3, ercefl, xks1b, unkkb
### Supplementary Table S2.4 Continued

<table>
<thead>
<tr>
<th>ribonuclease-protein complex subunit</th>
<th>33 of 829 genes, 0.4%</th>
<th>45 of 22409 genes, 0.2%</th>
<th>0.00237</th>
<th>0.000%</th>
<th>0</th>
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</thead>
<tbody>
<tr>
<td>DNA metabolic process</td>
<td>181 of 829 genes, 2.2%</td>
<td>367 of 22409 genes, 1.6%</td>
<td>0.0025</td>
<td>0.000%</td>
<td>0</td>
</tr>
</tbody>
</table>

| response to stress                  | 209 of 829 genes, 3.6% | 651 of 22409 genes, 2.9% | 0.0043 | 0.000% | 0 |

- tcri2, etf1d, etfi3b, ich, dicer1, eti3e, eti6d, eti6b, prp8p, rpa1, eIF3F, eIF6, eIF3B, eIF2m, eIF2g, eIF3E, slt1, surp, eIF3A, prmt7, surt3, eti3a, paf1d, coel, EFS3K, det2, pyp22b, ef51t, 30a, tby2, eti3a, npi7, ppr31, RPS17
- zgc110216, tdp2b, zgc194265, RFCA, sbpl1, ncm7, rml1, dsd2, inip, fazco, zgc77816, rad18, smcg1, rcm, cepl, slh1, dp65, nmse4a, bra2a, naplh1a, dmnt4, mgmt, mcm6b, rb4, smarcb1a, rfb4, pole, xrc2c, tripc13, zgc110277, ppb2pb2p, cmnp, uve2nb2, gfl2b1h, gmnn, apex1, scep2, psb1, plb, dnmnt3b, hfl5, xrc4, xmc1a6, gins2, tdrb6, teri, terfa, dnas2e, cepl45, zgc64675, psm1, nme11a, zgc110224, ncm23, fen1, int59, ORC4, seth, tdp1, rfc2, prmt1, xrc3e, pole2, smc5, hel5, TDO, mxd1j, tk1, MCM3, rad50, qag5, pola1, plcb2, nud1l1, hdfs5, apts, polb, top1, dnas2e123, rad28aa, hist2h21, top2a, dmnt1, rad51, actr5, mcm10, TOP1MT, DX1X1, pstock, GNS9, RM2e, smc2, actr8b, gfl2b2, rad52, INO80E, top6a, rnh1l, hfl5, xrc3e, narpb, oth1l, mre11, lew1d, rad51p1, uve2e, orel5, histh1, nacp3d, gins1, LIG3, napl1pa4, prmt5b, bre, cry4, rad5fd1, chaf1a, chdh2b, rigfb22b, WRNIP1, dt, nacp, ore1c, smarca1, gfl2b2h, mem2, mem6, rvod2, RNFS8, ore6b, zgc112406, tcri, pold3, tex15, smc4, ctb1, fam175a, top2b, dnas1p1, ore1c, gfl2b3h, atm, pold1, hlp1p3, POLH, ercc3, dec3c, setsl, pola2, zgc17159, H2AFY2, hflas5, rad2sb, bccip, rfc3, mem4, KIF22, nacp2l2, jmz, pcoa, nasp1, tipin, prmt7, dff1b, ogg1, pold2l, pot1, RBBP8, vep, rad1, lonp1, nacp2d2, npi11l, obfc1, setsa, wtm1, msnb1, pcs2
Supplementary Table S2.4 Continued

<table>
<thead>
<tr>
<th>Cellular component organization or biogenesis</th>
<th>603 of 8294 genes, 7.3%</th>
<th>1400 of 22409 genes, 6.2%</th>
<th>0.00267</th>
<th>0.000%</th>
<th>0</th>
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<td>nmem3, brd1, acelb, dna3, smac4f, ndc80, fts6, sclarb2, cys13, bcl1, bcl2, ercc5, csmo4-a, csepa, beca2, nup1, cslg, park7, smarch1a, cse5, tripl3, chch1, ube2n, gtfl2, cep53, apex1, pif1, polb, PIDD, mda1, xec4, smca1, foth1, vps74, mcl1, tric1, tripl5, cse2, cse5, met15, cat, TDX, smca2, dtl2, CEN, cni1, rap80, pol1, car4, tce, WIP1, mdr1, apc1, pola, plo2, mutd1, rad23a, rad51, atm, pold, POLH, ercc3, dixela, setz, h2af, rad23b, baxb, bccp, wdr45, KIF22, jmy, pen1, pm1, tip1, hbd1, rytb, oog1, RBBP8, yps3, vcp, td1, lon1, nup1, cdc94, wu, mms8, pm12</td>
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<table>
<thead>
<tr>
<th>Cellular response to stress</th>
<th>143 of 8294 genes, 1.7%</th>
<th>280 of 22409 genes, 1.2%</th>
<th>0.00297</th>
<th>0.000%</th>
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<td>wdr451, sde2, mad1, dut3, phk2, dna2, mirp, fav1, rad18, mdb3, strm3, ercc4, xrl1, del1, bcl210, prp19, neil1, bcl2, ercc5, nmoa4-a, csepa, beca2, nup1, csgl, park7, smarch1a, cse5, tripl3, chch1, ube2n, gtfl2, cep53, apex1, pif1, polb, PIDD, mda1, xec4, smca1, foth1, vps74, mcl1, tric1, tripl5, cse2, cse5, met15, cat, TDX, smca2, dtl2, CEN, cni1, rap80, pol1, car4, tce, WIP1, mdr1, apc1, pola, plo2, mutd1, rad23a, rad51, atm, pold, POLH, ercc3, dixela, setz, h2af, rad23b, baxb, bccp, wdr45, KIF22, jmy, pen1, pm1, tip1, hbd1, rytb, oog1, RBBP8, yps3, vcp, td1, lon1, nup1, cdc94, wu, mms8, pm12</td>
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<table>
<thead>
<tr>
<th>Formation of translation initiation complex</th>
<th>16 of 8294 genes, 0.2%</th>
<th>17 of 22409 genes, 0.1%</th>
<th>0.00414</th>
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Supplementary Table S2.5 Ingenuity Pathway Analysis Canonical Pathways in *Tg(flk1:RFP)*is18 dysplastic retina transcriptome.

<table>
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<tr>
<th>Ingenuity Canonical Pathways</th>
<th>log p-value</th>
<th>p-Ratio</th>
<th>z-score</th>
<th>Down-regulated</th>
<th>Up-regulated</th>
<th>No overlap</th>
<th>Molecules</th>
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<tr>
<td>Cavilcular-mediated Endocytosis Signaling</td>
<td>2.09E-06</td>
<td>2.11E-01</td>
<td>N/A</td>
<td>17/71 (2%)</td>
<td>0/71 (0%)</td>
<td>14/71 (20%)</td>
<td>56/71 (79%)</td>
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<tr>
<td>EIF2 Signaling</td>
<td>2.35E-01</td>
<td>4.08E-01</td>
<td>6.172</td>
<td>1/184 (0%)</td>
<td>0/184 (0%)</td>
<td>0/184 (0%)</td>
<td>0/184 (0%)</td>
</tr>
<tr>
<td>Endothelin-1 Signaling</td>
<td>4.87E-06</td>
<td>2.21E-01</td>
<td>2.694</td>
<td>11/172 (6%)</td>
<td>2/172 (1%)</td>
<td>27/172 (16%)</td>
<td>134/172 (78%)</td>
</tr>
<tr>
<td>GADD45 Signaling</td>
<td>1.33E-00</td>
<td>2.60E-01</td>
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<td>0/10 (0%)</td>
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<td>0/10 (0%)</td>
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<tr>
<td>Glial Inflammation Signaling</td>
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<td>1.792</td>
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<td>0/57 (0%)</td>
<td>0/57 (0%)</td>
</tr>
<tr>
<td>HIP1 Signaling</td>
<td>1.61E-00</td>
<td>1.76E-01</td>
<td>N/A</td>
<td>3/102 (3%)</td>
<td>0/102 (0%)</td>
<td>15/102 (15%)</td>
<td>84/102 (82%)</td>
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<tr>
<td>mTOR Signaling</td>
<td>7.64E-06</td>
<td>2.51E-01</td>
<td>2.840</td>
<td>6/187 (3%)</td>
<td>0/187 (0%)</td>
<td>41/187 (22%)</td>
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<td>5.47E-01</td>
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<td>0/53 (0%)</td>
<td>10/53 (19%)</td>
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<tr>
<td>VEGF Signaling</td>
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<td>1.74E-01</td>
<td>1.387</td>
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<td>0/92 (0%)</td>
<td>13/92 (14%)</td>
<td>76/92 (83%)</td>
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CHAPTER 3. EPIGENETIC MECHANISMS CONTROL ZEBRAFISH NEURAL DEVELOPMENT AND RB-DEFECTIVE BRAIN TUMOR ONCOGENESIS

Modified from a paper under review at the journal Disease Models and Mechanisms

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* L.E.S. aided in tumor transcriptome library preparation, generated rb1 Δ7/Δ7 embryonic transcriptome library, performed immunohistochemistry on rb1 Δ7/Δ7 larvae, isolated and performed immunohistochemistry on rbbp4 CRISPR-targeted and rbbp4 Δ4/Δ4 larvae, contributed to figures 2, 5, and 6, and aided in manuscript preparation and editing.

Abstract

In this study, we use comparative genomics and developmental genetics to identify epigenetic regulators driving oncogenesis in a zebrafish retinoblastoma1 (rb1) somatic-targeting model of RB-defective brain tumors. Zebrafish rb1-brain tumors caused by TALEN or CRISPR targeting are histologically similar to human central nervous system primitive neuroectodermal tumors (CNS-PNET). Like the human oligoneural OLIG2+/SOX10+-CNS-PNET subtype, zebrafish rb1-tumors show elevated expression of neural progenitor transcription factors olig2, sox10, sox8, and the receptor tyrosine kinase erbb3a oncogene. Comparison of rb1-tumor and rb1/rb1 germline mutant larval transcriptomes shows the altered oligoneural precursor signature is specific to tumor tissue. Elevated expression of histone modifier histone deacetylase1 (hdac1) was also unique to tumor cells, while chromatin adaptor retinoblastoma binding protein4 (rbbp4)
was upregulated in both \textit{rb1}-tumor and \textit{rb1/rb1} mutant. Analysis of germline mutants confirms zebrafish \textit{rb1}, \textit{rbbp4} and \textit{hdac1} are required during brain development. \textit{rb1} is necessary for neural precursor cell cycle exit and terminal differentiation, \textit{rbbp4} is required for survival of post-mitotic precursors, and \textit{hdac1} maintains proliferation of the neural stem cell/progenitor pool. We present an \textit{in vivo} assay using somatic CRISPR-targeting plus live imaging of histone-H2A.F/Z-GFP in developing larval brain to rapidly test the role of chromatin remodelers in neural stem and progenitor cells. Our somatic assay recapitulates germline mutant phenotypes and reveals a dynamic view of their roles in neural cell populations. Our study provides new insight into the epigenetic processes that drive pathogenesis in RB-defective brain tumors and identifies RBBP4 and its associated chromatin remodeling complexes as potential target pathways to induce apoptosis in RB-defective brain tumor cells.

\textbf{Introduction}

The retinoblastoma tumor suppressor RB plays distinct roles in regulating proliferation and differentiation in stem, progenitor, and lineage restricted cell populations (Fong and Slack, 2017; Julian and Blais, 2015; Sage, 2012). The canonical tumor suppressor role of RB is to regulate proliferation by transcriptional repression of E2F targets driving cell cycle entry (Dyson, 2016). RB may also function as a tumor suppressor by promoting differentiation of lineage committed cells (Calo et al., 2010), survival of post-mitotic neurons (Naser et al., 2016; Vandenbosch et al., 2016) and preventing cellular reprogramming (Kareta et al., 2015). RB transcriptional control is mediated in part through association with chromatin remodelers that modify posttranslational marks on histones to activate or repress gene expression (Beshiri et al.,
2012; Brehm et al., 1998; Lin et al., 2011). Like RB, many chromatin remodelers are mutated in human cancers (Vogelstein et al., 2013), implicating epigenetic control of gene expression as a significant contributor to oncogenesis (Conway et al., 2015; Suva et al., 2013). Recent analyses of the epigenome in human and mouse retinoblastoma show histone modification correlates with oncogenic gene expression (Aldiri et al., 2017; Benavente et al., 2013; Zhang et al., 2012). Understanding how RB loss affects epigenetic control in neural stem and progenitor cells during development and in brain cancer is important for identifying new pathways that contribute to carcinogenesis.

Two epigenetic regulators that directly interact with RB are the retinoblastoma binding protein RBBP4 (Qian and Lee, 1995; Qian et al., 1993) and the Class I histone deacetylase HDAC1 (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). RBBP4 is a WD40-repeat histone chaperone (Burkhart and Sage, 2008; Murzina et al., 2008; Nowak et al., 2011a; Song et al., 2008) and a chromatin adaptor component of multiple remodeling complexes, including the nucleosome remodeling and histone deacetylase complex NuRD (Alqarni et al., 2014) and the cell cycle regulatory DREAM complex (Harrison et al., 2006; Litovchick et al., 2007). RBBP4 expression is altered in human glioma (Forbes et al., 2017) and recently was shown to cooperate with the p300 histone acetyl transferase to activate DNA repair pathway gene expression in glioblastoma cells in response to temozolomide (Kitange et al., 2016). HDAC1 is a component of NuRD and other repressive complexes which control maintenance of stem cell pluripotency and cell differentiation (Hayakawa & Nakayama 2011). Studies in zebrafish show hdac1 is necessary for central and peripheral nervous system development (Henion et al., 1996; Ignatius et al., 2013), and is required for cell cycle exit
and differentiation of neural precursors in the retina (Stadler et al., 2005; Yamaguchi et al., 2005). The role of HDAC1 in promoting proliferation versus differentiation may depend on the type and location of the neural cell population examined (Jaworska et al., 2015). Examining the contribution of HDAC1 and RBBP4 to maintaining the progenitor-like state of RB-brain tumors would shed light on the mechanism of chromatin remodeling in epigenetic control of tumor suppression.

We previously demonstrated that genome editing nucleases can be used to model brain tumors in zebrafish by targeted somatic inactivation of the rb1 tumor suppressor gene (Solin et al., 2015). TALEN targeting of zebrafish rb1 leads to brain tumors with histological similarity to central nervous system primitive neuroectodermal tumors (CNS-PNET) (Solin et al., 2015). PNET encompasses a group of aggressive, poorly differentiated tumors that feature neuroblast-like cells, which suggests this class of tumors originate from a progenitor population that mirrors the embryonic neuroectoderm (Ostrom et al., 2017) Chan et al., 2015. Recently, other zebrafish PNET models have been created by somatic targeting or oncogene overexpression. Targeting rb1 in a tp53-mutant background produces medulloblastoma-like PNET tumors arising in the zebrafish hindbrain (Shim et al., 2017). Activation of RAS/MAPK signaling by NRAS overexpression in zebrafish oligoneural precursors leads to PNET (Modzelewska et al., 2016) that molecularly resembles the human oligoneural PNET subtype Olig2+/Sox10+ CNS-PNET (Picard et al., 2012; Sturm et al., 2016a), defined by elevated expression of the neural progenitor transcription factors Olig2, Sox10, Sox8 and Sox2. Together, these models suggest disruption of multiple cellular pathways can lead to formation of PNET. Whether epigenetic mechanisms also contribute to zebrafish
PNET oncogenesis, as suggested by genomic analyses of human and mouse tumors, remains to be examined.

Here we use transcriptomics, somatic and germline CRISPR/Cas9 mutagenesis, and live cell imaging in zebrafish to identify candidate RB-interacting chromatin remodelers and examine their role in neural stem and progenitor cells during development. Our studies provide new insight into the genomic processes that drive oncogenesis in RB-defective brain tumors. Comparative transcriptome analysis of zebrafish rb1-brain tumors with rb1/rb1 homozygous mutant tissue suggests elevated expression of oligoneural precursor transcription factors and chromatin remodelers distinguishes neoplastic from mutant tissue. Isolation of rb1 germline mutants shows that in the developing nervous system, rb1 is required cell autonomously to block cell cycle re-entry in neural precursors. We demonstrate that the chromatin remodeling adaptor and histone chaperone rbbp4 is necessary for survival of neural precursors and that in the absence of rbbp4 neural precursor cells undergo apoptosis. In contrast, hdac1 is necessary to maintain proliferation in neural stem/progenitor cells. CRISPR somatic targeting recapitulates germline mutagenesis phenotypes. Live cell imaging of histone H2A-GFP in mutant larvae reveals a dynamic view of the effect of gene loss on neural stem and progenitor cell division and survival. rb1-mutant neural precursors re-enter the cell cycle but appear to stall in early mitosis, indicating a requirement for Rb1 in initiating quiescence as well as progression through the cell cycle. Our genomic and phenotypic comparisons reveal that in rb1-neoplastic cells, elevated levels of rbbp4 and hdac1 may contribute to proliferation and survival. Together these results identify RBPP4
and its associated chromatin remodelers as a potential new target for inhibiting tumor cell survival by driving neural cancer stem cells into apoptosis.

Results

Zebrafish rb1-brain tumors model OLIG2+/SOX10+ CNS-Primitive Neuroectodermal Tumors

We previously developed a zebrafish rb1-brain tumor model that resembles poorly differentiated primitive neuroectodermal tumors by somatic targeting of zebrafish rb1 with TALENs (Solin et al., 2015). Targeting produces mosaic adults that develop brain tumors between 4-5 months of age (Figure 3.1A). To determine the molecular signature of the zebrafish rb1-brain tumors, 10 tumor bearing adults were dissected. Half of the tumor tissue was used for RNA isolation, and half was embedded for histological analysis (Figure 3.1A; Figure S3.1). RNA-Seq libraries were prepared from the 10 tumor biological replicates and two pools of three normal adult brains (Figure S3.2) and analyzed for differentially expressed genes (Table S3.1). E2F targets driving cell cycle entry were highly upregulated, and Ingenuity Pathway Analysis (IPA) revealed activation of mitosis, cell cycle, and DNA replication, recombination and repair pathways (Figure S3.3). Gene Set Enrichment Analysis (GSEA) analysis also indicated a positive correlation with RB-E2F oncogenic cell signaling and transcriptional regulation pathways and a negative correlation with neurological differentiation (Figure S3.4). These results are consistent with mis-regulation of RB-dependent pathways driving oncogenesis in the rb1-brain tumors.
Figure 3.1 Zebrafish rb1-brain tumors express an oligoneural precursor signature similar to human OLIG2+/SOX10+ CNS-PNET. A Zebrafish rb1-brain tumor model created by somatic targeting of rb1 exon 2 with CRISPR-Cas9 or TALEN genome editing nucleases. Gross brain tumors develop between 4-5 months of age. For transcriptome analysis, ½ of tumor tissue was dissected from 10 tumor-positive fish for RNA isolation. The remaining tissue was embedded for pathology and immunolabeling. B Heat maps of log2(FPKM) show differential gene expression in zebrafish rb1-brain tumor transcriptome of 120 genes in the human OLIG2+/SOX10+ CNS-PNET subtype. rb1 Tumor, RNA-Seq of 10 biological replicates from 10 individual tumor positive adults. C, RNA-Seq of two biological replicates of three pooled normal adult zebrafish brain. C-L Histological and immunolabeling of wildtype adult zebrafish pretectum/diencephalon (C-G) and tumor containing brain tissue from transcriptome individual T2 (H, I) and T8 (J-L). Neoplastic cells in T2 tumor show small densely basophilic nuclei, consistent with primitive
(Figure 3.1 continued) neuroectodermal-like tumors (H, I). In normal adult brain Olig2, Sox2, and Sox10 labeling is restricted to cells at or adjacent to the ventricle (E-G). High levels of Olig2, Sox2 and Sox10 are detected in the neoplastic tissue in tumor T8. Phosphohistone-H3 mitotic cells are scattered throughout the lesion (J-L). Scale bars C, H 200µm; E, F, G, J, K, L 50µm.

We next compared the zebrafish rb1-brain tumor transcriptome to other zebrafish brain tumor models and to human CNS-PNET. A set of 120 genes most highly up- and downregulated in the human \( \text{OLIG2}^{+}/\text{SOX10}^{+} \) CNS-PNET subtype (Picard et al., 2012) was previously used to analyze differential gene expression in a zebrafish NRAS-CNS-PNET model (Modzelewska et al., 2016). The zebrafish rb1-brain tumors showed a similar pattern of differential gene expression across the 60 up- and 60 downregulated genes. Defining factors of the human oligoneural CNS-PNET subgroup, \( \text{olig2} \), \( \text{sox10} \), \( \text{sox8} \) and \( \text{erbb3a} \), and the stem/progenitor marker \( \text{sox2} \) (Figure 3.1B) were upregulated in the zebrafish rb1- tumors. Histopathological analyses of the remaining tumor tissue from the individuals used for transcriptome analyses were consistent with a proliferative, primitive neuroectodermal-like tumor (Figure 3.1 CL, Figure S3.1). In the diencephalon/pretectal region of wild type adult zebrafish brain, Olig2, Sox2 and Sox10 immunolabeling is normally restricted to cells lining the ventricles and regions adjacent to the ventricular zone (Figure 3.1 C-G), where stem and progenitor cells are located. Immunolabeling of zebrafish rb1-tumors confirmed high levels of expression of Olig2, Sox2, and Sox10 and numerous phosphohistone H3-positive mitotic cells scattered throughout the lesion (Figure 3.1 H-L). Together these results demonstrate the zebrafish rb1- brain tumors have a highly proliferative, poorly differentiated state with an oligoneural precursor molecular signature found in human \( \text{OLIG2}^{+}/\text{SOX10}^{+} \)-CNS-PNET and zebrafish NRAS-CNS-PNET tumors.
Zebrafish rb1 is required cell-autonomously for neural precursor cell cycle exit and terminal differentiation during development

The transcriptome and molecular analysis of zebrafish rb1- tumors indicated an oligoneural precursor phenotype drives tumor proliferation. To identify additional molecular and genomic changes that underlie transformation and oncogenesis in the absence of RB, we generated RNA-Seq libraries from larval zebrafish homozygous for a recessive loss of function rb1 mutation. The rb1/rb1 mutant transcriptome was used for comparative analysis with the tumor transcriptome to identify molecular pathways that distinguish transformed rb1- tumor cells from non-transformed rb1/rb1 mutant cells.

We previously isolated a 7-base pair frameshift mutation in rb1 exon 2, rb1Δ7/Δ7, and found the allele is homozygous lethal during the larval stage between five and ten days post-fertilization (dpf) (Solin et al., 2015). To generate the rb1Δ7/Δ7 transcriptome, 5 dpf larva were collected from an incross between two heterozygous rb1Δ7/+ adults. rb1Δ7/Δ7 homozygotes do not show a dramatic gross morphological difference from wildtype or heterozygous siblings, other than the absence of a swim bladder. To confirm larval genotypes, larvae were dissected through the hindbrain; the head was placed in TRIzol and the trunk tissue used for genotyping (Figure 3.2A). Five confirmed wildtype +/- and homozygous rb1Δ7/Δ7 heads were pooled in triplicate and used to prepare RNA-Seq libraries (Figure 3.2A, Figure S3.4, Tables S3.2). Differential gene expression analysis of the rb1/rb1 mutant larval transcriptome showed upregulation of E2F targets driving cell cycle entry and IPA pathways controlling cell division, mitosis, and DNA replication, recombination and repair (Figure S3.6), as expected for activation of E2F-
Figure 3.2 Zebrafish rb1Δ7/Δ7 homozygous mutant larval brain transcriptome and neurogenic phenotype. A Zebrafish rb1/rb1 mutant transcriptome was generated from rb1Δ7/Δ7 homozygous and +/+ wildtype siblings from a cross between heterozygous rb1Δ7/+ adults. Heads from 5dpf larva were dissected and trunk tissue genotyped. 3 pools of 5 heads of each genotype were used to generate RNA-Seq libraries. Wildtype B, and rb1Δ7/Δ7 C 5 dpf larva. D Diagram of larval midbrain and retina with location of neural stem and progenitor cells at brain ventricle (V) in the optic tectum (OT) and thalamic region (Th) and at the retina ciliary marginal zone (cmz). Mature,
(Figure 3.2 continued) post-mitotic neurons are located in the lateral brain parenchyma and inner retina. E-L Immunolocalization with neural differentiation marker HuC/D and mitotic M-phase marker phosphohistone-H3. E, F, I, J Wild type brain and retina show the organization of mature neurons in dorsal brain optic tectum, ventral brain thalamus, and laminated retina. Only one mitotic cell is detected at the brain ventricle (F, green). G, H, K, L mutant brain shows M-phase cells scattered throughout the midbrain tectum and thalamus (G, H). In the retina, numerous M-phase cells are present across the entire inner nuclear layer (K, L), with occasional cells in the outer nuclear and ganglion cell layer. Scale bars B, C 200µm; E, I, G, K 50µm; F, J, H, L 20µm.

dependent pathways in the absence of RB. The results were remarkably similar to the
rb1-brain tumor transcriptome, suggesting activation of molecular pathways driving cell proliferation in rb1/rb1-mutant and rb1-transformed cells are controlled by similar mechanisms.

To confirm the proliferative phenotype of rb1/rb1-mutant cells, brain sections of 5dpf rb1/rb1 mutant larvae were labeled with proliferation and differentiation markers and showed an increase in mitotic-phase cells in the developing brain and retina (Figure 3.2 B-L). In wildtype midbrain, mitotic cells are normally restricted to proliferative zones at the dorsal surface, ventricles, and lateral edges (Figure 3.2 E, F), and the ciliary marginal zone at the periphery of the retina (Figure 3.2 I, J). Phosphohistone-H3 labeled cells are absent from the brain parenchyma (Figure 3.2 F) and mature retina (Figure 3.2 J) where the cell bodies of post-mitotic differentiated neurons expressing the neuronal RNA binding protein HuC/D-Elavl3 are located in wild type brain tissue. In contrast, ectopic phosphohistone H3-positive cells were detected in the rb1/rb1 mutant larvae throughout the midbrain parenchyma in the optic tectum and thalamus (Figure 3.2 G, H), and in the inner and outer nuclear layers of the mature retina (Figure 3.2 K, L). Ectopic proliferation in the rb1/rb1 mutant was also detected in the forebrain, cerebellum and hindbrain, along
the entire anterior-posterior axis (Figure S3.7). Close examination of the \( rb1/rb1 \) mutant phosphohistone-H3 positive cells showed no labeling with HuC/D (Figure S3.8), suggesting the cells were in a cycling progenitor-like state. These results are consistent with a requirement for RB in neural precursor cell cycle exit and terminal differentiation.

To examine whether the requirement for RB in neural precursor cell cycle exit was cell autonomous, genetic mosaics were created by transplanting \( rb1/rb1 \) mutant cells from blastula stage embryos into wildtype host embryos and examining their behavior in larval brain. Embryos from heterozygous \( rb1Δ7/+ \) crossed to heterozygous \( rb1Δ7/+;Tg(Tol2<ubi:DsRed2>) \) adults carrying a ubiquitous RFP reporter transgene were collected, and cells from blastula stage embryos were transplanted into a casper host embryo. The remaining donor embryo tissue was used to determine the \( rb1 \) genotype (Figure S3.9). Three host larva containing cells from a \(+/+\) or \( rb1Δ7/Δ7 \) donor embryo were analyzed. At 5dpf, host larva were sectioned and co-labeled with antibodies to DsRed2, to identify the descendants of transplanted cells, and to the proliferation marker phosphohistone H3 or the neuronal differentiation marker HuC/D (Figure 3). The descendants of \(+/+\) wildtype (Figure 3.3A, B, E, F) and \( rb1Δ7/Δ7 \) mutant (Figure 3.3 C, D, G, H) transplanted cells were incorporated into the developing brain and could be detected in the midbrain parenchyma. None of the \(+/+\) wildtype cells labeled with phosphohistone H3 (Figure 3.3 A, B), and each cell was positive for the neuronal marker HuC/D (Figure 3.3 E, F). The majority of the \( rb1Δ7/Δ7 \) mutant cells were phosphohistone H3-negative. However, in each section one or two phosphohistone H3-positive mutant cells with highly condensed chromatin was detected (Figure 3.3 C, D), indicating the cells were in M-phase. Each of the \( rb1Δ7/Δ7 \) mutant cells expressed
Figure 3.3  Cell-autonomous requirement for RB in blocking cell cycle entry in zebrafish neural precursors. Immunolabeling of 5dpf host larval brain sections containing descendants of +/+; ubi:DsRed2 and rb1Δ7/Δ7; ubi:DsRed2 transplanted cells. A-D phosphohistone H3 and DsRed2 labeled sections. Wildtype (A, B) and rb1Δ7/Δ7 mutant (C, D) cells present in a wildtype host optic tectum. A few phosphohistone H3-positive rb1Δ7/Δ7 mutant cells can be detected (D, arrows). E-H Neuronal marker HuC/D and DsRed2 labeled sections. Wildtype (E, F) and rb1Δ7/Δ7 mutant (G, H) cells in a host optic tectum express HuC/D. A number of rb1Δ7/Δ7 mutant cells with highly condensed chromatin lack HuC/D labeling (H, arrows). I-J Boxed region in F, showing HuC/D and DsRed2-positive wildtype cells with interphase chromatin. M-P Boxed region in H shows DsRed2-positive rb1Δ7/Δ7 mutant cells with interphase chromatin also express HuC/D. HuC/D is absent from
HuC/D, except for the M-phase cells with condensed chromatin (Figure 3.3 G, H arrows). Higher magnification images show that each wild type (Figure 3.3 I-L) or \( rb1\Delta 7/\Delta 7 \) mutant (Figure 3.3 M-P) cell that labels positively for HuC/D and DsRed2 contains chromatin characteristic of interphase cells. In contrast, the \( rb1\Delta 7/\Delta 7 \) mutant cells with highly condensed chromatin do not co-label with HuC/D (Figure 3.3 M-P). The presence of \( rb1\Delta 7/\Delta 7 \) cells that express HuC/D/Elavl3 and \( rb1\Delta 7/\Delta 7 \) cells with condensed chromatin in M phase, suggests the mutant neural precursors are able to re-enter the cell cycle. These results demonstrate the requirement for RB to block cell cycle entry in neural precursors is cell autonomous.

**Comparison of zebrafish rb1-transformed and rb1/rb1 mutant transcriptomes support epigenetics drive rb1-tumor growth**

The \( rb1\Delta 7/\Delta 7 \) mutant transcriptome and phenotypic analyses showed \( rb1/rb1 \) mutant cells can re-enter the cell cycle and proceed into M phase, but lack continuous unregulated proliferation characteristic of \( rb1 \)-transformed tumor cells. To gain insight into the molecular differences between \( rb1/rb1 \) mutant and \( rb1 \)-transformed cells, we performed additional comparative analyses between the zebrafish \( rb1 \) mutant and brain tumor transcriptomes. We focused on transcriptional regulators which include epigenetic chromatin remodelers and transcription factors, since a number of studies indicate regulation of the epigenome is a critical component of oncogenesis (Suva et al., 2013).

Of the 3302 transcriptional regulators in the zebrafish genome (Armant et al., 2013), 1191 were differentially expressed in the \( rb1 \)-tumor transcriptome, and 125
differentially expressed in the \(rb1/rb1\) mutant transcriptome. The majority of the differentially expressed transcriptional regulators in both transcriptomes were transcription factors; approximately one fifth were chromatin regulators (Figure 3.4A). However, elevated expression of stem and neural progenitor transcription factors sox2, sox8, sox10, olig2, and ascl1b, and downregulation of proneurogenic transcription factors pax6a, pax2a, neurod1, and neurod6a, was unique to the tumor (Figure 3.4B).

The chromatin adaptor \(rbbp4\), a component of multiple chromatin remodelers controlling gene expression including NuRD, PRC2, histone acetyltransferase p300, and the cell cycle DREAM/MuvB complex, was upregulated in both \(rb1/rb1\) mutant and \(rb1\)-tumor transcriptomes (Figure 3.4B). In contrast, the catalytic component of NuRD, histone deacetylase 1 \(hdac1\), and the DNA binding subunit \(mbd3a\), showed differential expression only in the tumor. \(rbbp4\), \(hdac1\), \(olig2\) and \(ascl1b\) were examined by qRT-PCR to validate the similarity and differences between the tumor and \(rb1\) mutant transcriptomes (Figure 3.4C). The differences in gene expression between the tumor and mutant transcriptomes suggest alteration of chromatin remodeler activity correlates with transformation of \(rb1\)-mutant cells, maintenance of the tumor progenitor-like state, and tumor oncogenesis.

**Distinct requirements for chromatin remodelers \(rbbp4\) and \(hdac1\) in neurogenesis**

To investigate how \(rbbp4\) and \(hdac1\) drive zebrafish \(rb1\)-brain tumorigenesis we isolated stable germline loss of function mutations in each and examined their role in neural development. CRISPR-Cas9 gene editing was used to generate a frameshift mutation in exon two of \(rbbp4\) (Figure 3.5A-C) and exon five of \(hdac1\) (Figure 3.5D-F). A recessive-lethal loss of function four base pair deletion mutation line was established
Figure 3.4 Differential Gene Expression of Transcriptional Regulators in \(rb1\)-tumor and \(rb1/rb1\) mutant transcriptomes. A Comparative analysis of all differentially expressed Transcriptional Regulators in \(rb1\)-tumor and \(rb1/rb1\) transcriptomes. B Heat maps showing relative expression of components of the NuRD chromatin remodeler, neural progenitor and neurogenic transcription factors in \(rb1\)-tumor and \(rb1/rb1\) mutant transcriptomes. C Quantitative RT-PCR validates elevated expression of \(rbbp4\) in the tumor and mutant transcriptome, while upregulation of \(hdac1, olig2,\) and \(ascl1b\) is unique to the tumor. \(metap1\), housekeeping gene used as a standard control for heat maps and qRT-PCR.

for each, designated \(rbbp4\Delta4^{660}\) and \(hdac1\Delta4^{is70}\). Homozygous mutant \(rbbp4\Delta4/\Delta4\) larvae are lethal between 5 and 10 days post fertilization and show a severe neurogenic phenotype with microcephaly and microphthalmia (Figure 3.5C). Similar to the previously published \(colgate\) mutant (Henion et al., 1996; Ignatius et al., 2008),
Figure 3.5 Requirement for Chromatin Remodelers *rbbp4* and *hdac1* in zebrafish neurogenesis. A CRISPR target site in exon 2 of *rbbp4* used to isolate 4 base pair frameshift mutation *rbbp4Δ4-is60*. PAM sequence is underlined. *SmlI* restriction enzyme site overlapping the target site is shown in red. B 5dpf wildtype (+/+) larva. C Gross phenotype of 5dpf homozygous mutant *rbbp4Δ4/Δ4* larva showing microcephaly and microphthalmia. D CRISPR target site in exon 5 of *hdac1* used to isolate 4 base pair frameshift mutation *hdac1Δ4-is70*. PAM sequence is underlined. E 3dpf wildtype larva. F Gross phenotype of 3dpf homozygous mutant *hdac1Δ4/Δ4* larva showing microcephaly and retinal coloboma (arrow). G Diagram of 2dpf larval midbrain and retina. Sections of wildtype (H), *rbbp4Δ4/Δ4* (I), and *hdac1Δ4/Δ4* (J) 2dpf larval heads labeled with neural differentiation marker HuC/D (red) and apoptosis marker activated caspase3 (green). I In *rbbp4Δ4/Δ4*, apoptosis is present in the dorsal and lateral tectum (arrows), and throughout the inner retina (brackets). J *hdac1Δ4/Δ4* larval brain and retina are smaller than wildtype, but few apoptotic cells are detected in the brain or retina. OT optic tectum; Th thalamus; R retina. Scale bars B, C 500 µm; E, F 200µm, H-J 100µm.
homozygous mutant \textit{hdac1Δ4/Δ4} are also larval lethal, and at 3 days post fertilization show a reduced body size, curved trunk, microcephaly, and coloboma in the retina (Figure 3.5F, arrow).

Homozygous mutant larvae were examined at 2dpf to determine the defect underlying the reduced size of the brain and retina (Figure 3.5 G-J). In 2dpf wildtype larval brain and retina only a few apoptotic cells were labeled with an antibody to activated-caspase3 (Figure 3.5H). \textit{rbbp4Δ4/Δ4} mutants showed extensive apoptosis in the midbrain optic tectum and retina, regions of active proliferation as the embryo transitions to larval neurogenesis, demonstrating RBBP4 is required for survival of neural precursors (Figure 3.5I). The failure of \textit{rbbp4Δ4/Δ4} neural precursors to survive explains the reduced eye and microcephaly observed in mutant larvae. In contrast to \textit{rbbp4}, apoptosis was not detected in the midbrain or retina of \textit{hdac1Δ4/Δ4} mutants at 2dpf. However, the size of the tectum and thalamus was reduced compared to wildtype, with a decrease in the amount of HuC/D expressing neurons in the parenchyma (Figure 3.5J). The absence of apoptosis combined with an overall reduced size suggests \textit{hdac1} is required for persistent proliferation of stem cells to generate the neural progenitor pool and new neurons. These results show a distinct requirement for \textit{rbbp4} in neural progenitor or precursor survival, whereas \textit{hdac1} may be required upstream for maintaining proliferation of the neural stem and/or progenitor pool. Similar roles in neural progenitor proliferation and survival may drive unregulated tumor growth in zebrafish \textit{rb1}-brain tumors.
Somatic targeting assay to examine requirement of chromatin remodelers in neural stem and progenitor cells during development

CRISPR-Cas9 somatic targeting in zebrafish provides a simple and rapid method to investigate the function of a gene during embryonic and larval development. We developed an in vivo live animal imaging assay to examine the role of chromatin remodelers and other candidate tumor suppressor genes in neural stem and progenitor cells during brain development (Figure 3.6A). CRISPR-Cas9 somatic targeting is performed in the Tg(H2A.F/Z-GFP) reporter line (Pauls et al., 2001) which expresses a histone H2A.F/Z-GFP fusion protein that labels chromatin in all nuclei. We validated the assay by somatic targeting of rb1, rbbp4, and hdac1, and comparing the phenotype in live 5dpf larva to the fixed immunohistochemical analysis of homozygous germline mutants. CRISPR targeting of rbbp4 and hdac1 resulted in gross defects in the head (Figure 3.6B) consistent with the phenotype of stable germline loss of function mutants described above.

To examine the effect of somatic targeting on neurogenesis at the cellular level, we imaged the optic tectum in live Tg(H2A.F/Z-GFP) 5dpf larva (Figure 3.6C). Larva were mounted with dorsal side upright. A confocal z-stack of 11 slices was collected at 3 um steps, beginning ~30um below the surface of the tectum, moving ventrally towards the thalamic region. In this ventral region of the tectum the rate of stem cell division has slowed dramatically compared to the actively proliferative zone at the dorsal side. In wild type larva, a single confocal slice through the ventral tectum shows the highly organized radial pattern of neuronal nuclei extending laterally away from the midline. A clear demarcation of cells lining the ventricle marks the midline (Figure 3.6C uninjected). In
Figure 3.6 CRISPR somatic targeting-live imaging assay to test chromatin remodeler function in neural cell populations during brain development. Somatic inactivation recapitulates germline mutants and reveals distinct requirements for rb1, rbbp4, and hdac1 in neural progenitor proliferation, differentiation, and survival. A CRISPR targeting in histone H2A.F/Z-GFP fusion line and dorsal view, confocal live imaging of GFP in the brain at 5dpf. B Gross defects in rb1, rbbp4 and hdac1 CRISPR targeted larvae at 5dpf recapitulates germline phenotypes. C Confocal imaging in the optic tectum in the brain of live 5dpf larva. Section through ventral region of optic tectum. Mitotic figures are rarely captured in uninjected larva at this level of the tectum (red arrowhead). rb1 CRISPR targeted, cells will nuclei containing condensed chromatin scattered throughout the post-mitotic region of the tectum.
(Figure 3.6 continued) CRISPR targeted, intense, punctate fluorescence in presumed apoptotic nuclei (red arrowheads) and abnormally large, irregular nuclei (asterisks). *hdac1* CRISPR targeted, Overall reduction in the size/cellular number of the tectum. Enlarged nuclei with faint GFP line the ventricle (red arrowheads). D Quantification of M-phase phosphohistone-H3 positive nuclei in optic tectum in three uninjected control, *rb1*, *rbbp4* and *hdac1* targeted larvae. Targeting experiments were replicated three times for *rb1*, and two times for *rbbp4* and *hdac1*. Plot shows mean number of positive nuclei in 11 sections, in 3 biological replicates, with error bars showing standard error mean. The number of M-phase nuclei in *rb1* targeted larvae is significantly different than control. Two-tailed unpaired t-test, *rb1* \( p = 0.0145; \) *rbbp4* \( p = 0.3024; \) *hdac1* \( p = 0.1841. \) Middle and Right panels, PCR genotyping shows highly efficient mutagenesis in the 3 CRISPR-targeted individuals used for quantification. C, control uninjected individuals. *rb1* exon 2, sequence shows location of CRISPR gRNA and *ClaI* enzyme site overlapping Cas9 cut site. *rb1* targeting, U=undigested PCR amplicon, D=*ClaI* digested PCR amplicon surrounding exon 2. *rbbp4* and *hdac1* targeting was performed with the gRNAs used to isolate loss of function alleles described in Figure 5. *rbbp4* targeting, U=undigested exon 2 PCR amplicon, D=*SmlI* digested exon 2 PCR amplicon. *hdac1* targeting, diffuse band for PCR amplicon in targeted individuals correlates with efficient mutagenesis.

the larval midbrain stem cells line the ventricles and the anterior-lateral surface of the tectum. In the confocal section shown, a single metaphase mitotic figure is present near the anterior side of the uninjected wild type tectum (Figure 3.6C uninjected, arrowhead). Quantification of mitotic figures in all 11 wildtype sections showed 14-23 dividing cells total per tectum (n=3, Figure 3.6D, Table S3.4).

Live imaging of the tectum in *rb1* CRISPR targeted larva showed a dramatic increase in the number of cells with condensed chromatin (Figure 3.6C *rb1* CRISPR arrowheads), consistent with the phosphohistone-H3 immunolabeling results from *rb1/rb1* mutant fixed, sectioned tissue. The M-phase cells were located near the ventricle and also distributed throughout the tectum, where the regular array of postmitotic neuron cell bodies are located. The total number of cells with condensed chromatin in *rb1*-targeted embryos was significantly higher, ranging from 76-133 (n=3, \( p = 0.0145, \) Figure
3.6D; Table S3.4), compared to 14-23 cells observed in wild type. The difference in total number may be due to the degree of mosaicism and formation of bi-allelic indel mutations in the rb1-targeted larva, although genotyping indicated each was efficiently mutagenized at the CRISPR target site (Figure 3.6D). These results suggested that rb1/rb1 mutant neural precursors can re-enter the cell cycle after migration into the post-mitotic region of the tectum.

To examine the behavior of rb1 mutant neural precursors in real time, time-lapse confocal images were captured of H2A-GFP in wildtype and homozygous mutant rb1Δ7/Δ7 larva brains (Fig. S3.9B). Live imaging of the dorsal surface of the optic tectum in wildtype at 3dpf and 5dpf shows the tissue is highly proliferative, with multiple stem cells entering and completing mitosis. At 5dpf in more ventral regions of the tectum where tissue growth has slowed, a two-hour time lapse showed quiescent neural precursors, and only one stem cell at the periphery was captured undergoing mitosis. In contrast, in the rb1Δ7/Δ7 mutant, multiple cells throughout the tectum underwent chromosome condensation. Over the course of three hours, cells that had entered the cell cycle failed to progress through metaphase, and frequently appeared to be removed by microglia. These analyses confirmed in real time the requirement for zebrafish Rb1 in blocking neural precursor cell cycle re-entry, and support an additional role for rb1 in promoting metaphase progression and completion of mitosis.

The role of zebrafish Rbbp4 and Hdac1 in neural cell populations during neurogenesis was also examined by live imaging in CRISPR targeted larva. Quantification confirmed that proliferation in the tectum was not significantly different than wild type after targeting rbbp4 (1-9 cells per larva; n=3, p=0.3024; Figure 3.6D;
Table S3.4). In *rbbp4* targeted larvae cell nuclei throughout the tectum appeared fragmented or hyper-condensed (Figure 3.6C, *rbbp4* CRISPR, arrowheads). Frequently, very enlarged nuclei or cells were detected near the tectum posterior border (Figure 3.6C, *rbbp4* CRISPR, asterisks). Like the fixed tissue analysis in homozygous mutants, live imaging of the *rbbp4* targeted larval brain revealed *rbbp4* is required for survival of newborn or postmitotic neurons.

*hdac1* CRISPR targeted larvae also showed a consistent neurogenic phenotype but a range of severity. In some larvae the optic tectum was reduced in size, while in others the midbrain ventricle was completely open and tectal structures entirely missing. Imaging of *hdac1* targeted larvae that had developed an optic tectum revealed no significant difference in the number of mitotic cells compared to wildtype (3-17 cells per larva, n=3, *p*=0.1841, Figure 3.6D; Table S3.4). However, the nuclei in cells lining the ventricle appeared enlarged, with diffuse GFP expression (Figure 3.6C, *hdac1* CRISPR, arrowheads), indicating the chromatin was decondensed. The reduced size of the brain, together with decondensed chromatin in cells lining the ventricle, suggests the neural stem cells in the *hdac1* targeted larvae had exited the cell cycle, and supports a requirement for *hdac1* in maintenance of a proliferating neural stem cell population.

Our CRISPR somatic targeting-live imaging assay is a simple, rapid method to test the function of transcriptional regulators in neural cells and gain additional insight into their roles in tumor cell proliferation and oncogenesis. The somatic targeting assay confirms the germline mutant phenotype and reveals a dynamic view of proliferation and commitment in neural cell populations in the developing midbrain (Figure 3.7). Our
Figure 3.7  Roles for *rb1*, *rbbp4* and *hdac1* in proliferation, survival and terminal differentiation of neural cell populations during development. Diagram of spatial organization of neurogenesis in the zebrafish midbrain. In the midbrain stem cells in the optic tectum (OT) and thalamus (Th) line the ventricles and undergo self-renewing divisions to produce progenitors. Progenitors exit the cell cycle, and neural precursors migrate to their final destination in the parenchyma where they differentiate into mature neurons. Germline mutant and CRISPR somatic targeting analyses showed zebrafish *rb1* is required cell-autonomously to prevent neural precursor cell cycle re-entry. *rbbp4* is required for survival of neural precursors. *hdac1* is necessary to maintain proliferation in the stem cell pool, possibly by preventing premature cell cycle exit and differentiation, and/or quiescence.
analyses suggest \( rb1 \) is necessary to block re-entry of neural precursors into the cell cycle. In the absence of \( rbbp4 \), neural precursors fail to survive and activate caspase-3 mediated apoptosis. The production of new neural precursors is dependent on \( hdac1 \) maintaining proliferation of the neural stem cell pool. Together these results suggest elevated expression of \( rbbp4 \) and \( hdac1 \) after \( rb1 \) loss may drive unregulated proliferation and growth in zebrafish \( rb1 \)-brain tumors.

**Discussion**

In this study, we compared the transcriptome of somatically engineered zebrafish \( rb1 \)-primitive neuroectodermal-like brain tumors with homozygous \( rb1/rb1 \) mutants and identified epigenetic regulators that may contribute to the mechanism of RB-defective oncogenesis. Our results suggest transcriptional regulation by epigenetic modifiers and neural progenitor transcription factors distinguishes transformed RB-defective tumor cells from homozygous mutant brain tissue harboring a simple loss of function mutation in \( rb1 \). We next performed phenotypic mutant analysis in the developing zebrafish larval brain to understand the role of \( rb1 \) and the chromatin remodelers \( rbbp4 \) and \( hdac1 \) in normal neurogenesis. As part of this analysis we developed a novel *in vivo* assay to examine RB and chromatin remodelers in neural precursor proliferation, survival and differentiation. The results show a cell autonomous requirement for \( rb1 \) in blocking cell cycle re-entry in neural precursors. While \( rbbp4 \) is required for neural progenitor and/or precursor survival, \( hdac1 \) is necessary for maintaining proliferation of the neural stem/progenitor pool. The requirements for \( rbbp4 \) and \( hdac1 \) in regulating neural progenitor proliferation and survival may contribute to oncogenesis after \( rb1 \) loss in zebrafish \( rb1 \)-brain tumors. Our study provides novel insights into the tumor suppressive
role of zebrafish Rb1 and its candidate interacting proteins Rbbp4 and Hdac1 in driving persistent tumor growth, and identifies Rbbp4 as a potential target to inhibit tumor cell survival.

Our zebrafish rb1-brain tumor model histologically and molecularly resembles human CNS-PNET, an embryonal tumor composed of proliferative neuroblast cells. Conditional RB knockout in mice neural precursors in combination with TP53 and PTEN tumor suppressors favors formation of CNS-PNET over glioma (Chow et al., 2011; Jacques et al., 2010). Although our zebrafish model results from inactivation of a single tumor suppressor rb1, tumor pathway analysis identified TP53 dependent signaling, DNA mismatch, base excision and homologous recombination repair pathways as altered in rb1-tumors and may contribute to transformation and tumorigenesis. In humans, CNS-PNET has been genetically linked to mutation or alteration of RB/E2F, TP53, and DNA mismatch repair pathways (reviewed in (Larson and Largaespada, 2012). 4-15% of children carrying RB mutations have been reported to develop PNET outside of the retina (Blach et al., 1994; de Jong et al., 2015; Kivela, 1999) and silencing of CDKN2A, a negative regulator of RB inhibiting cyclin dependent kinases, occurs in sporadic PNETs (Li et al., 2005; Pfister et al., 2007). Sporadic CNS-PNET has also been associated with TP53 mutation and overexpression (Eberhart et al., 2005) and TP53 germline mutation in Li Fraumeni syndrome (Orellana et al., 1998). A number of tumors types including CNS-PNET arise in individuals with Turcot syndrome Type I, which is linked to mutations in DNA mismatch repair genes hMLH1, hMSH2, hPMS1 and hPMS2 (De Vos et al., 2004). Altered expression of RB/E2F, TP53 and DNA mismatch repair pathways in the
zebrafish *rb1*-brain tumor model is consistent with the genetic origin of human CNS-PNET.

Comparative genomics revealed the zebrafish RB-defective brain tumors are most similar to the human *OLIG2*/+*SOX10*/-CNS-PNET subtype (Picard et al., 2012; Sturm et al., 2016a). We confirmed Olig2 and Sox10 protein are highly expressed in tumor tissue, supporting a mechanism in which oligoneural precursor transcription factors contribute to gene expression driving tumor cell proliferation. The previous observations that epigenetic mechanisms drive cell transformation and tumorigenesis in human and mouse retinoblastoma (Aldiri et al., 2017; Benavente et al., 2013; Zhang et al., 2012) suggested epigenome changes may also underlie oncogenesis in the zebrafish *rb1*-tumor model. Differential gene expression showed >25% of all transcriptional regulators, including >200 chromatin remodelers, are altered in the *rb1*-tumor transcriptome, supporting epigenetic control of gene expression drives tumorigenesis. RBBP4, a chromatin adaptor for multiple chromatin remodeling complexes, is an E2F target and, as expected, was increased in the zebrafish *rb1*-tumor and *rb1/rb1* homozygous mutant transcriptome. Upregulation of *hdac1*, which is not a direct target of E2F transcriptional activation, was specific to the *rb1*-tumor. This suggests *hdac1* is one of many chromatin remodelers whose activity may distinguish transformed tumor cells from mutant cells. Gene regulatory network analysis and simulation of tumorigenesis in a human cell line model has suggested chromatin remodelers cooperate with transcription factors as cells progress to transformation (Malysheva et al., 2016). A similar epigenetic mechanism in which Hdac1 and Rbbp4 associate with oligoneural precursor transcription factors may drive zebrafish *rb1*-brain tumor oncogenesis.
Our analysis of zebrafish loss of function rb1/rb1 mutants in neural development reveals a cell-autonomous requirement for rb1 in suppressing cell cycle re-entry in neural precursors. This is consistent with the original mouse RB knockout models demonstrating ectopic proliferation in the embryonic mouse brain (Clarke et al., 1992; Ferguson and Slack, 2001; Jacks et al., 1992; Lee et al., 1992; Lipinski and Jacks, 1999). More recently, conditional RB knockout in embryonic and adult mouse neural progenitors has shown RB is required to control neural precursor proliferation and for the long-term survival of newborn neurons in the olfactory bulb and hippocampus (Naser et al., 2016; Vandenbosch et al., 2016). Although our results do not indicate zebrafish Rb1 functions in long-term neuron survival, analysis of rb1/rb1 mutant cells over an extended time would be required to rule out this role. Our live imaging of histone H2A-GFP in zebrafish larval brain allowed a dynamic view of rb1/rb1 mutant neural precursor behavior and cell cycle entry. Following chromatin condensation, rb1/rb1 mutant cells failed to progress through mitosis and appeared to idle in prometaphase, indicating RB is also required to complete the cell cycle. A delay in cell cycle exit of retinal ganglion cell precursors was previously reported in the zebrafish rb1 mutant space cadet (Gyda et al., 2012). Whether rb1/rb1 mutant neural precursors remain arrested in M-phase, eventually exit the cell cycle, or undergo continuous rounds of inappropriate cell cycle entry will require additional analyses.

Recently RB has been shown to cooperate with the DREAM complex subunit Lin37 to block cell cycle entry and transition cells from G1 to G0/quiescence (Mages et al., 2017). In budding yeast the transition from proliferation to quiescence is mediated by DREAM recruitment of the histone deacetylase HDAC1 to S phase genes to repress cell
cycle entry (Miles and Breeden, 2017), again connecting RB with chromatin remodeling in cell cycle regulation. RB has also been shown to interact genetically with the chromatin remodeler histone demethylase UTX in cell fate control in *C. elegans* (Wang et al., 2010b). Zebrafish *rb1/rb1* mutant neural precursors fail to initiate quiescence, but may also lack the ability to respond to differentiation signals in the environment. RB in association with HDAC1 and other chromatin remodelers may be required to shift the gene expression program in G1 neural precursors to a neural differentiation program. The disruption of RB-dependent transcriptional mechanisms controlling neural precursor quiescence and differentiation may be a part of oncogenesis in RB-defective brain tumors.

The zebrafish *rbbp4* and *hdac1* mutants we isolated and characterized have provided new insight into their roles in neural cell fate decisions. RBBP4 has not previously been studied in the developing vertebrate nervous system, and our analysis shows *rbbp4* is necessary for neural precursor survival in zebrafish. RBBP4 acts as a chromatin adaptor for multiple chromatin regulatory complexes, including HDAC1/NuRD (Alqarni et al., 2014), PRC2 (Kuzmichev et al., 2002; Muller et al., 2002), DREAM/MuvB (Harrison et al., 2006; Litovchick et al., 2007), and the transcriptional activator p300/Histone Acetyl Transferase (Kitange et al., 2016). In zebrafish neural precursors Rbbp4 could assist in genome maintenance through p300 activation of gene expression in DNA repair pathways, as was reported in glioblastoma cells (Kitange et al., 2016). Additional phenotype and genetic interaction studies of zebrafish *rbbp4* are necessary to dissect the contribution of RBBP4 and its chromatin remodeling complexes to preventing neural precursor apoptosis and facilitating neural
differentiation. Like RBBP4, HDAC1 is a component of multiple chromatin remodeling complexes that may function separately to control proliferation and differentiation, depending on the cell type and context. The zebrafish \textit{hdac1} mutant phenotype would support a role for HDAC1 in maintaining stem cell proliferation and the production of neural progenitors, either by blocking quiescence or premature differentiation in the stem cell population. The decondensed, enlarged nuclear phenotype of zebrafish \textit{hdac1} mutant stem cells in the brain suggest histone deacetylation is important for maintaining a proliferative stem cell pool. Global loss of histone acetylation due to overexpression of HDACs is a common feature in several human cancers (Brien et al. 2016; Haery et al. 2015). Together with the survival promoting activity of RBBP4, HDAC1 and RBBP4 may cooperate to drive persistent proliferation and growth of tumor cells in RB-defective brain cancer.

Future studies to investigate the cooperation of RBBP4, HDAC1 and RB in specific neural precursor cell types will increase understanding of the mechanism by which RBBP4 prevents programmed cell death and HDAC1 maintains a balance between proliferation and differentiation and/or quiescence. Since the first description of somatic targeting in zebrafish using custom genome editing nucleases (Bedell et al., 2012), CRISPR/Cas9 and TALENs have been widely adopted to examine developmental mechanisms and model disease (Peng et al., 2014; Shah et al., 2016). Combined with the histone-GFP reporter live imaging assay we developed, our somatic screen provides a rapid method to test chromatin remodelers and further dissect the mechanism controlling RB-dependent proliferation and survival in neurogenesis and tumor suppression. The recent discovery of a WD40 Repeat inhibitor that binds the MLL subunit of PRC2 (Song
et al., 2017) suggests other WDR proteins, such as RBBP4, are strong candidates for chemical compound screening to identify therapeutic drugs. Given the requirement for RBBP4 in neural precursor survival, RBBP4 and its associated chromatin remodeling complexes represent a potential new target to inhibit tumor cells and induce neural cancer cell apoptosis.

**Materials and Methods**

**Zebrfish care and husbandry**

Zebrfish were raised on an Aquatic Habitat housing system from Pentair Aquatic Eco-systems (Apopka, Florida) and maintained at 27°C on a 14hr light/10hr dark cycle. The zebrafish WIK wildtype strain and transparent *casper* mutant line (White et al., 2008) were purchased from the Zebrafish International Resource Center ([https://zebrafish.org/home/guide.php](https://zebrafish.org/home/guide.php)). Transgenic line *Tg(H2A.F/Z-GFP)* (Pauls et al., 2001) was received from Dr. Brian Link, Medical College of Wisconsin. Isolation of the zebrafish *rb1* 7 base pair exon 2 frameshift mutation *rb1Δ7is54* was described previously (Solin et al., 2015). All experimental protocols were approved by the Iowa State University Animal Care and Use Committee (Log#11-06-6252) and performed in compliance with American Veterinary Medical Association and National Institutes of Health guidelines for humane use of laboratory animals in research. The zebrafish *rb1*-brain tumor model was generated by injection of *rb1* TALENs targeting exon 2 (Solin et al., 2015). Animals were monitored according to the guidelines for endpoint in neoplasia studies as outlined in the NIH/Office of Animal Care and Use/Animal Research Advisory committee (ARAC) Guidelines for endpoint in neoplasia studies (oacu.od.nih.gov/ARAC/Guidelines for Endpoints in Animal Study Proposals). Adult fish
were monitored daily during feeding for general appearance, size, length, viability and morbidity relative to control siblings. Fish were monitored bi-weekly for evidence of cranial tumors and sacrificed before tumor burden reached 3 mm in size/25mg in weight, constituting less than 10% of the total body weight of an adult fish (300-500mg).

**RNA-Seq libraries, sequencing, and analysis**

*Zebrafish rb1-brain tumor model RNA-Seq libraries:* Tissues for isolation of total RNA were dissected from 10 adult fish with brain tumors and 6 control wild type siblings between the ages 6.5 to 10.5 months. One sagittal half of each brain from the diencephalon to behind the cerebellum was isolated for RNA extraction. The remaining half was processed and embedded in either paraffin blocks or OCT media (Fisher). Total RNA was isolated using TRIzol (Invitrogen), followed by DNase I (Qiagen) digestion and Qiagen RNeasy MinElute Cleanup (Qiagen). RNA quality was analyzed with the Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer. 1 µg of total RNA input was used for each library preparation. Replicate cDNA libraries were synthesized from 10 individual brain tumor RNA samples and 2 pools of 3 wild type brain RNA samples using the Illumina TruSeq RNA Library Prep Kit v2., for a total of 24 barcode indexed libraries. The 24 cDNA libraries were multiplex 50bp single-end sequenced in 2 lanes (12 per lane) on an Illumina HiSeq 2500 instrument at the Genome Sequencing Core, Center for Molecular Analysis of Disease Pathways, University of Kansas.

*Zebrafish rb1Δ7/Δ7 mutant 5 dpf larval brain RNA-Seq libraries:* Heterozygous rb1Δ7/+ adults were in-crossed and embryos aged to 5 dpf for tissue isolation. Individual 5dpf larvae were dissected in two; the head was placed in TRIzol for storage at -80°C; the trunk was placed in 50mM NaOH, heated at 95°C for 30 minutes, and used for PCR
genotyping. 3 pools of 5 genotyped heads were used to prepare 3 wild type +/+ and 3 
\( rb1A7/\Delta 7 \) homozygous mutant barcoded indexed RNA-Seq libraries, as described above. 
The 6 indexed libraries were multiplex 50bp single-end sequenced in 2 lanes on an 
Illumina HiSeq 2500 instrument at the Genome Sequencing Core, Center for Molecular 
Analysis of Disease Pathways, University of Kansas.

Reads were aligned to the GRCz10 zebrafish reference genome using GSNAP 
version 20150723 with the following parameters “-N 1 -t 8 -B 4 -m 5 -A sam --split-
output” to account for exon-exon splice junctions during alignment and a maximum of 5 
mismatches. The “uniq” SAM output files from GSNAP were funneled through Cufflinks 
2.2.1 for normalization. Python HT-Seq 0.9.1 was used to generate the count table from 
the GSNAP “uniq” SAM files. Gene exonic length was calculated from the 
Danio_rerio.GRCz10.89.gtf file in R using the “GenomicFeatures” package from 
Bioconductor. Fragments Per Kilobase of transcript per Million mapped reads (FPKM) 
was calculated using the following equation 
\[ \text{FPKM} = \frac{10^9 \times \text{Number of mapped reads to a gene}}{(\text{Gene exonic length} \times \text{Total mapped reads in the experiment})}. \]
Count data 
generated from HT-Seq was analyzed for differential gene expression detection using 
DESeq2. Differentially Expressed Genes were defined by an adjusted \( p \)-value of less than 
or equal to 0.01 and a 1.5-fold change in either direction.

Correlation coefficient plots were computed using FPKM in Microsoft Excel with 
the CORREL function. GSEA and E2F target gene analysis was performed at the Broad 
Institute website \texttt{http://software.broadinstitute.org/gsea/index.jsp}. Ingenuity Pathway 
Analysis (Qiagen 830036) was performed with software licensed to Iowa State 
University. For IPA, human homologs of zebrafish genes were identified at Biomart
Differential gene expression of 3302 transcriptional regulators was analyzed using the gene list identified in (Arman et al., 2013). Heatmaps were generated in R with gplots heatmaps.2 or with Broad Institute GENE-E software (https://software.broadinstitute.org/GENE-E/). Venn diagrams were drawn at http://www.venndiagrams.net.

Quantitative RT-PCR

5dpf larva from an in-cross between rb1Δ7/+ adults were collected and dissected into head and trunk tissue. Head tissue was placed in TRIzol. Trunk tissue was digested in 50mM NaOH at 95°C for thirty minutes, then PCR genotyped to identify homozygous rb1Δ7/Δ7 and wildtype +/- siblings. 33 homozygous rb1Δ7/Δ7 heads and 40 +/- heads were pooled, and total RNA extracted. mRNA was purified from total RNA using a magnetic isolation kit (NEB E7490S), and checked for quality using an Agilent 2100 Bioanalyzer. First strand cDNA was reverse transcribed with SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen #11752-050). qPCR reactions were set up with SYBR Green (Applied Biosystems A25742) and a final primer concentration 200nM. qPCR was run with standard temperature settings on a Roche Lightcycler 480 instrument at the ISU Genome Technology Facility.

Histology and Immunolocalization

For histological analysis tumor tissue was fixed in 10% formalin, paraffin-embedded, and 6µm microtome sections stained with Hematoxylin 7211 Richard-Allan Scientific (Fisher) and 3% Eosin Y (Argos Organics 152880250). For immunolocalization, tumor tissue and larval heads were fixed in 4% paraformaldehyde, embedded in Tissue-Tek OCT 4583 (Fisher) and sectioned at 16-20µm. Cryosections
were labeled with the following antibodies: rabbit polyclonal anti-Phosphohistone-H3 (Cell Signaling Technology 9701) at 1:1000; rabbit polyclonal anti-SOX2 (EMD Millipore AB5603) at 1:200; rabbit polyclonal anti-SOX10 (GeneTex GTX128374) at 1:200; rabbit polyclonal anti-OLIG2 (IBL America 18953) at 1:200; mouse monoclonal anti-HuC/HuD (Invitrogen A-21271) at 1:300; rabbit polyclonal Living Colors DsRed (Clonetech 632496) at 1:200; rabbit anti-activate-human-caspase-3 (BD Pharmingen 559565) at 1:500; Alexa-488 (Invitrogen A-11008) and Alexa-594 (Invitrogen A-11005) conjugated secondary antibodies were used at a dilution of 1:500. Sections were stained with DAPI and mounted in Fluoro-Gel II containing DAPI (Electron Microscopy Sciences 17985-50). Immunolabeled sections were imaged on a Zeiss LSM700 laser scanning confocal microscope with 10X, 20X, 40X C-apochromat and 63X Plan-Apochromat objectives. Images and figures were assembled in Adobe Photoshop.

**Isolation of Tol2<ubi:DsRed2> transgenic line**

The *Tol2<ubi:DsRed2>* transgene was assembled in the mini-*pTol2* vector (Balciunas et al., 2006) by cloning in the zebrafish *ubiquitin* promoter (Mosimann et al., 2011) followed by the *DsRed2* cDNA (Clonetech) and the zebrafish β-actin 3’UTR (Dr. Darius Balciunas, Temple University; (McGrail et al., 2011) ). 1µg of linearized *pT3TS-Tol2* transposase plasmid (Balciunas et al., 2006) was used for *in vitro* synthesis of *Tol2* transposase capped, polyadenylated mRNA using the T3 mMessage mMachine High Yield Capped RNA transcription kit (Ambion AM1348). mRNA was precipitated with LiCl and resuspended in RNase, DNase-free molecular grade water. The *Tol2<ubi:DsRed2>* transgenic line was isolated by co-injection of 25pg *Tol2* vector plus 100pg *Tol2* mRNA into 1 cell-stage zebrafish embryos. Two founder fish were screened
for germline transmission of ubiquitously expressed DsRed2. The

*Tg(Tol2<ubi:DsRed2>)* line was established from a single F1 adult.

**Blastula cell transplants to create genetic mosaic zebrafish embryos**

Transplant of cells from blastula-stage embryos was performed as described previously (Wang et al., 2010c). 30-50 cells from blastula stage embryos from adult

*rb1Δ7/+* crossed to *rb1Δ7i/+*; *Tg(Tol2<ubi:DsRed2>)* were transplanted into blastula stage *casper* host embryos, and the remaining donor blastula tissue was used for genotyping. Host embryos were screened for DsRed2-positive fluorescence at 24 and 48 hpf. Three host embryos containing DsRed2-expressing transplanted cells from three individual donor embryos of wildtype +/+ or homozygous mutant *rb1Δ7/Δ7* genotype were aged to 5dpf. Larval heads were dissected, fixed, embedded, sectioned and immunolabeled for confocal imaging as described above.

**CRISPR-Cas9 gRNAs and isolation of loss of function rbpp4 and hdac1 mutations**

The following CRISPR gRNAs were used for somatic targeting of zebrafish *rb1, rbpp4 and hdac1*. *rbpp4* and *hdac1* gRNAs were also used to isolate the stable germline 4bp frameshift mutations *rbpp4Δ4-is60* and *hdac1Δ4-is70*. The *rb1* exon 2 CRISPR site 5’-GGAGAGGGAGATCAGATCGA-3’ contains a *ClaI* restriction enzyme site overlapping the Cas9 cut site upstream of the PAM. The *rbpp4* exon 2 CRISPR site 5’-GATGACCCACGCCCTTGAG-3’ has an *SmlI* restriction enzyme site overlapping the Cas9 cut site. For *hdac1* the exon 5 CRISPR site 5’-GACAGACAGTGCCATTAAC-3’ was used for targeting. CRISPR gRNAs were prepared by the cloning-free single-guide RNA synthesis method (Varshney et al., 2015) using Ambion T7 MEGAscript Kit (Ambion AM1334M) and purified with the Qiagen miRNeasy Kit (Qiagen 217004).
Cas9 mRNA was synthesized in vitro using the mMessage mMmachine T3 kit (Life Technologies AM1348). 5-10ug of pT3TS-nlsCas9nls plasmid (Addgene 46767) was linearized with XbaI and purified with Qiaquick Spin kit (Qiagen 28104). 500ng-1ug purified linear pT3T3-nlsCas9nls DNA was used as template for mRNA synthesis, and the capped nls-Cas9-nls mRNA purified with the miRNeasy kit (Qiagen 217004). Primers for amplicons to test for mutagenesis were as follows: rb1 F 5’-
TTTCCAGACACAAGGACAAGGATCC-3’, R 5’-
GCAGATATCAGAAGAAAGGTACATTTGTCTT-3’; rbhp4 F 5’-
GCGTGATGACAGATCTCATATTGTTTTCC-3’, R 5’-
CTGGTGACATCTGGCAACCCTTCT-3’; hdac1 F 5’-GCGGTGAAACTCAACAAACA-3’, R 5’-GAATGGCCAGTACAATGTCG-3’.

To isolate stable germline mutations, 25pg rbhp4 or hdac1 CRISPR guides were coinjected with 150pg Cas9 mRNA into WIK embryos. 8-10 targeted embryos were tested for mutagenesis by PCR genotyping, and sibling founders raised to adulthood. Adult founder fish were outcrossed to wildtype WIK fish and F1 embryos screened for inheritance of stable germline mutant alleles. Sibling F1 progeny were raised to adulthood and heterozygotes identified by genotyping of fin clip tissue. F2 families of heterozygous mutant fish were established by outcrossing to wild type WIK.

**CRISPR somatic targeting and larval brain live imaging**

CRISPR gRNAs were co-injected with 150pg Cas mRNA into 1 cell Tg(H2A.F/Z-GFP) embryos. At 12hpf embryos were placed in (0.003%) 30mg/liter Phenyl-Thio-Urea (PTU) to inhibit melanin synthesis. 5dpf larvae were mounted in 1.2% low-melt agarose containing 160ug/ml Tricaine methanesulfonate ventral side down on coverslip-bottom
35mm petri dishes. The dish was filled with embryo water (Westerfield, 1995) containing Tricaine and PTU. Live larvae were imaged on a Zeiss LSM700 laser scanning confocal microscope using a 40X W-N Achroplan water immersion objective. Z series containing 11 sections were captured beginning ~30um below the dorsal side of the optic tectum and descending ventrally at 3 um intervals. Three control un-injected Tg(H2A.F/Z-GFP) larvae and three CRISPR gRNA injected Tg(H2A.F/Z-GFP) larvae for each gene were imaged. After imaging larvae were digested in 50mM NaOH and genotyped by PCR. Mitotic figures in each section were counted in ImageJ. Mitotic figure quantification was plotted and the significance calculated using a two-tailed unpaired t test in GraphPad Prism 7.0 (GraphPad Software).

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References


Figure S3.1  Histological analysis of neoplastic populations in TALEN induced rb1-brain tumor T3 and T4 is consistent with primitive neuroectodermal-like tumor. H&E stained sections of tissue remaining after dissection of tumors T3 (A, B) and T4 (C, D) used for transcriptome analysis. Sections exhibit an unencapsulated, multifocally infiltrative neoplastic population within the neuropil. (Figure S3.1 continued) Neoplastic cells are small with deeply basophilic, round to oval to wedge-shaped nuclei, densely clumped chromatin, and scant amounts of cytoplasm. Scale bars A, C 200µm; B, D 50 µm.
Figure S3.2 Correlation coefficients for RNA-Seq libraries from 10 *rb1*-brain tumor and two pools of 3 wildtype adult brains show high correlation between technical and biological replicates.
Figure S3.3 Differentially expressed E2F target genes and Ingenuity Pathway Analysis of zebrafish *rb1*-brain tumor transcriptome. A Heat map of differentially expressed E2F cell cycle target genes in *rb1*-tumor transcriptome (*rb1*-Tumor, T1-T10) compared to normal adult brain (C, C1-C2). B Ingenuity Pathway Analysis Canonical Pathways represented in the *rb1*-tumor transcriptome.
Figure S3.4 Gene Set Enrichment Analysis of the rb1-tumor transcriptome. GSEA shows positive correlation with RB-E2F, ES cell, pediatric tumor marker, ERB2, mTOR, SHH, ATF2, PRC2 and SNF5 pathways, and negative correlation with neurological systems.

Figure S3.5 Correlation coefficients for RNA-Seq libraries from 3 rb1Δ7/Δ7 and 3 +/- 5dpf larva show high correlation between technical and biological replicates.
Figure S3.6 Differentially expressed E2F target genes and Ingenuity Pathway Analysis of zebrafish *rb1Δ7/Δ7* homozygous mutant 5pdf transcriptome. A Heat map (Figure S3.6 continued) of differentially expressed E2F cell cycle target genes in *rb1Δ7/Δ7* mutant transcriptome (M1-M3) compared to +/- wildtype siblings (WT1-3). B Ingenuity Pathway Analysis Canonical Pathways represented in the *rb1Δ7/Δ7* mutant transcriptome.
Figure S3.7 Ectopic M-phase phosphohistone-H3 positive cells in the telencephalon/forebrain and hindbrain of \(rb1\Delta7/\Delta7\) mutant 5dpf larva. A wildtype, +/- and B \(rb1\Delta7/\Delta7\) mutant 5dpf larvae labeled with antibodies to the neuronal marker HuC/D (red) and mitotic M-phase marker phosphohistone-H3 (green). Sections through the telencephalon (C-F) and hindbrain (G-J) show that in wild type mitotic cells are absent or rare, but in the \(rb1\Delta7/\Delta7\) mutant many ectopic phosphohistone-H3 positive cells are scattered throughout the region containing HuC/D-positive cell bodies of neurons.
Figure S3.8  *rb1Δ7/Δ7* mutant cells in M-phase lack expression of the neuronal differentiation marker HuC/D. A, B High magnification image of phosphohistone-H3 positive nuclei and HuC/D-positive cells in the tectum of a 5dpf *rb1Δ7/Δ7* mutant brain. No overlap is detected between cells expressing HuC/D (C, red) and phosphohistone-H3 (D, green, arrows, dashed outlines). The chromatin in the phosphohistone-H3 positive cells appears highly condensed (E), consistent with cells in S phase or prophase/prometaphase of the cell cycle.
A. $rb1$ genotype of donor blastula embryos used for cell transplants to create genetic mosaic animals presented in Figure 3.

![Genotyping Image]

<table>
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B. $rb1$ genotype of 5dpf $H2.A.F/Z-GFP$ larva imaged in time lapse Movies S2-S4.

![Time Lapse Movies]

Figure S3.9 Genotyping of blastula donor embryos used for cell transplants and 5dpf larva used for time lapse imaging. A PCR genotyping of remaining tissue in blastula stage donor embryos from $rb1^{Δ7/+} \times rb1^{Δ7/+}; Tg(Tol2<ubi:DsRed2>)$ after transplanting cells into host *casper* embryos. Genotype of individual embryos is shown below. Sections of host 5dpf larvae containing transplanted cells from $+/+$ embryo #13 and $rb1^{Δ7/Δ7}$ embryos #6 and #12 are shown in Figure 3. B PCR genotyping of 5dpf $+/+; Tg(H2.A.F/Z-GFP)$ and $rb1^{Δ7/Δ7}; Tg(H2.A.F/Z-GFP)$ larva imaged in time lapse Movies S2-S4.
Table S3.1 Reads and quality control statistics from rb1-brain tumor and normal adult brain RNA-Seq libraries.

<table>
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<th>Sample</th>
<th>Yield (Mbases)</th>
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Library replicates A and B were sequenced by 50pb single read HiSeq Rapid Run in 2 separate lanes.

Table S3.2 Reads and quality control statistics from zebrafish homozygous mutant rb1Δ7/Δ7 and wildtype +/+ 5dpf larval head RNA-Seq libraries.

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Libraries were sequenced by 50pb single read HiSeq Rapid Run in each of 2 separate lanes, A and B.
Table S3.3  qRT-PCR primers.

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Table S3.4  Quantification of phosphohistone H3-positive M-phase cells in live 5dpf larva.

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CHAPTER 4. RBBP4 MAINTAINS CYCLCING STEM CELLS AND BLOCKS NEURAL PRECURSOR TP53-DEPENDENT DNA DAMAGE AND APOPTOSIS IN ZEBRAFISH

Modified from a paper to be submitted to the journal Development

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\(^1\) Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA

*L.E.S. performed all experiments, generated figures, and wrote manuscript with editing by M.M.

Abstract

Retinoblastoma-Binding Protein 4 (RBBP4) is a chromatin adaptor protein that associates with numerous activating and repressive chromatin regulatory complexes. RBBP4 is overexpressed in human gliomas, and has been shown to promote survival of GBM cells and p300-dependent expression of DNA repair genes (Kitange et al., 2016). We previously established a zebrafish RB-defective neural progenitor-like brain tumor model (Solin et al., 2015) and recently found Rbbp4 is highly upregulated in the RB-brain tumors. Here we demonstrate Rbbp4 is essential for zebrafish brain development and show it has distinct requirements in neural stem and progenitor cells. CRISPR/Cas9 targeting was used to isolate a 4 base-pair frameshift mutation in the zebrafish homolog rbbp4. Homozygous mutant rbbp4\(^{Δ4/Δ4}\) are lethal at approximately 5-7 days post fertilization and show a dramatic reduction in the size of the brain and retina. Overexpression of rbbp4 cDNA in a Tg(Tol2<ubiquitin:rbbp4>) transgenic line rescues
the *rbbp4* mutant phenotype, demonstrating disruption of neurogenesis in *rbbp4Δ4/Δ4* homozygotes is due to lack of functional Rbbp4. Beginning at 2 days post fertilization, γ-H2AX and activated caspase labeling was detected in post-mitotic regions in the larval midbrain and retina, suggesting RBBP4 is required to prevent DNA-damage and apoptosis in neural progenitors or newborn neurons. γ-H2AX and caspase were not detected in the stem cell niches at the brain ventricle or retinal ciliary marginal zone, however, stem cell morphology was abnormally large. Pulse chase BrdU labeling experiments confirmed a delay in stem cell proliferation and a lack of survival of post-mitotic neural progenitors. TP53 morpholino knockdown in *rbbp4Δ4/Δ4* homozygotes transiently suppressed neural progenitor apoptosis, indicating the absence of Rbbp4 activates TP53-dependent programmed cell death. Together these results suggest distinct requirements for Rbbp4 in neural stem cell proliferation and neural progenitor survival. Overall, our results indicate RBBP4 may drive RB-defective brain tumor growth by promoting proliferation and preventing DNA damage induced apoptosis of tumor cells.

**Introduction**

Chromatin modification is an important and diverse mechanism employed by the cell to control gene expression beyond the level of the basal transcriptional machinery. Chromatin is comprised of an octamer of histones, two each of H2A, H2B, H3 and H4, encompassed by 146 bp of DNA which together make up the nucleosome. Densely packed heterochromatin is generally associate with decreased gene expression, while open euchromatin is associated with increased gene expression. Several modifications to the N- or C-terminal tails of histones are known such as alterations such as adenylation, acetylation, methylation, phosphorylation, and ubitiylation. There is a wide array of
chromatin modifying complexes that are responsible for generating and reading these modifications (reviewed in (Clapier and Cairns, 2009)). While the role of chromatin modification and epigenetic regulation is well understood in a developmental paradigm, how these mechanisms are altered in transformed cells and promote tumorigenesis is less well understood.

The zebrafish embryo, with its transparency and rapid development, and large numbers, makes an excellent model to study neurogenesis (reviewed in (Schmidt et al., 2013)). As such the development of the central nervous system (CNS) of the zebrafish has been well defined. The CNS is specified as early as the 16-cell stage in which four blastomeres are generated that will eventually give rise to the entire nervous system. During gastrulation, the neural plate or neural ectoderm is specified and then undergoes neurulation to form a solid neural keel unique among zebrafish. Neurogenesis begins shortly after gastrulation and the early embryonic phase of zebrafish development is defined by rapid proliferation and neurogenesis leading to the appearance of distinct brain and retina structures by 2 days post fertilization (dpf). Indeed by 2 dpf zebrafish embryos are already able to move and respond to stimuli. The next larval stage of development is marked by a decrease in proliferative activity and the increased neural specification and formation of specific brain structures and retinal layers. The retina starts as an optic cup containing a pseudostratified epithelium. At 24 hpf a wave of neurogenesis originating at nasal retina progresses to the temporal retina such that by 60 hours post fertilization (hpf) all retinal cell types are specified. By adulthood, zebrafish contain neurogenic regions along the entire A-P axis and in fact parts of the brain and retina can regenerate (Kizil et al., 2012) (Than-Trong and Bally-Cuif, 2015).
Neurogenesis begins with stem cells located at specific niches in the brain and retina which give rise to proliferative progenitors that generate an expanded progenitor pool. This pool of progenitors is maintained by lateral inhibition through Notch/Delta (Appel et al., 2001). The process of becoming a neuron starts with the expression of proneural transcription factors, such as acscl1a, neurog1, and neuroD, which promote progressive steps of specification and commitment in progenitor cells toward a neural fate. This culminates in final cell cycle exit and the process of differentiation whereby the cell adopts the morphology and function of the cell type it has been specified to become. Concomitant with this process of specification is the migration of progenitor cells away from neurogenic niches in both the brain and retina, usually along the processes of radial glia, to their final specified location in the brain or retina (Wullimann and Puelles, 1999) (Mueller and Wullimann, 2003).

RBBP4 (RB Binding Protein 4), along with its homolog RBBP7 (RB Binding Protein 7) (Qian and Lee, 1995), is highly conserved in organisms ranging from plants to invertebrates to mammals (Ach et al., 1997) (Guitton and Berger, 2005) (Souza et al., 2002) (Wen et al., 2012). Structurally RBBP4 is a WD40 domain protein exhibiting a 7 blade β-propeller structure. While it does not have any enzymatic activity, RBBP4 binds with histone H4 (and NuRD complex member MTA1) through a binding pocket on the side of the β-propeller and histone H3 (and transcriptional regulator FOG1) through a binding pocket on the top of the β-propeller (Song et al., 2008; Torrado et al., 2017) (Nowak et al., 2011a) (Zhang et al., 2013) (Lejon et al., 2011). RBBP4 was first discovered as a binding partner of the tumor suppressor RB1 in yeast (Qian et al., 1993). RBBP4 is a component of several major chromatin modifying complexes, and it is
thought that the main functionality of RBBP4 is through providing specificity and stability of these complexes to chromatin.

Included among these are the co-repressor complexes NuRD (Xue et al., 1998) (Zhang et al., 1999) and SIN3 (Vermaak et al., 1999) which repress gene expression via de-acetylation by HDAC1 and HDAC2. The NuRD complex also remolds chromatin through chromodomain ATP dependent helicase CHD4 and is involved in DNA damage repair, stem cell biology, and development (Basta and Rauchman, 2015; Denslow and Wade, 2007; O'Shaughnessy and Hendrich, 2013). Additionally, RBBP4 is a component of PRC2 which is involved in gene silencing through H3 lysine 27 di- and tri-methylation (H3K27me2/3) via Ezh1 or Ezh2 (Kuzmichev et al., 2002) (Vaute et al., 2002) (Margueron et al., 2008). In addition to its role in repressive complexes, RBBP4 is also a member of CAF-1 (Tyler et al., 1996) (Verreault et al., 1996) and CBP/p300 (Zhang et al., 2000) which are involved in nucleosome assembly after replication and transcriptional co-activation respectively. Finally, RBBP4 is plays a role in the DREAM complex which controls cell cycle and entry into quiescence (Litovchick et al., 2007).

Early work in *C. elegans* revealed that the RBBP4 homolog *lin-53* is ubiquitously expressed during embryogenesis, loss of *lin-53* is embryonic lethal, and it is required to negatively regulate the expression of genes involved in vulval induction (Lu and Horvitz, 1998). Additionally, studies in planarians have shown that the RBBP4 homolog *DjRbAp48* is expressed in neoblasts and is required for functional regeneration after wounding in the adult (Bonuccelli et al., 2010). While these limited studies suggests a definite role for RBBP4 in development, many recent studies have also implicated
RBBP4 in the progression of tumorigenesis as well as the response of transformed cells to different cancer treatments (Zheng et al., 2013) (Wu et al., 2017) (Kitange et al., 2016). We recently found that Rbbp4 is upregulated in a zebrafish model of rb1-primitive neuroectodermal brain tumors and showed that Rbbp4 is required for neural precursor survival during neural development (Schultz et al., 2017, Submitted). Here we examine the expression of Rbbp4 in neural cells in the developing zebrafish brain and retina and provide a molecular characterization of the mutant phenotype. Our results show that Rbbp4 is expressed in the brain and retina of embryonic and larval zebrafish. Histological analysis reveals this cause of this gross phenotype to be the apoptosis of neural precursors in the dorsal midbrain and retina prior to differentiation. Additional experiments indicate the accumulation of DNA damage and a tp53-dependent apoptotic response leading to tissue loss in the absence of Rbbp4.

**Results**

**Rbbp4 is expressed throughout the zebrafish embryonic brain**

In order to elucidate the role of RBBP4 in zebrafish neural development, we first characterized its expression in the wildtype embryonic midbrain and retina. Immunolocalization with an antibody against Rbbp4 was used to label sectioned zebrafish heads. We examined heads at 2 days post fertilization (dpf) during rapid proliferation and differentiation as well at the post-embryonic stage at 5 dpf. Western blot on whole protein extract from 5 dpf larvae showed a single band of approximately 47 kDa, confirming the specificity of the antibody in zebrafish (Supplemental Figure S4.1A).
At 2 dpf the zebrafish midbrain consists of a highly proliferative dorsal optic tectum and a ventral region, both of which contain proliferating cells lining the ventricles juxtaposed by regions of neural specification and differentiation (Mueller and Wullimann, 2003). Immunolabeling revealed Rbbp4 is present in the nuclei throughout all cells of the midbrain and retina (Figure 4.1A’,B’,C’). Furthermore, Rbbp4 co-labeling with PCNA, an S-phase marker of proliferating cells, was observed at the brain ventricle and CMZ of the retina (Figure 4.1A,A’’,B,B’,C,C’’). These results indicate that at this stage RBBP4 is expressed in both proliferating precursor and stem cells and early-differentiated neural cells.

**Rbbp4 expression levels vary in stem cells, precursors and neurons in the larval midbrain**

After the transition from embryonic to larval neurogenesis, growth in the brain becomes asymmetric (Mueller and Wullimann, 2003) (Stigloher et al., 2008). By 5 dpf regions of proliferation are confined to the stem cell niches at the ventricles and the medial tectal proliferative zone in the brain (Figure 4.2A-C’’’) and the ciliary marginal zone (CMZ) in the retina (Figure 4.2D-E’’’) (Wullimann and Puelles, 1999) (Schmitt and Dowling, 1999). At this stage the level of Rbbp44 in the midbrain begins to vary in intensity in the proliferative zones which contain stem and progenitor cells. Specifically, there appears to be a decrease in the level of Rbbp4 in cells in the stem cell niches in the brain and retina, and varying levels in nuclei of post-mitotic neurons. In the medial tectal proliferative zone in PCNA+ cells Rbbp4 expression is absent or very faint (Figure 4.2B-B’’’ outlined area). At the midbrain ventricles in the tectum and dorsal thalamus, expression is greatly reduced or completely absent in most cells (Figure 4.2C-C’’’
Figure 4.1  Rbbp4 is ubiquitously expressed in the embryonic zebrafish brain and retina. (A-A’’’) Representative transverse cryosection (n=3) through the 2 dpf wildtype zebrafish midbrain labeled with antibodies to Rbbp4 (green), proliferation maker PCNA (red), nuclear stain DAPI (blue). Boxes outline the ventricle at the top of the thalamus in the midbrain (B) and the ciliary marginal zone (CMZ) of the retina (C). (B-B’’’) Higher magnification view of the midbrain ventricle at the thalamus. (C-C’’’) Higher magnification view of the ciliary marginal zone of the retina (C-C’’’). Scale bars: 100 um (A-A’’’), 20 µm (B-C’’’).

outlined area). However, there are a few cells in which co-labeling with Rbbp4 and PCNA does occur (Figure 4.2B-B’’’, C-C’’’ arrows). Lateral to the proliferative zones in the optic tectum and the thalamus in the regions containing post-mitotic neurons, Rbbp4 expression is relatively ubiquitous though present at varying levels (Figure 4.2A-A’’’).
Figure 4.2  Rbbp4 expression levels vary in neural precursors and neurons in the larval zebrafish brain and retina. Representative transverse cryosection (n=3) of 5 dpf wildtype zebrafish midbrain (A-A''') and retina (D-D''') labeled with antibodies to Rbbp4 (green), proliferation maker PCNA (red), nuclear stain DAPI (blue). Higher magnification view of the medial tectal proliferative
(Figure 4.2 continued) zone (B-B'''), brain ventricle (C-C'''), ciliary marginal zone (E-E'''), and central laminated layers of the retina (F-F'''). The highly proliferative, PCNA+ population of cells are outlined; arrows indicate cell co-labeled with Rbbp4 and PCNA. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars: 100 µm (A-A'''') (D-D'''), 10 µm (B-B'''') (C-C'''') (E-E'''') (F-F''').

In the retina at 5 dpf the proliferative CMZ containing stem and progenitor cells, like the proliferative regions of the midbrain, shows a reduction in the level of Rbbp4 compared to the more mature regions of the inner retina (Figure 4.2D-D''',E-E''' outlined area). Expression in the ganglion cell layer (GCL) is similar to post-mitotic regions of the midbrain with ubiquitous expression at varying intensities (Figure 4.2F-F'''). On the vitreal side of the inner nuclear layer (INL) where the cell bodies of amacrine cells and Mueller glia are located, high level expression is seen in all cells, while expression is seen only in a subpopulation of cells in the portion of the INL containing the bipolar cells (Figure 4.2F-F'''). In the outer nuclear layer (ONL) a low level of Rbbp4 is detected in the photoreceptor cells (Figure 4.2F-F'''). In summary, during the developmentally active and dynamic period at 2 dpf, Rbbp4 is expressed ubiquitously in the stem, precursor, and newly born neural cells in the midbrain and retina. However, by 5 dpf Rbbp4 expression is enriched in neurons in post-mitotic regions of the brain and retina, while the level of RBBP4 is reduced in proliferative regions containing stem and progenitor cells. These results suggest Rbbp4 is required in post-mitotic or newly differentiated cells in the zebrafish brain and retina.

Rbbp4 is essential for development of brain and neural crest derived structures

Previously we isolated a 4 base pair frameshift deletion \( \text{rbbp4}\Delta^{460} \) allele using CRISPR/Cas9 targeting and demonstrated the requirement for Rbbp4 in normal neural
development (Schultz et al., 2018, Submitted). Homozygous rbbp4Δ4/Δ4 larvae display defects in cellular texture in the midbrain that can be observed at 2 dpf under a stereomicroscope (data not shown). By 4-5 dpf, rbbp4Δ4/Δ4 homozygotes show gross neural developmental defects including microcephaly and microphthalmia as compared to wildtype (Figure 4.3A,B). Western Blot analysis and Rbbp4 immunolabeling on 5 dpf embryos confirmed that the rbbp4Δ4/Δ4 mutants lack Rbbp4 protein (Supplemental Figure S4.1A,B,C). Additional characterization of the gross rbbp4Δ4/Δ4 mutant phenotype by alcian blue staining revealed several abnormalities formation of cartilage structures in the head. This included abnormal positioning of the ceratohyal cartilage (Figure 4.3C-F), compression of the ceratobranchial cartilage (Figure 4.3E,F), and shrinkage of the Meckel’s cartilage (Figure 4.3E,F). These results indicate that RBBP4 is essential for proper development of the central nervous system.

We performed overexpression rescue experiments to confirm that the observed rbbp4Δ4/Δ4 mutant phenotype is due to lack of functional Rbbp4. A Tol2<ubi:rbbp4-2A-GFPpA> transposon construct was built containing the zebrafish ubiquitin promoter (Mosimann et al., 2011) cloned in front of the full length rbbp4 cDNA, followed by the viral 2A peptide and the green fluorescent protein (GFP) cDNA (Figure 4.3G). Transgenics were isolated by Tol2 transposase-mediated transgenesis and a single copy insertion line Tg(Tol2<ubi:rbbp4-2A-GFPpA>)is61 was established. rbbp4Δ4/Δ4 embryos containing the Tg(Tol2<ubi:rbbp4-2A-GFPpA>) transgene as indicated by the ubiquitous expression of GFP were viable through larval stages and showed a rescue in gross mutant morphology. Comparisons of eye width (Figure 4.3A, white arrow) and midbrain height (Figure 4.3A, black arrow) were performed between wildtype and rbbp4Δ4/Δ4 embryos at 5
Figure 4.3  rbbp4 cDNA rescue of rbbp4<sup>44/44</sup> homozygotes demonstrates requirement for Rbbp4 in brain and neural crest development. (A) Lateral view of 5 dpf wildtype zebrafish indicating location of measurements for brain height (black arrow) and eye width (white arrow). (B) Lateral view of 5 dpf rbbp4<sup>44/44</sup> homozygote showing gross defects including microcephaly and microphthalmia. Lateral view of 5 dpf wildtype (C) and rbbp4<sup>44/44</sup> homozygous (D) larval stained with alcian blue to reveal cartilage structures. (E) Ventral view of wildtype and (F) rbbp4<sup>44/44</sup> homozygote stained with alcian blue. (G) Schematic of Tol2<sub>ubi</sub>:rbbp4-2A-eGFP cDNA rescue construct driving expression of rbbp4-2A-eGFP by the ubiquitin promoter. (H) 5 dpf transgenic Tg(Tol2<sub>ubi</sub>:rbbp4-2A-eGFP) and (I) 5 dpf transgenic rbbp4<sup>44/44</sup>; Tg(Tol2<sub>ubi</sub>:rbbp4-2A-eGFP) larva showing ubiquitous GFP expression. Ubiquitous expression of rbbp4 rescues the rbbp4<sup>44/44</sup> homozygous mutant phenotype. (J) Measurement of brain height in non-transgenic (GFP-) and transgenic (GFP+) +/+ and rbbp4<sup>44/44</sup> siblings. Non-transgenic rbbp4<sup>44/44</sup>, GFP- homozygotes (n=6) have a significantly smaller head height compared to wildtype (n=3) (p=0.0015). Comparison of +/+ GFP+ (n=4) and transgenic rbbp4<sup>44/44</sup> GFP+ (n=4) shows no significant difference in head height (p=0.4595). (K) Measurement of eye width in non-transgenic (GFP-) and transgenic (GFP+) +/+ and rbbp4<sup>44/44</sup> siblings. Non-transgenic rbbp4<sup>44/44</sup> GFP- homozygotes (n=8) have a significantly smaller eye width compared to wildtype (n=5) (p=0.0001). Comparison of +/+ GFP+ (n=8) and rbbp4<sup>44/44</sup> GFP+ (n=5) shows no significant difference in eye size (p=0.6293). Graphs plot measurement in individual larvae with mean ± s.e.m. p values were calculated with one-tailed Student's t-test. ch, ceratohyal cartilage; m, Meckel's cartilage; cbs, ceratobranchial cartilage. Scale bars: 100 µm.
dpf (Figure 4.3H,I). The homozygous embryos lacking the overexpression transgene show a statistically significant decrease in the size of both the eye and brain (Figure 4.3A,B,J,K). However, homozygotes containing the overexpression transgene showed no significant difference in the size of the eye or height of the midbrain in comparison to wildtype (Figure 4.3H-K, Supplemental Figure S4.2). \( Tg(Tol2<ubi:rbbp4-2A-GFPpA>) \) containing animals displayed no defects in growth, viability, or fertility (Supplemental Table S4.1). Furthermore, \( rbbp4^{Δ4/Δ4}; Tg(Tol2<ubi:rbbp4-2A-GFPpA>) \) individuals are viable and fertile into adulthood. These results indicate that the observed defects in neural development are caused by a loss of Rbbp4.

**Loss of Rbbp4 leads to stem cell hypertrophy and disrupts midbrain and retina development**

We next examined homozygous \( rbbp4^{44/44} \) mutants for the effect of loss of Rbbp4 on neural cell populations in the developing brain and eye. 5 dpf larvae were labeled with antibodies to stem/progenitor cell marker Sox2 and the proliferating cell marker PCNA. In the wildtype brain Sox2-positive cells are found lining the ventricle and scattered through the dorsal tectum and may represent a stem and/or progenitor cells (Figure 4.4A,C). Intense PCNA labeling is apparent at the ventricle and in cells in the dorsal medial tectal proliferative zone where the proliferating stem and progenitors are located (Figure 4.4A,C). In the midbrain the \( rbbp4^{44/44} \) mutant shows a dramatic reduction in the size of the optic tectum on the dorsal side while the ventral portion of the brain remains relatively normal (Figure 4.4A-D, brackets). The PCNA-positive proliferative progenitor cells in the dorsal medial tectal proliferative zone are missing (Figure 4.4C,D). With the exception of the specific population of cells comprising the subcommissual organ, the
Figure 4.4  Neurogenesis is disrupted in the midbrain tectum and retina in \textit{rbbp4}^{Δ4/Δ4} mutant larvae. (A-H) Transverse sections of midbrain and retina from wild type +/+ (n=5) and homozygous mutant \textit{rbbp4}^{Δ4/Δ4} (n=4) 5 dpf zebrafish larvae labeled with antibodies to proliferative marker PCNA (red) and
(Figure 4.4 continued) stem/progenitor marker Sox2 (green) and the nuclear stain DAPI (blue). (A, C) In wildtype PCNA and Sox2-positive stem cells line the midbrain ventricle (A, C dashed outline). Sox2-positive progenitors lie adjacent to the ventricle in the tectum (C bracket). (B, D) PCNA and Sox2-positive cells with enlarged morphology line the midbrain ventricles in \( rbhp4^{A4/A4} \) mutant (B, D outline). The dorsal tectum is significantly reduced in height in the \( rbhp4^{A4/A4} \) mutant (D bracket). (E, G) In wild type retina PCNA-positive stem cells are restricted to the outer edge of the ciliary marginal zone (G outline). Sox2-positive amacrine and displaced amacrine cells are distributed throughout the inner nuclear and ganglion cell layers. (F, H) PCNA-positive stem cells with enlarged, hypertrophic morphology are present at the ciliary marginal zone in \( rbhp4^{A4/A4} \) mutant retina (F, H outline). Arrow shows missing tissue adjacent to the ciliary marginal zone. Sox2-positive cells representing early born amacrine cells are present in the retinal layers. (I-P) Transverse section of midbrain and retina from wild type +/+(n=3) and \( rbhp4^{A4/A4} \) homozygous (n=3) labeled with antibodies to glial markers Gfap (red) and Blbp (green) and nuclear stain DAPI (blue). (I, K) In wild type midbrain Blbp and Gfap label radial glia cell bodies and projections in the tectum (K bracket) and thalamic regions, respectively. (J, L) The \( rbhp4^{A4/A4} \) mutant midbrain contains mature radial glia in the thalamic region (J). Intense Blbp labeling is present where hypertrophic stem cells are located at the ventricle (L bracket). (M, O) Blbp and Gfap label Mueller glia cells bodies and projections across the inner plexiform layer of the wild type retina. (N, P) Early born radial glia can be detected with Blbp and Gfap in the \( rbhp4^{A4/A4} \) mutant retina. (Q-X) Transverse section of midbrain and retina from wild type +/+(n=3) and \( rbhp4^{A4/A4} \) mutant (n=3) 5 dpf larvae labeled with antibodies to the synaptic vesicle marker Sv2 (red) and interneuron and ganglion cell marker Calretinin (green) and the nuclear stain DAPI (blue). (Q, S) Sv2 labels the neuropil of wild type brain and Calretinin labels the cell bodies of a subset of mature neurons in the thalamus and tectum (S bracket). (R, T) The \( rbhp4^{A4/A4} \) mutant shows a reduction in the tectal neuropil and number of Calretinin-positive neurons (T bracket). (U, W) Sv2 and Calretinin labeling reveal lamination of the wild type retina. (V, X) Early born neurons maintain Calretinin labeling and Sv2 labeled projections across the inner and outer plexiform layers in the \( rbhp4^{A4/A4} \) mutant retina. Scale bars: 50 µm (A, B, E, F, I, J, M, N, Q, R, U, V); 20 µm (C, D, G, H, K, L, O, P, S, T, W, X).

Sox2 expressing cells are also absent from the dorsal midbrain in the \( rbhp4^{A4/A4} \) mutant (Figure 4.4C,D). PCNA/Sox2-positive cells remained present in the stem cell niche lining the brain ventricle of the \( rbhp4^{A4/A4} \) mutant, however they appeared hypertrophic (Figure 4.4D, outlined).
The wildtype retina consists of the CMZ containing proliferative stem cells (Figure 4.4E,G, outlined). This niche gives rise to the cells that eventually mature and populate the ordered layers of the laminated central retina consisting. In the retina of the \textit{rbbp4}^{	ext{D4/D4}} mutants some lamination is still present in the central retina though overall the retina is reduced in size (Figure 4.4F,N,V). Similar to the brain, the PCNA/Sox2-positive presumed stem cells at the CMZ of the retina are present and again show an enlarged hypertrophic morphology (Figure 4.4H, outlined). Adjacent to the CMZ at the site of neurogenesis and progenitor cell commitment and differentiation there is an obvious area of missing tissue (Figure 4.4H, arrows).

We next examined whether the loss of RBBP4 affected neuronal and glial populations. In wildtype brain Blbp and Gfap are expressed in radial glial cells along the ventricle of the dorsal midbrain (Figure 4.4I,K) and in Müller glia extending from the ganglion cell layer (GCL) to the inner nuclear layer (INL) in the retina (Figure 4.4M,O). Gfap labels glial processes in the ventral midbrain (Figure 4.4I) and emanating from the GCL in the retina (Figure 4.4M,O). In the \textit{rbbp4}^{	ext{D4/D4}} mutants there is continued Blbp and Gfap labeling in the brain and retina (Figure 4.4J,L,N,P). However, the Blbp labeling in particular appears decreased in areas where there is a dramatic loss of tissue such as the dorsal midbrain and under-developed central retina (Figure 4.4L,P). Antibody labeling with the synaptic vesicle marker SV2 and neuronal calcium binding protein Calretinin was performed to examine populations of mature neuronal cells and grey matter. In wildtype Sv2 labels the acellular regions of the peripheral midbrain (Figure 4.4Q) and the plexiform layers of the retina (Figure 4.4U). Calretinin labels cells in the brain at the peripheral regions away from the ventricles (Figure 4.4Q,S) and cell bodies and processes
in the GCL, IPL, and INL of the retina (Figure 4.4U,W). In the \textit{rbbp4}^{Δ4/Δ4} mutant where mature tissue is still present in the midbrain and central retina Calretinin and Sv2 labeling is still apparent (Figure 4.4R,T,V,X). The fact that the ventral midbrain and central retina in the \textit{rbbp4}^{Δ4/Δ4} mutants appear somewhat normal may be due to the presence of maternal \textit{rbbp4} RNA. This early and temporary \textit{rbbp4} expression may protect the earliest born progenitors and neurons in the eye and brain, allowing them to migrate to their final location where they undergo normal differentiation. Together, the above results indicate that Rbbp4 is not required for survival of stem cells or differentiated neurons and glial cells. However, Rbbp4 is necessary for survival of neural precursors that become the mature glia and neurons in the growing brain and retina during development. This defect in neural precursor survival accounts for the observed microcephaly and microphthalmia seen in the \textit{rbbp4}^{Δ4/Δ4} mutant.

\textbf{Loss of Rbbp4 leads to neural precursor arrest and apoptosis at the terminal cell division}

Examination of \textit{rbbp4}^{Δ4/Δ4} mutants with stem/progenitor, neural, and glial markers suggested that the defect in dorsal brain and retina development is due to the loss of neural precursors in the absence of Rbbp4. To explore the timing of \textit{rbbp4} mutant neural precursor loss, we examined expression of markers of progressive neural cell fate commitment in the post-embryonic zebrafish retina. Neurogenesis in the zebrafish retina is spatially organized such that specific neural cell populations can be finely resolved into distinct regions. Stem cells are located at the ciliary marginal zone (CMZ) at the periphery while mature glia and neurons are located inwards towards the central part of the retina (Mosimann et al., 2011). Retinal neurogenesis has been described as a
conveyor belt in which cells generated at the CMZ undergo progressive steps of differentiation as they migrate inward toward the central retina (Cerveny et al., 2012) (Devès and Bourrat, 2012).

We first used BrdU labeling to examine the fate of newborn cells at the CMZ in the \textit{rbbp4}\textsuperscript{Δ4/Δ4} mutants. At 2 pdf embryos were exposed to a 3 hour BrdU pulse and collected immediately or chased in embryo media until 3 dpf and 5 dpf. Immediately following the pulse, in 2 dpf wildtype embryos BrdU labeling is present in cells at the CMZ and in the inner nuclear layer and photoreceptor layer of the retina (Figure 4.5A). In the 2dpf \textit{rbbp4}\textsuperscript{Δ4/Δ4} mutant retina BrdU labeling at the cmz region appears similar to wildtype, but the inner nuclear layer appears slightly disorganized (Figure 4.5B). At 3 dpf in the wildtype retina the classic conveyor belt pattern of neurogenesis is observed. BrdU labeling is no longer detected in cells at the CMZ as BrdU positive cells generated during the pulse at 2 dpf have migrated away from the CMZ with the newest cells born post-labeling filling in behind them (Figure 4.5C, outline). In the \textit{rbbp4}\textsuperscript{Δ4/Δ4} mutants at 3 dpf, cells in the CMZ are no longer labeled, and a small population of BrdU-positive cells can be detected adjacent to the cmz migrating away from the periphery (Figure 4.5D, outline). This limited population of BrdU positive cells at 3 dpf could indicate that the stem cells are continuing to divide but at a reduced rate. At 5 dpf in the wildtype retina the conveyor belt pattern of movement is again seen as BrdU positive cells have migrated even further toward the central retina leaving stem cells and newly born unlabeled cells at the peripheral most CMZ (Figure 4.5E, outline). In the \textit{rbbp4}\textsuperscript{Δ4/Δ4} mutant by 5 dpf the population of BrdU labeled cells appears to be almost completely gone (Figure 4.5F) suggesting that the progeny of the stem cells, either committed progenitors or newborn
Figure 4.5  BrdU pulse-chase labeling to examine the fate of newborn neurons in the \textit{rbbp4\textsuperscript{44/44}} mutant retina. (A, B) 2 dpf zebrafish embryos treated with a 2.5 hour BrdU pulse and immediately sacrificed. Transverse sections of retina from wildtype (n=4) and \textit{rbbp4\textsuperscript{44/44}} mutants (n=5) were labeled with antibodies to BrdU and mitotic marker phosphohistoneH3 (pH3). BrdU labels proliferating cells at the retina ciliary marginal zone and cells scattered throughout the laminating retina. (C,D) Pulse-chased larvae at 3 dpf. In wildtype retina (n=8) BrdU labeled cells are located in the region adjacent to the cmz where neural precursors and newly differentiated neurons reside (C outline). A small section of BrdU labeled cells remains at the ciliary marginal zone in the \textit{rbbp4\textsuperscript{44/44}} mutant retina (n=6) (D outline). (E,F) Pulse-chased larvae at 5 dpf. In wildtype (n=7) the BrdU labeled cells are now more centrally located in an older region of the growing retina (E outline). \textit{rbbp4\textsuperscript{44/44}} mutant retina (n=7) older born neurons in the central retina maintain BrdU labeling, however, BrdU-labeled newborn neurons are absent. BrdU-negative stem cells persist at the ciliary marginal zone in the \textit{rbbp4\textsuperscript{44/44}} mutant (F arrow). Scale bars: 50 µm.

neurons, failed to survive after leaving the CMZ. The population of stem cells at the CMZ however remains intact (Figure 4.5F, arrow). The BrdU labeled cells in the central part of the mutant retinas are most likely rod progenitors derived from early born Mueller
glia in the INL (Figure 4.5F). Together, these results suggest that in the absence of Rbbp4 neural stem cells continue to cycle, but at a slower rate than in wildtype. Progenitors or newborn neurons fail to survive, possibly due to an inability to commit to or initiate neuronal differentiation.

In order to identify the stage at which retinal neural precursors die in the rbbp4Δ4/Δ4 mutant we performed in situ hybridization with markers that distinguish retinal progenitors progressively more committed toward a neuronal cell fate. In the wildtype CMZ at 3 dpf, the peripheral-most cells label with the stem cell marker mz98 (Pujic et al., 2006) (Figure 4.6A, arrow). The next population of cells are the ccnD1-expressing proliferating progenitors (Das et al., 2009) (Figure 4.6B, bracket). Following these are the ath5-expressing neural precursors committed to neural cell fate (Masai et al., 2000) (Poggi et al., 2005) (Figure 4.6C, bracket). Finally are the cdkn1-expressing cells which have exited the cell cycle, arrested in G1 and are in the process of transitioning to a differentiated cell (Ohnuma et al., 1999) (Shkumatava and Neumann, 2005) (Figure 4.6D, arrow). In the rbbp4Δ4/Δ4 mutant retina mz98-expressing stem cells are restricted to a small population at the edge of the CMZ (Figure 4.6E, arrow). The population of ccnd1- and ath5-expressing cells appears expanded in the mutant (Figure 4.6F,G brackets). Finally, whereas there is a clear population of cdkn1c-expressing cells in the wildtype retina, this population of cells is absent in the rbbp4Δ4/Δ4 mutant retina (Figure 4.6H, arrow). These results suggest Rbbp4 is necessary for neural precursor committed cells to complete terminal cell division and exit the cell cycle in G1. The failure of ath5-expressing cells to differentiate likely underlies neural precursor cell death and the observed tissue loss in the retina of the rbbp4Δ4/Δ4 mutant.
Figure 4.6 Neural precursors fail to differentiate in the \textit{rbbp4}^{Δ4/Δ4} homozygous mutant retina. Transverse sections of 3 dpf zebrafish retina labeled by \textit{in situ} hybridization to examine expression of the stem cell marker \textit{mz98} (A, E arrows), proliferating progenitor marker \textit{ccnD1} (B,F brackets), committed progenitor marker \textit{ath5} (C,D brackets) and differentiating precursor marker \textit{cdkn1c} (D,H arrows). (A-D) Wildtype retina (n=5) shows location of progressively committed precursor cell populations extending away from the stem cells at the ciliary marginal zone. In the \textit{rbbp4}^{Δ4/Δ4} homozygous mutant retina (n=3) the proliferating and committed neural progenitor cell populations are expanded compared to wildtype. Expression of the differentiating neural precursor marker \textit{cdkn1c} is absent in the \textit{rbbp4}^{Δ4/Δ4} mutant retina (H arrow). Scale bars: 50 µm.

\textbf{Rbbp4 is required to prevents apoptosis and persistent DNA damage in neural precursor cells}

To determine if Rbbp4 is required to prevent programmed cell death in neural precursors we examined apoptosis in the \textit{rbbp4}^{Δ4/Δ4} mutant using an antibody to activated Caspase-3. At 2 dpf in the wildtype dorsal midbrain and retina there is almost a complete absence of Caspase-3 labeling (Figure 4.7A). At 2 dpf in the \textit{rbbp4}^{Δ4/Δ4} mutant there is extensive Caspase-3 labeling in the dorsal midbrain and retina (Figure 4.7B). At 3 dpf there is a limited amount of random Caspase-3 labeling in the midbrain and retina (Figure
Figure 4.7. Neural precursor apoptosis underlies defects in *rbbp4Δ4/Δ4* midbrain and retina neurogenesis. Transverse sections of zebrafish midbrain and retina labeled with antibodies to the neural marker HuC/D (red), apoptosis marker activated Caspase-3 (green), and nuclear label DAPI (blue). (A, C) Few activated Caspase-3 labeled cells are detected in wildtype at 2 dpf (n=4) and 3 dpf (n=3). (B, D) Extensive activated-Caspase-3 labeling is present throughout the midbrain tectum and retina in the *rbbp4Δ4/Δ4* mutant at 2 dpf (n=7) and 3 dpf (n=5). E, F Counts of activated Caspase-3 positive cells plotted for individual larva show a significant difference between wildtype and *rbbp4Δ4/Δ4* mutant at 2 dpf in the midbrain (E, p=0.0022) and in the retina (F, p=0.0010). At 3 dpf the difference is no longer significant in the midbrain (E, p=0.2569) and only slightly significant in the retina (F, p=0.0358), due to loss of cells by
(Figure 4.7 continued) apoptosis. Graphs plot counts in individual larvae with mean ± s.e.m. *p* values were calculated with one-tailed Student’s t-test. (G,H) Higher magnification view of nuclear morphology shows extensive nuclear fragmentation and pyknosis in the \( \text{rbbp4}^{\Delta4/\Delta4} \) mutant 2dpf retina (H). Scale bars: 100 µm (A, B, C, D); 10 µm (G,H).

4.7C). At 3 dpf in the in the \( \text{rbbp4}^{\Delta4/\Delta4} \) mutant, as the rate of proliferation in the brain and retina slows, the amount of apoptosis also appears to progressively decrease (Figure 4.7D). Counts of Caspase-3 positive cells in the midbrain and retina confirm a statistically significant increase in apoptotic cells at 2 dpf in the \( \text{rbbp4}^{\Delta4/\Delta4} \) mutant compared to wildtype (Figure 4.7E,F). A significant increase in apoptotic cells was also observed at 3 dpf in the retina with a trending increase at 3 dpf in the midbrain (Figure 4.7E,F). Furthermore, the location of the activated Caspase-3 labeling at 2 and 3 dpf is restricted to the regions of the midbrain and retina which normally contain actively dividing progenitors (Figure 4.7B,D). Neuronal HuC/D labeling did not overlap with activated Caspase-3, again suggesting the affected cells are neural progenitors or newborn neurons rather than differentiated neurons (Figure 4.7B,D; Supplemental Figure S4.3). Apoptosis was also apparent in the presence of DAPI-labeled nuclei undergoing pyknosis and fragmentation which is completely absent from the wildtype (Figure 4.7G,H). Together these results suggest that in rbbp4 mutant, neural precursors undergo apoptosis before initiating the process of differentiation, and is the likely cause of the observed \( \text{rbbp4}^{\Delta4/\Delta4} \) mutant phenotype in which loss of cells in the dorsal midbrain and retina leads to microphthalmia and microcephaly.

To begin to elucidate the underlying cause of neural precursor apoptosis in the \( \text{rbbp4}^{\Delta4/\Delta4} \) mutant midbrain and retina, we examined 2 and 3 dpf zebrafish larvae for the accumulation of DNA damage. DNA damage was detected with an antibody to \( \gamma \)-H2AX,
which recognizes phosphorylated histone H2AX that forms at DNA double strand breaks (DSB) and more broadly labels damaged chromatin in nuclei in apoptotic cells (Burma et al., 2001). Similar to activated Caspase-3 labeling, there is little γ-H2AX labeling in the wildtype midbrain or retina at 2 dpf (Figure 4.8A). In the *rbbp4Δ4/Δ4* mutant at 2 dpf there is an increase in γ-H2AX labeling (Figure 4.8B). Again, while little to no γ-H2AX labeling is seen in the wildtype at 3 dpf (Figure 4.8C), in the *rbbp4Δ4/Δ4* mutant at 3 dpf there is an increase in labeling (Figure 4.8D). This observation of increased γ-H2AX labeling and therefore DNA damage response is confirmed by counts of γ-H2AX positive cells which are significantly higher in the *rbbp4Δ4/Δ4* mutant than wildtype at 2 dpf in the midbrain and retina and trending higher in the mutant as compared to wildtype at 3 dpf in the midbrain and retina (Figure 4.8E,F). Of note is the observation that the location of γ-H2AX staining roughly approximates that of the Caspase-3 labeling, indicating the same cells that are showing signs of DNA damage are also undergoing apoptosis.

Interestingly, the pattern of γ-H2AX staining varied in the *rbbp4Δ4/Δ4* mutant cells. In some cells it resembled the dotted foci pattern of DSB labeling in the nucleus (Figure 4.8H, dotted arrow), while in others it appeared as a defined ring around the nucleus (Figure 4.8H, open arrow) or was overall pan-nuclear (Figure 4.8H, closed arrow). The majority of γ-H2AX labeled cells showed rings or pan-nuclear pattern and contained small fragmented nuclei characteristic of cells actively undergoing apoptosis (Figure 4.8H). The varied patterns of γ-H2AX observed in *rbbp4Δ4/Δ4* mutant may capture multiple steps in DNA damage induced programmed cell death. Nuclear foci may represent labeling at DSB to induce the initial DNA damage response, while pan-nuclear labeling occurs in cells during the process of nuclear fragmentation and apoptosis.
Figure 4.8 γ-H2AX labeling reveals persistent DNA damage in rbbp4 Δ4/Δ4 mutant neural precursors. (A-D) Transverse sections of zebrafish midbrain and retina labeled with an antibody to the DNA damage marker γ-H2AX (green) and nuclear label DAPI (blue). (A, C) Few γ-H2AX labeled cells are present in wildtype midbrain or retina at 2 dpf (n=3) and 3 dpf (n=3). (B, D) In the rbbp4 Δ4/Δ4 mutants (n=3) at 2 dpf a significantly greater number of cells with γ-H2AX labeling was present in the brain (E, *p* =0.0008) and retina (F, *p* =0.0032). By 3 dpf (n=3) the number was not significantly different than in wildtype (midbrain E, *p* =0.1211; retina F, *p* =0.0537), due to loss of cells by apoptosis. Graphs plot counts in individual larvae with mean ± s.e.m. *p* values were calculated with one-tailed Student’s t-test. (G,H) Higher magnification view of nuclear morphology and γ-H2AX labeling in the rbbp4 Δ4/Δ4 mutant 2dpf retina (H). Closed arrow shows pannuclear staining; open arrow shows nuclear ring staining; dotted arrow shows foci pattern of staining. Scale bars: 100 µm (A, B, C, D); 10 µm (G, H).
Alternatively, this labeling may represent an alternative pathway to apoptosis rather than a DNA damage response.

**Apoptosis due to loss of Rbbp4 is dependent on the Tp53 pathway**

We next wanted to investigate the mechanism of apoptosis occurring in the \( rbbp4^{Δ4/Δ4} \) mutant midbrain and retina. To address this question we injected \( tp53 \) antisense morpholino into the \( rbbp4^{Δ4/Δ4} \) mutant to suppress \( tp53 \) function. The \( tp53 \) morpholino had little to no effect on the wildtype midbrain or retina at any age as seen by counts of activated Caspase-3 labeled cells (Figure 4.9A,B,E,F,I,J). Where there is a statistical difference between injected and un-injected wildtype at 2 dpf in the midbrain and 3 dpf in the retina appears to be an artifact of low overall count numbers and comparison to 0. At 2 dpf injection of the \( tp53 \) morpholino into the \( rbbp4^{Δ4/Δ4} \) mutant led to a near complete and statistically significant suppression of activated Caspase-3 labeling, and thus apoptosis, in the midbrain and retina (Figure 4.9C,D,I,J). At 3 dpf injection of the \( tp53 \) morpholino into the \( rbbp4^{Δ4/Δ4} \) mutant caused a trending and significant decrease in Caspase-3 labeling in the midbrain and retina respectively (Figure 4.9G,H,I,J). The fact that the inhibition of Caspase-3 labeling is attenuated by 3 dpf is most likely do to the fact that the antisense morpholino does not persist and suppression of \( tp53 \) is not maintained passed 2 or 3 dpf. Overall, these results indicate that loss of Rbbp4 leads to apoptosis that is dependent on the activation of a Tp53-dependent pathway.

We next tested the effect of knockdown of \( tp53 \) expression on \( γ-H2AX \) labeling in the wildtype and \( rbbp4^{Δ4/Δ4} \) mutant. \( tp53 \) knockdown had no effect on \( γ-H2AX \) labeling in the wildtype midbrain or retina at 2 or 3 dpf (Figure 4.9K,L,O,P,S,T).
Figure 4.9  Tp53-dependent activation of DNA damage response and apoptosis in *rbbp4^{Δ4/Δ4}* mutant neural precursors. 2 dpf and 3 dpf wildtype and *rbbp4^{Δ4/Δ4}* larval midbrain sections after *tp53* antisense morpholino knockdown. Uninjected siblings were used as a control. (A-H) Activated Caspase-3
(Figure 4.9 continued) (green), HuC/D (red) and DAPI (blue) labeling at 2 dpf (A-D) and 3 dpf (E-H). (I) Counts of Caspase-3 positive cells in the midbrain were compared between un-injected and p53 MO injected wildtype at 2 dpf (un-injected n=4; p53 MO injected n=4; one-tailed p-value=0.017), rbbp4Δ4/Δ4 homozygotes at 2 dpf (un-injected n=2; p53 MO injected n=4; one-tailed p-value=0.0112), wildtype at 3 dpf (un-injected n=3; p53 MO injected n=3; one-tailed p-value=0.3349), and rbbp4Δ4/Δ4 homozygotes at 3 dpf (un-injected n=4; p53 MO injected n=6; one-tailed p-value=0.1736). (J) Counts of Caspase-3 positive cells in the retina were compared between un-injected and p53 MO injected wildtype at 2 dpf (un-injected n=4; p53 MO injected n=4; one-tailed p-value=0), rbbp4Δ4/Δ4 homozygotes at 2 dpf (un-injected n=2; p53 MO injected n=4; one-tailed p-value=0.0032), wildtype at 3 dpf (un-injected n=3; p53 MO injected n=3; one-tailed p-value=0.0285), and rbbp4Δ4/Δ4 homozygotes at 3 dpf (un-injected n=4; p53 MO injected n=6; one-tailed p-value=0.0037). (K-R) γ-H2AX labeling (green), and nuclear stain DAPI (blue) at 2 dpf (K-N) and 3 dpf (O-R). (S) Counts of γ-H2AX positive cells in the midbrain were compared between un-injected and p53 MO injected wildtype at 2 dpf (un-injected n=4; p53 MO injected n=3; one-tailed p-value=0.2344), rbbp4Δ4/Δ4 homozygotes at 2 dpf (un-injected n=2; p53 MO injected n=4; one-tailed p-value=0.0024), wildtype at 3 dpf (un-injected n=3; p53 MO injected n=5; one-tailed p-value=0.1101), and rbbp4Δ4/Δ4 homozygotes at 3 dpf (un-injected n=6; p53 MO injected n=10; one-tailed p-value=0.0657). (T) Counts of γ-H2AX positive cells in the retina were compared between un-injected and p53 MO injected wildtype at 2 dpf (un-injected n=4; p53 MO injected n=3; one-tailed p-value=0), rbbp4Δ4/Δ4 homozygotes at 2 dpf (un-injected n=2; p53 MO injected n=4; one-tailed p-value=0.0001), wildtype at 3 dpf (un-injected n=3; p53 MO injected n=5; one-tailed p-value=0.5784), and rbbp4Δ4/Δ4 homozygotes at 3 dpf (un-injected n=6; p53 MO injected n=10; one-tailed p-value=0.0128). Scale bars: 100 µm.

However, at 2 dpf and 3 dpf there is a significant decrease in the number of γ-H2AX labeled cells in the morpholino injected rbbp4Δ4/Δ4 mutant retina, and a significant (2 dpf) and trending (3 dpf) decrease in the midbrain (Figure 4.9M,N,Q,R,S,T). Interestingly, phosphorylation of H2AX by ATM due to double strand breaks occurs independently/upstream of p53 activation. Together, our analysis shows DNA damage and neural precursor apoptosis in the absence of Rbbp4 is due to activation of Tp53 dependent programmed cell death. Therefore, the fact that the γ-H2AX labeling observed
in the rbbp4Δ4/Δ4 mutant is dependent on tp53 expression suggests that it is a read out of an alternate type of DNA damage or a different physiological process altogether.

**Discussion**

In this study we characterized the expression of Rbbp4 in vivo and demonstrate its requirement in vertebrate neurogenesis. Results show that Rbbp4 is expressed in the developing zebrafish nervous system. Expression is widespread in the brain and retina at the early stages of embryonic development. At the later larval stages expression of Rbbp4 becomes more restricted in the stem and progenitor populations while remaining widely expressed in post-mitotic and mature cells. This pattern suggests a requirement for Rbbp4 in properly differentiated neurons. A frameshift allele was isolated and examination of rbbp4Δ4/Δ4 mutants revealed distinct roles for Rbbp4 in neural stem and progenitor cells during larval neurogenesis. While neural stem cell survival is unaffected, an alteration in morphology suggests defects in cell cycle progression. Additionally, we have shown that Rbbp4 is required for the survival of certain populations of cells, most likely differentiating precursor cells in the brain and retina. Apoptosis of this population of cells is p53-dependent and due in part to the accumulation of DNA damage.

An interesting finding of our experiments is the differential effect of the loss of Rbbp4 on stem versus progenitor or precursor cells. In particular, the survival of the stem cells located at the ventricles of the midbrain and the CMZ of the retina is unaffected. Lineage tracing with BrdU indicates that these cells are still able to progress through the cell cycle and divide. However, by 5 dpf the stem cells appear enlarged and abnormal in morphology. This enlarged morphology could represent the onset of senescence which is commonly characterized by cellular hypertrophy (Dulic, 2013) (Blagosklonny, 2006).
Other research has shown that knockdown of Rbbp4 in vitro leads to induced cellular senescence which the authors argue is caused by impaired nuclear transport efficiency (Tsujii et al., 2015). Additional experiments in vitro have shown that high levels of Rbbp4 are associated with increased pluripotency (O'Connor et al., 2011). These observations are consistent with our results suggesting that Rbbp4 is necessary to maintain a normal rate division and function in neural stem cells. Rbbp4 is known to be a component of the DREAM complex, specifically as a part of the MuvB core complex, which has been shown to regulate progression through the cell cycle (Sadasivam et al., 2012). Findings in C. elegans have shown that the MuvB complex and Rbbp4 is required for progression through the cell cycle, specifically from G1 through S phase, and without it cells become prematurely senescent. It is possible that the fact that the stem cells in the rbbp4Δ4/Δ4 mutant continue to cycle, although at a slower rate, is due to remaining maternal rbbp4. Examination of these cells at later time points is necessary to determine the exact nature of the cell cycle defects. Further experiments such as β-galactose labeling and testing for increased expression of cyclin-dependent kinase inhibitors could be performed to confirm whether or not the stem cells in the rbbp4Δ4/Δ4 mutants are in fact in senescence.

A major finding of our experiments is the extensive, p53-dependent apoptosis that occurs in the progenitor populations of the brain and retina in the absence of Rbbp4. While there are many possible causes of the observed apoptosis, DNA damage is highly likely to be one of the main causes as is evident by the presence of γ-H2AX labeling. Of note is our observation of several different patterns of γ-H2AX labeling in addition to the classical foci present at the sites of DSB (Burma et al., 2001). There is a growing body of
evidence suggesting that γ-H2AX labeling can represent more than just foci labeling DSBs. Specifically, pan-nuclear (Fragkos et al., 2009) (Ding et al., 2016) (Meyer et al., 2013) and nuclear ring labeling patterns (Solier and Pommier, 2009) (Solier and Pommier, 2014), and even in some cases the classic focal labeling pattern that don’t co-label with 53bp1 (de Feraudy et al., 2010), may label early apoptotic events subsequent to the initial DSB induced DDR. Several different mechanisms have been proposed for this altered γ-H2AX labeling including ring like labeling that occurs in response to extrinsic TRAIL induce apoptotic stimuli (Wang and El-Deiry, 2003), JNK1-mediated phosphorylation of tyrosine 142 of H2AX (Cook et al., 2009), and phosphorylation of H2AX by ATM/DNAPK in early apoptosis that is required to completely fragment DNA during apoptosis preventing tumorigenesis and autoimmune responses (Rogakou et al., 2000) (Mukherjee et al., 2006) (Lu et al., 2006). Therefore, the fact that we observed multiple patterns of γ-H2AX labeling could represent a snapshot of cells at different temporal stages of apoptosis, ranging from initial DNA damage to early apoptosis when chromatin begins to fragment. Additionally, the fact that γ-H2AX labeling is dependent on p53 highly suggests that in this instance it is not indicative of early labeling of DSBs which is upstream and independent of p53 activation. Labeling experiments with Rad51 or other markers specific to DSBs could be performed to confirm these observations.

Similar p53-dependent γ-H2AX pannuclear labeling has also been observed in human peripheral blood lymphocytes after X-ray irradiation (Ding et al., 2016). The atypical γ-H2AX labeling pattern in rbbp4A4/Δ4 mutants could be a result of accumulation of extensive, persistent DNA damage leading to the bypassing of the usual attempts to repair the DNA and instead activation of p53-induced apoptosis.
There are several ways in which the loss of Rbbp4 could lead to defects in DNA integrity and in the DDR. Specifically, Rbbp4 is a member of the CAF-1 complex which functions to maintain genome integrity during replication (Tyler et al., 1996) (Verreault et al., 1996). Studies have found that RBBP4, likely through this association with the CAF-1 complex, is necessary to prevent unequal segregation in anaphase (Hayashi et al., 2004) or accumulation of mitotic defects \textit{in vitro} (Satrimafitrah et al., 2016). In addition to genome integrity, Rbbp4 likely plays a role in the DNA damage response through its association with CBP/p300 (Zhang et al., 2000). A recent study revealed that in cultured Glioblastoma Multiforme cells Rbbp4 associates with CBP/p300 to acetylate and activate the promoters of DNA damage response genes such as MGMT and Rad51 in response to treatment with temozolomide (Kitange et al., 2016). Further testing for the presence of abnormal spindle formation by labeling with alpha-tubulin could be performed to confirm the presence of genome instability.

In addition to DNA damage, another possible cause of apoptosis in precursor cells could be a failure to initiate quiescence and expression of neural differentiation program as Rbbp4 is associated with several complexes involved in proper differentiation such as the NuRD and PRC2 complexes. One of the few other \textit{in vivo} studies of Rbbp4 to date has been performed in planarians. Results from these experiments demonstrated that the loss of Rbbp4 prevents regeneration and led to apoptosis in neoblast progeny, possibly due to a failure of these cells to commit or differentiate (Bonuccelli et al., 2010). Our \textit{in situ} results suggest that in the retina of the \textit{rbbp4}^{Δ4/Δ4} mutant there is an expanded population of \textit{ath5} expressing cells and a loss of \textit{cdkn1c} expressing cells. Previous studies of the \textit{flotte lotte/Elys} mutant in zebrafish have shown neural precursors that fail to exit
the cell cycle undergo apoptosis in the zebrafish retina (Cerveny et al., 2010). However, unlike the flotte lotte/Elys mutant, the rbbp4Δ4/Δ4 mutant retina still contain ath5 expressing cells at the CMZ but lack cdkn1c expressing cells which have exited the cell cycle. As ath5 is associated with the terminal cell division (Masai et al., 2000), this could indicate that the defects in the rbbp4Δ4/Δ4 mutants are specific to differentiation and not cell cycle exit. Additionally, this defect may not be intrinsic; rather the lack of proper environmental signals from the missing adjacent cdkn1c expressing cells could contribute to the failure of committed ath5 progenitors to exit the cell cycle (Mu et al., 2005) (Cerveny et al., 2010) (Neumann and Nuesslein-Volhard, 2000). This would lead to observed expansion of the ath5-positive cell population in the rbbp4 homozygous retina. As the use of morpholinos provides a transient suppression of gene expression, the generation of an rbbp4; tp53 double mutant would prevent apoptosis and allow us to examine the fate of rbbp4 mutant precursors in the absence of tp53 activated programmed cell death and test their ability to differentiate.

Our results indicate that mature neurons and glia are largely unaffected by the loss of Rbbp4, suggesting it does not play a major role in the maintenance of differentiation status. However, Rbbp4 is associated with several chromatin remodeling complexes, many of which function to maintain epigenetic marks on chromatin in differentiated cells. It is conceivable that if we were able to follow the mutant cells past 5 dpf with the use of conditional knockouts we may see an effect on maintenance of differentiation. For instance, a study of the Arabidopsis homolog AtMSII revealed a progressive de-differentiation phenotype in primary shoot apical meristem that is likely due to loss of epigenetic repression of developmental gene programs (Hennig et al., 2003).
Rbbp4 has been implicated in the pathogenesis of numerous different cancers including brain cancers such as medulloblastoma, glioma and Glioblastoma Multiforme (Pauty et al., 2017) (Kong et al., 2007) (Pacifico et al., 2007) (Wu et al., 2017) (Gao et al., 2017) (Vavougios et al., 2015) (Li et al., 2015a) (Song et al., 2004) (Haskins et al., 2012) (Lohavanichbutr et al., 2009) (Leivo et al., 2005) (Bai et al., 2015) (Casas et al., 2003) (Bunt et al., 2013). Our own previous studies identified Rbbp4 as being upregulated in zebrafish primitive neuroectodermal-like tumors resulting from somatic inactivation of rb1 (Solin et al., 2015) [Schultz et al., submitted]. Studies have shown that expression of Rbbp4 is specifically induced in fibroblasts by the binding of deregulated E2F to GC repeats in its promoter (Kitamura et al., 2015). These results provide a possible explanation as to how Rbbp4 becomes overexpressed in rb1-deficient tumors. How dysregulated Rbbp4 contributes to tumorigenesis remains to be fully elucidated, which complexes mediate its activity in oncogenesis in neural cancer cells. Our data suggests it is likely that Rbbp4 plays an important role in several of the fundamental processes that are altered in cancer such as genome maintenance and differentiation status. It is possible to imagine how altered Rbbp4 along with further accumulation of mutations, such as in the tp53 apoptotic pathway, could lead to cells in which DNA damage or an undifferentiated state is tolerated, resulting in cellular transformation. Future studies focusing on the role of Rbbp4 specifically in stem and progenitor cells are necessary and can be achieved using conditional alleles generated with targeted gene editing [Wierson et al., in preparation]. As recent studies have shown that WD40 repeat proteins, such as Rbbp4, may make druggable targets for novel cancer treatments and our results suggest that inhibiting Rbbp4 may lead to apoptosis in tumor cells, this gene
product represents a valuable target for future work in the development of cancer treatments.

Materials and Methods

Zebrafish care and husbandry

Zebrafish were reared in an Aquatic Habitat system (Aquatic Ecosystems, Inc., Apopka, FL). Fish were maintained on a 14-hr light/dark cycle at 27°C. Transgenic lines were established in a WIK wild type strain obtained from the Zebrafish International Research Center (http://zebrafish.org/zirc/home/guide.php). The zebrafish \textit{rbbp4}\textsubscript{Δ4}\textsubscript{is60} 4 bp deletion mutant was isolated by CRISPR/Cas9 mutagenesis as previously described (Schultz et al., under review.) For \textit{in situ} hybridization and immunohistochemistry experiments, embryos were collected and maintained at 28.5°C in fish water (60.5 mg ocean salts/l) until harvesting. Embryos were staged according to published guidelines (Kimmel et al., 1995). All experimental protocols were approved by the Iowa State University Institutional Animal Care and Use Committee (Log # 11-06-6252-I) and are in compliance with American Veterinary Medical Association and the National Institutes of Health guidelines for the humane use of laboratory animals in research.

\textbf{rbbp4 genotyping and isolation of transgenic line Tg(Tol2<Ubi:rbbp4-2AGFP>)\textsubscript{is61}}

Primers to amplify \textit{rbbp4} exon 2 containing the \textit{rbbp4}\textsubscript{Δ4}\textsubscript{is60} mutation: Forward 5' GCGTGATGACAGATCTCATATTGTTTTCCC 3'; Reverse 5' CTGGTGACATCTGGCAACCACT 3'. The \textit{Tol2<ubi:rbbp4-2AGFP>} transgene was assembled in the mini-\textit{pTol2} vector (Balciunas et al., 2006) by cloning the zebrafish \textit{ubiquitin} promoter (Mosimann et al., 2011) followed by an in-frame 2A viral peptide GFP cassette (Trichas et al., 2008) and the zebrafish \textit{β-actin} 3’UTR (Dr. Darius
Balciunas, Temple University; (McGrail et al., 2011)). 1 µg of linearized pT3TS-Tol2 transposase plasmid (Balciunas et al., 2006) was used as template for in vitro synthesis of Tol2 transposase capped, polyadenylated mRNA with T3 mMessage mMACHINE High Yield Capped RNA transcription kit (Ambion AM1348). Synthesized mRNA was precipitated with LiCl and resuspended in RNase, DNase-free molecular grade water. The Tg(Tol2<ubi:rbbp4-2AGFP>)js61 transgenic line was isolated by co-injection into 1-cell WIK zebrafish embryos of 25pg Tol2 vector plus 100pg Tol2 mRNA. 14 founder fish were screened for germline transmission of ubiquitously expressed GFP. A single F1 adult that showed Mendelian transmission of the Tol2<ubi:rbbp4-2AGFP> transgene to the F2 generation was used to establish the line.

**Immunohistochemistry**

For immunohistochemistry, embryonic (2 dpf) or larval (3-5 dpf) zebrafish were anesthetized in MS-222 Tricaine Methanesulfonate and head and trunk dissected. Trunk tissue was placed in 20µl 50mM NaOH for genotyping. Heads were fixed in 4% paraformaldehyde overnight at 4°C, incubated in 30% sucrose overnight at 4°C, then processed and embedded in Tissue-Tek OCT (Fisher 4583). Tissues were sectioned at 14-16 µm on a Microm HM 550 cryostat. For BrdU labeling experiments, 2 dpf embryos were incubated in 5 µM BrdU (Sigma catalog #B5002) in embryo media (Westerfield, 1995) for 2.5 hours, placed in fresh fish water, then sacrificed immediately or at 3 dpf and 5 dpf. Primary antibodies used for labeling : rabbit polyclonal anti-RB Binding Protein 4 RBBP4 1:200 (Bethyl; A301-206A-T); rabbit polyclonal anti-SOX2 1:200 (EMD Millipore; AB5603); mouse monoclonal anti-proliferating cell nuclear antigen PCNA 1:300 (Sigma; P8825); mouse monoclonal anti-glial fibrillary acid protein GFAP
1:1000 obtained from the Zebrafish International Research Center (ZIRC; zrf-1); rabbit polyclone anti-phospho-histone H3 PH3 1:1000 (Cell Signaling Technology; 9701); rabbit polyclonal anti-Brain Lipid Binding Protein BLBP 1:200 (Abcam; ab32423); mouse monoclonal anti-hu antigen D HUC/D 1:500 (Invitrogen; A-21271); rabbit polyclonal anti-gamma H2A histone family, member X γ-H2AX 1:200 (GeneTex; GTX127342); rabbit polyclonal anti-CASPASE-3 1:500 (BD Biosciences; 559565); mouse monoclonal anti-SV2 1:100 (Developmental Studies Hybridoma Bank; AB_2315387); rabbit polyclonal anti-CALRETININ 1:1000 (Millipore; AB5054); anti-BrdU 1:500 (Bio-Rad; MCA2483). Alexa-594 (Invitrogen; A-11005) and Alexa-488 (Invitrogen; A-11008) conjugated secondary antibodies were used at a dilution of 1:500. Tissues were counterstained with 5 µg/ml DAPI and mounted in Fluoro-Gel II containing DAPI (Electron Microscopy Sciences 17985-50). To aid antigen retrieval before BrdU labeling tissues were pretreated with 2 M HCl. Sections were imaged on a Zeiss LSM700 laser scanning confocal microscope and assembled in Adobe Photoshop CC.

in situ hybridization and alcian blue staining

cDNA was amplified by reverse transcription-polymerase chain reaction out of total RNA isolated from wild-type 5 day post-fertilization embryos and cloned into the pCR4-TOPO vector (Invitrogen). Primers for amplification were as follows: mz98 forward 5’CCGGACACTACACTCAATGC3’, mz98 reverse 5’GTGCTGGATGTAGCTGTTCTCG3’; ccnD1 forward 5’GCGAAGTGGATACCATAAGAAGC3’, ccnD1 reverse 5’GTCCTGATGTATAGGCAGTTTGG3’; ath5 forward 5’GATTCCAGAGACCCGGAGAAG3’, ath5 reverse
5′CAGAGGCTTTTCGTAGTGATTAGGAG3′; cdkn1c forward
5′CGTGGACGTATCAAGCAATCTGG3′, cdkn1c reverse
5′GTCTGTAATTTCGCGCGTGC3′. Digoxigenin-labeled probes for in situ hybridization were synthesized using T3 RNA polymerase (Roche #11031163001) and DIG RNA labeling mix (Roche #11277073910) according to the manufacturer’s instructions and stored in 50% formamide at −20°C. Embryonic and larval zebrafish tissues were fixed in 4% paraformaldehyde and embedded in Tissue-Tek OCT (Fisher 4583). in situ hybridization on 14–16 μm cryosections of head tissue was performed as described (Trimarchi et al., 2007). For alcian blue staining of cartilage, zebrafish larvae were anesthetized and fixed in 4% paraformaldehyde overnight at 4°C and incubated in 0.1% alcian blue solution overnight at 4°C. Embryos were rinsed in acidic ethanol and stored in 70% glycerol. Whole larvae and tissue sections were imaged on a Zeiss Discovery.V12 stereomicroscope or Zeiss Axioskop 2 microscope, respectively, and photographed with a Nikon Rebel camera.

**Western blotting**

Whole protein extract was generated by dounce homogenization of ~70 5 dpf embryos in 200 uL Cell Extraction Buffer (ThermoFisher catalog # FNN0011). Lysates were run on a NuPAGE™ 12% Bis-Tris Protein Gel (Invitrogen catalog # NP0342BOX) and blotted onto PVDF membrane (BioRAD catalog # 1620177). After blocking, membranes were incubated in either rabbit polyclonal anti-RB Binding Protein 4 RBBP4 (Bethyl A301-206A-T) at 1:800 or mouse monoclonal anti-β-actin (Sigma catalog #A2228) at 1:5000 overnight at 4°C. Membranes were incubated in Anti-Rabbit IgG F(ab’)2-Alkaline Phosphatase (Sigma catalog #SAB3700833) or Anti-Mouse IgG
F(ab′)2-Alkaline Phosphatase (Sigma catalog #SAB3700994) at 1:500 for one hour at room temperature. Membranes were developed with CSPD (Roche catalog #11655884001) and imaged on a BioRAD Molecular Imager ChemiDoc XRS System.

References


Supplemental Figures and Tables

Supplemental Figure S4.1 Absence of Rbbp4 protein in *rbbp4*Δ4/Δ4 homozygous mutant larvae at 5dpf. A Western blot of whole protein lysate from wildtype +/+ and homozygous mutant *rbbp4*Δ4/Δ4 5 dpf larvae
(Supplemental Figure S4.1 continued) probed with a rabbit polyclonal anti-Rbbp4 antibody. The antibody recognizes a single polypeptide band of ~50kDa, close to the Rbbp4 predicted size of 48kDa. The band is absent from the rbbp4Δ4/Δ4 mutant protein extract. Lower panel, loading control blot probed with mouse monoclonal antibody β-actin shows similar levels in wildtype and rbbp4Δ4/Δ4 mutant extracts. (B, C) Immunolabeling with Rbbp4 and proliferative marker PCNA antibodies on transverse sections through the head of 5 dpf wildtype (B) and rbbp4Δ4/Δ4 homozygous mutant (C) zebrafish larvae. Confocal images for each larva were captured at approximately the same gain settings, with a slightly higher gain setting for the homozygote, to confirm the absence of Rbbp4 signal. Images were adjusted equally for brightness in Photoshop. In wildtype brain (B) high levels of Rbbp4 are present in the nucleus of neurons in the tectum and thalamic regions. In the rbbp4Δ4/Δ4 homozygote (C) Rbbp4 levels are nearly absent. Scale bars: 50 µm.

Figure S4.2 Genotype confirmation of rbbp4Δ4/Δ4; Tg(Tol2<ubi:rbbp4-2A-GFP>) rescue experiment presented in Figure 3. rbbp4 genotyping primers amplify a 196 bp amplicon surrounding the 4 bp deletion mutation in exon 2. The rbbp4Δ4 allele overlaps a SmI restriction enzyme site. SmI digestion of wild type (WT) DNA generates 150 and 46 bp fragments, while the rbbp4Δ4 DNA is resistant to digestion. After imaging, the larvae shown in Figure 3A, B, H, I were lysed in 50mM NaOH to release genomic DNA used as template for genotyping. The digested bands in lanes 2 and 6 confirm the
(Supplemental Figure S4.2 continued) GFP+ animals in Figure 3A and 3H are wild type +/+ , while the resistant bands in lanes 4 and 8 confirm the GFP+ animals in Figure 3B and 3I are homozygous for the rbbp4Δ4 allele. U, un-digested; D, digested.

Supplemental Figure S4.3  Caspase-3 labeled rbbp4Δ4/Δ4 mutant retinal cells undergoing apoptosis do not express the neuronal marker HuC/D. (A-H) Transverse sections of 3 dpf zebrafish retinas labeled with antibodies to the apoptosis marker activated caspase-3 (green), neuronal marker HuC/D (red), and nuclear stain DAPI (blue). A-D In wildtype HuC/D is detected in neuron cell bodies in the inner nuclear layer and photoreceptor layers of the retina. E-H In the rbbp4Δ4/Δ4 homozygous retina cells expressing activated caspase-3 do not co-label with HuC/D. Arrow points to an activated caspase-3-negativ/HuC/D-positive cell surrounded by activated caspase-3-positive/HuC/D-negative cells. Scale bars: 100 µm.

Supplemental Table S4.1  Frequency of progeny genotypes and phenotypes from heterozygous rbbp4Δ4/+ crossed to heterozygous rbbp4Δ4/+;Tg(Tol2<ubi:rbbp4-2A-GFP>) zebrafish. Three independent crosses were set up between female rbbp4Δ4/+ heterozygotes and male rbbp4Δ4/+;Tg(Tol2<ubi:rbbp4-2A-GFP>) transgenic heterozygotes. Embryo clutches were sorted for the presence or absence of GFP expression at 2 dpf, and
Supplemental Table S4.1 continued) phenotype and genotyped at 5 dpf. “Total” column shows approximately 50% GFP- and GFP+ in each clutch, indicating the Tg(Tol2<ubi:rbbp4-2A-GFP>) line contains a single transgene insertion. Scoring of GFP negative embryos revealed close to Mendelian segregation of the rbbp4/4 gross mutant phenotype in one quarter of the progeny (28.2%, 21.3%, 29%). A random sampling of embryos from clutches 2 and 3 were genotyped and confirmed Mendelian segregation of the phenotype with the rbbp4 allele. In each clutch, none of the GFP positive embryos showed the rbbp4 mutant phenotype. Random sampling and genotyping confirmed ~ one quarter of the morphologically normal GFP+ larvae were homozygous mutant rbbp4/4 (32.4%, 20.8%, 20.8%).

| Clutch | GFP Negative | | | | | Total | | | | | TOTAL |
|--------|--------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|        | Total        | Normal Phenotype | Mutant Phenotype | Rbbp4 +/- | Rbbp4 +/- | Rbbp4 +/- | | | | | | | |
| Clutch 1 | 38/76 | 20/39 | 11/39 | n/a | n/a | n/a | | | | | | | |
|          | (5.1%) | (71.8%) | (28.2%) | | | | | | | | | | |
| Clutch 2 | 61/110 | 40/61 | 14/51 | 5/24 | 11/24 | 9/24 | | | | | | | |
|          | (55.5%) | (68.4%) | (21.3%) | (20.8%) | (45.4%) | (33.3%) | | | | | | | |
| Clutch 3 | 155/307 | 110/155 | 45/155 | 18/48 | 17/48 | 13/48 (27%) | | | | | | | |
|          | (53.9%) | (71%) | (29%) | (47.6%) | (56.4%) | (42.3%) | | | | | | | |

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CHAPTER 5. CONCLUSION

Summary

In the first paper of this dissertation, an adult zebrafish retinal tumor model was used to examine the different signaling pathways involved in promoting and maintaining excessive proliferation in non-malignant, glial-originating tumors. The dysplastic tumors analyzed showed sustained proliferation in cell populations that are not normally proliferative. Furthermore, transcriptome analysis of tumor samples indicate that hypoxia inducible factor HIF-1α and its downstream targets angiogenic growth factor VEGF and the cytokine Leptin are upregulated in the tumor. These results were confirmed with in situ analysis which further showed that vegfab appears to be upregulated in neuronal cells while lepb expression is upregulated in glial cells suggesting a cell specific response to HIF-1α signaling. Additionally, mTOR signaling was found to upregulated in the tumor, an observation confirmed by immunolabeling of tumor samples, indicating a possible mechanism in which overproliferation is sustained once initiated by other signaling pathways. Overall, this study is a good example of two of the hallmarks of cancer, namely the ability to evade growth suppression and sustain proliferative signaling. Furthermore, this is an excellent example of how normal developmental paradigms can become re-activated and dysregulated in tumorigenesis. Specifically, adult zebrafish retain populations of glia that can de-differentiate and re-program giving rise to proliferative progenitors in response to injury. We propose a mechanism whereby sustained signaling due to hypoxia and chronic inflammation drive transformation of glial-derived progenitors leading to un-regulated proliferation and tumor growth. This
work is important in highlighting the way different signaling pathways when
dysregulated can converge to drive the transformation of normal cells.

With the growing appreciation of the role of pRB a multifunctional regulatory
protein rather than just cell cycle gatekeeper, the second paper of this dissertation
examines the role of pRB in chromatin modification through interaction with different
chromatin associated proteins. Transcriptome analysis of poorly differentiated brain
tumors generated by somatic inactivation of *rb1* demonstrates that these tumors display a
similar expression signature as human oligoneural CNS-PNET subgroup in which *olig2*,
*sox10, sox8, sox2* and *erbb3a* were upregulated. In order to better understand how loss of
pRB drives tumorigenesis in the zebrafish brain, we generated a germline *rb1-/-* mutant
allele to compare phenotype and transcriptome signatures that define transformed vs.
mutant tissue. The *rb1* mutant embryos display ectopic proliferation in the brain and
retina at 5 dpf but are otherwise largely normal indicating that certain cells in the mutant
can re-enter the cell cycle and proceed into M phase but lack continuous unregulated
proliferation characteristic of *rb1*-transformed tumor cells. Transcriptome analysis of
*rb1-/-* mutant embryo heads showed similar upregulation of pathways controlling cell
division, mitosis, and DNA replication as would be expected upon upregulation of E2F.
However, the tumor transcriptome showed more transcriptional regulators were
dysregulated (1191) than were in the mutant (125). Additionally, the expression of
several stem and neural progenitor transcription factors were upregulated and several
proneurogenic transcription factors were downregulated in the tumor but not the mutant
transcriptomes. We specifically analyzed the role of two genes in particular that were
found to be dysregulated in the tumor and are known to interact with pRB, *rbbp4* and
both genes are involved in chromatin remodeling and thus may provide insights into how epigenetic dysregulation may lead to transformation. Histological analysis of rbbp4-/- and hdac1-/- mutant embryos suggest rbbp4 is required for neural progenitor survival while hdac1 is required for maintaining proliferation of the neural stem cell pool. In addition to identifying underlying chromatin modifying mechanisms that may be driving transformation, another important output of this study is the generation of CRISPR/Cas9 somatic mutagenesis coupled with histone-GFP live imaging to rapidly and effectively screen potential pRB interacting genes driving tumor formation.

Finally, the third paper in this dissertation contains an in-depth examination of the mutant phenotype associated with loss of rbbp4 in zebrafish embryos. Results show that loss of rbbp4 causes microcephaly, microphthalmia, and cartilage defects of the jaw visible by 5 dpf and eventually leading to lethality at ~9 dpf. Histological examination of rbbp4-/- mutant embryos revealed severe loss of tissue in the dorsal midbrain and retina by 5 dpf. Immunolabeling at 2 and 3 dpf confirms that the loss of tissue is due to apoptosis in regions of progenitor/precursor cells in the retina and midbrain. Additional in situ and immunolabeling suggest that excessive DNA damage and failures in cell cycle exit/differentiation in progenitor/precursor populations are contributing factors to the observed apoptosis. Finally, p53 morpholino experiments confirm that the apoptosis observed is dependent on p53 signaling. These results are significant in that RBBP4 has been the subject of several studies in recent years, including many suggesting a role in cancer. Furthermore, this is the first study to date analyzing the loss of RBBP4 in a vertebrate model. Recent studies suggest WD40 domain containing proteins such as RBBP4 may be druggable targets for cancer therapeutics. However, given the prevalence
of RBBP4 in so many different regulatory complexes it is important to understand how its interaction with each of these complexes contributes to its function \textit{in vivo}.

Overall, the studies presented in this dissertation help advance our knowledge of how the dysregulation of certain genes and pathways can contribute to the initiation of cellular transformation. Novel insights include discovering the over-expression of Leptin in a retinal tumor which is not a tissue it is normally associated with, the development of novel techniques that will allow for rapid screening of candidate genes contributing to tumor initiation, and the finding that loss of a non-enzymatic chromatin adaptor protein is lethal in zebrafish and thus highly important to normal neurogenesis and development. These studies also highlight the utility of a multi-faceted approach, both adult and embryonic, and the versatility of the zebrafish in modeling cancer and developmental defect. Finally, the hope is that these studies and the novel mechanisms they uncover will help lead to novel treatments and diagnoses of human tumors.

\textbf{Future Directions}

\textbf{AIM 1: Clarify the role that RBBP4 plays in normal stem cell function vs. progenitor/precursor cell function.}

\textbf{Background:} Initial experiments suggest that RBBP4 plays very distinct roles in different populations of cells. Specifically, upon loss of RBBP4, stem cells survive but they appear to be enlarged and possibly display a slower rate of division. However, in precursor cells, the loss of RBBP4 leads to apoptosis likely due to a combination of DNA damage and failure of the cells to complete normal commitment, cell cycle exit, and/or differentiation. \textbf{Question/Hypothesis:} The goal of these experiments is to answer the question of how specifically stem and progenitor cells are affected by the loss of RBBP4. We hypothesize
that the enlarged stem cell morphology is indicative of induced senescence and delays in cell cycle. We also hypothesize that defects in proper DNA replication due to CAF-1 dysfunction lead to replicative stress in cycling progenitor cells and DNA damage. Additionally, based on in situ results we hypothesize that defects in commitment/cell cycle exit/differentiation of progenitor cells is occurring and resulting in apoptosis.

Experiments:

Testing for senescence in Rbbp4-/- mutant stem cell populations. Cellular hypertrophy, similar to that observed in the neural stem cell populations of the Rbbp4-/- mutant embryo, is a classic hallmark of senescence which is the permanent and irreversible exit from the cell cycle usually due to cellular stress. To confirm that these cells are in fact senescent, tests for several signs of senescence can be performed including beta-galactosidase staining, qPCR or immunolabeling to detect increases in the cyclin dependent kinase inhibitors p21 and p16, DAPI labeling to detect Senescence-associated heterochromatin foci, immunolabeling to detect H3K9me3 and H4K20me3. Previous studies have suggested that loss of RBBP4 is associated with defects in nuclear import leading to senescence (Tsujii et al., 2015). Therefore, to examine nuclear import efficiency in vivo, transgenic animals containing a NLS-GFP construct could be examined in a wildtype versus mutant Rbbp4-/- background.

BrdU labeling. Initial BrdU experiments were performed in which embryos were incubated in BrdU for several hours and then chased in fish water for one to three days. The purpose of this experiment was to track the movement of newly born/labeled cells. However, to address the issue of whether or not there is a delay in S-phase, a different experiment can be performed in which embryos receive a short 15 minute pulse with
BrdU and then are collected at several different time points and labeled with Ph3 to determine the number of BrdU labeled cells that have progressed to M-phase. This quantification can be compared between wildtype and Rbbp4-/- mutant embryos beginning at 2 dpf.

Analysis of p53/Rbbp4 double mutants. Rbbp4-/- mutant progenitor/precursor populations normally undergo apoptosis beginning at 2 dpf making the analysis of the defects in these cell populations difficult. Therefore, by examining the Rbbp4-/- mutant phenotype in a p53-deletion background, we can suppress apoptosis and examine the behavior of the progenitor/precursor cells beyond 2 dpf. Experiments to perform in the p53/Rbbp4 double mutant would include repeating the Sox2/PCNA, Blbp/Gfap, and Sv2/Calretinin immunolabeling, the in situ hybridization with mz98, ccnD1, ath5, and cdkn1c, as well as the 2.5 hour BrdU pulse-chase experiments. The in situ hybridization experiments will tell us what stage in the commitment/cell cycle exit/ differentiation process the aberrant cells are and the immunolabeling experiments will tell us whether or not in the suppression of apoptosis is these cells are able to differentiate into normal glial or neural cells. Finally, by preventing apoptosis, we can fully track the movement of newly born mutant cells labeled with BrdU.

Transplant experiments to test cell autonomy. To answer the question of whether or not the Rbbp4-/- mutant phenotype is cell autonomous, cells from homozygous animals containing the H2A-gfp transgene can be implanted into wildtype blastocysts. If the mutant cells still undergo apoptosis even in a wildtype background then the phenotype is cell autonomous and not dependent on external cues.
Conditional knockouts to examine spatial and temporal effects of loss of RBBP4. The difficulty in studying the function of a gene product with an embryonic/larval lethal phenotype is the inability to examine mutant cells at later time points in development or even in the adult. Therefore, conditional Rbbp4-/- alleles that can be inactivated by Cre driven by different spatial or temporal drivers will allow us to study the function of RBBP4 in later developmental time points or in a tissue-specific manner in the adult.

**A IM 2: Determine which interacting partners/complexes are dysregulated upon loss of RBBP4 and how that dysregulation contributes to the observed Rbbp4-/- mutant phenotype.**

**Background:** RBBP4 is an interacting partner of several regulatory and chromatin modifying, complexes such as NuRD, Sin3, RB1/E2F, CAF-1, PRC2, DREAM, and CBP/p300. Loss of RBBP4 in the developing zebrafish leads to failures in differentiation of precursor populations in the brain and retina leading to p53 dependent apoptosis. However, this exact phenotype is not phenocopied when components of some of its interacting partners are mutated. For example, inactivation of RB1 leads to ectopic proliferation and no apoptosis, while inactivation of HDAC1, a subunit of NuRD and Sin3, leads to a more severe phenotype likely resulting from a failure of stem cells to proliferate. Therefore, the phenotype observed when RBBP4 is inactivated is likely due to a combinatorial, synergistic effect of the dysregulation of several different complexes.

**Question/Hypothesis:** The main question at hand is how the function of the different interacting complexes of RBBP4 are affected when RBBP4 is lost. The hypothesis is that programs of gene expression that are controlled by NuRD, PRC2, and RB1/E2F will be altered in the absence of RBBP4. Additionally, it is hypothesized that dysfunction of the
CAF-1 complex upon loss of RBBP4 will lead to genomic instability of newly synthesized DNA in proliferating cell populations.

**Experiments:**

*Transcriptome analysis.* To help elucidate the physiological processes contributing to the observed Rbbp4-/- mutant phenotype, transcriptome analysis on mutant tissue can be performed. The patterns of gene expression changes observed in the mutant can help us determine why certain populations of cell are undergoing apoptosis. For example, if genes involved in proper neuronal commitment, cell cycle exit, or differentiation are downregulated we can infer that defects in this process are contribution to apoptosis. Additionally, we can analyze which DNA damage response genes are activated as well as which apoptosis-related genes are altered to help clarify the triggers of the observed apoptosis. Because the apoptosis phenotype is most pronounced at 2-3 dpf, the heads of 2 dpf wildtype as well as Rbbp4-/- mutant embryos would be pooled and analyzed.

*Acetylome analysis.* One of the primary functions of the NuRD and Sin3 complexes is to deacetylate histone tails via the action of HDAC1/2. Therefore, we want to analyze global changes in histone acetylation patterns upon loss of RBBP4 to determine its role in proper histone deacetylation. ChIPSeq can be performed to obtain the gene sequences associated with particular histone acetylation marks in the wildtype and Rbbp4 mutant embryos. Specifically we would analyze H3K27 and H3K9 which are both deacetylated by the NuRD complex and H4K5, H4K12, and H4K16 which are deacetylated by the Sin3 complex. These experiments would again be performed in 2 dpf pooled embryo head samples from wildtype and Rbbp4-/- mutant.
**Methylome analysis.** PRC2 is known to function by controlling patterns of DNA methylation, especially during development. We propose to perform MBDCap-seq experiments (Li et al., 2015b) on 2 dpf embryo heads to analyze global changes in DNA methylation patterns in the wildtype vs. Rbbp4-/- mutant samples.

**ChIP-seq analysis.** To determine the specific effect loss of RBBP4 has on the localization of RB1/E2F, ChIP-seq experiments using an antibody to different E2F proteins to analyze their interaction with the genome in the wildtype versus mutant embryo can be carried out. We will analyze pooled samples of embryonic heads at 2 dpf. To further analyze the role of the interaction between RB1 and RBBP4 on E2F localization, we will also perform these experiments in double Rb1/Rbbp4 homozygous embryonic heads at 2 dpf.

**Analysis of chromosomal defects.** CAF-1 is involved in assembly of newly synthesized DNA into chromatin, and therefore defects in CAF-1 function upon loss of RBBP4 could cause chromatin-specific defects. Experiments to test for these defects include immunolabeling for H3K9me3 foci which label the formation of normal heterochromatin, FISH (fluorescence in situ hybridization) to test for aneuploidy, and alpha-tubulin and gamma-tubulin immunohistochemistry to label mitotic spindles and centrosomes respectively.

**Analysis of replicative stress.** It has been demonstrated that CAF-1 is necessary for efficient replication fork progression (Hoek and Stillman, 2003) and imperfect DNA replication can result in replicative stress and subsequent DNA damage response. Therefore, we want to test for the presence of replicative stress which could possibly be leading to DNA damage/DDR in the Rbbp4-/- mutant. Experiments to test for this would
include a new method known as DNA combing (Iyer and Rhind, 2017) to detect replication fork stall and BrdU labeling to test for delayed s-phase progression.

*Live-imaging to test for genetic interactions.* Initial experiments analyzing static sections of tissue from double Rb1/Rbbp4 homozygous mutants revealed no alteration to either phenotype in the double mutant. However, real-time analysis of double homozygotes containing a H2A-GFP transgene may allow us to detect alterations, for example in timing or quantity, in the observed phenotypes in the double mutant. Therefore, we can analyze the double mutants in real time at 2, 3, and 5 dpf time points and look for changes in timing of mitoses or apoptosis.

**AIM 3: Examine the role of RBBP4 in tumorigenesis and response to therapeutics including DNA damaging agents.**

**Background:** Several studies have indicated that the expression of Rbbp4 is altered in different tumor types. However, whether Rbbp4 is up or down regulated depends on the tumor or cell type. Our analysis of poorly differentiated, PNET-like tumors caused by the somatic inactivation of Rb1 showed that Rbbp4 expression was increased 6-fold in the tumor as compared to wildtype. This increase in expression may be due to the fact that deregulated E2Fs have been shown to bind to the promoter of Rbbp4. Somatic inactivation of Rbbp4 did not lead to tumor formation in F0 animals despite the high efficiency of the CRISPR designed against Rbbp4. While our data shows that loss of Rbbp4 leads to an increase in apoptosis, and studies in tumor cell lines have shown that loss of Rbbp4 also leads to increased apoptosis in response to temozolomide due to the lack of DNA repair proteins.
**Question/Hypothesis:** The main question is how altered RBBP4 contributes to tumor formation and progression as well as response to therapeutic treatments. Based on our transcriptome analysis, we hypothesize that overexpression of RBBP4 will lead to a more severe phenotype in tumors formed by somatic inactivation of pRB. We also hypothesize that overexpression of RBBP4 will lead to a decrease in sensitivity to DNA damaging agents while the loss of RBBP4 will have the opposite effect.

**Experiments:**

*Overexpression of RBBP4 in a tumor background.* Transgenic animals have been generated which express RBBP4 linked to GFP with H2A. After quantification of the amount of overexpressed protein as compared to wildtype, these animals can then be used to generate Rb1 somatically inactivated tumors via the microinjection of TALENs or CRISPRs. Tumors can then be analyzed for changes in severity, timing, or phenotype as compared to the original Rb1-targeted tumors.

*Temporal-specific conditional knockout of Rbbp4.* Even though somatic targeting of Rbbp4 did not lead to tumor formation in F0 animals, it is still worth testing whether or not disrupting Rbbp4 in stem and progenitor populations in the adult brain and retina will produce tumors. This can be achieved by constructing tamoxifen-inducible Cre drivers targeted to neural stem and progenitor cells and a floxed conditional Rbbp4 allele. Animals can then be monitored for the formation of tumors.

*Analysis of DNA damage response.* To analyze response to DNA damage in the presence or absence of RBBP4, embryos either homozygous for the Rbbp4-/- mutant allele or containing the overexpression transgene, can be treated with a DNA damaging agent such as UV irradiation and then the subsequent DNA damage response can be evaluated. This
can be done with immunolabeling to DNA damage response proteins such as gamma-H2AX or with qPCR analysis of the expression of specific DNA damage response genes. Finally, apoptosis can also be analyzed with the use of Caspase-3 immunolabeling.
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