A Cancer Screen in Zebrafish Identifies Many Ribosomal Proteins as Haploinsufficient Tumor Suppressors

by

Kevin Lai

B. S. Biochemistry

Tufts University, 2000

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

JUNE 2006

Signature of Author: _____
Department of Biology
May 26, 2006

Certified by: _____
Jacqueline A. Lees
Professor of Biology
Thesis Supervisor

Certified by: _____
Nancy Hopkins
Professor of Biology
Thesis Supervisor

Accepted by: _____
Stephen P. Bell
Professor of Biology
Chairman, Committee for Graduate Students

A Cancer Screen in Zebrafish Identifies Many Ribosomal Proteins as Haploinsufficient Tumor Suppressors

by

Kevin Lai

Submitted to the Department of Biology on May 26, 2006
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Biology

Abstract

A collection of over 500 lines of zebrafish (*Danio rerio*), each heterozygous for a recessive embryonic lethal mutation caused by a retroviral insertion, was screened for lines that displayed early mortality and externally visible tumors. Among such tumor-prone lines identified was one in which the known human tumor suppressor gene, neurofibromatosis type 2 (*NF2*), was mutated. This validated the screening approach, as well as confirmed the human relevance of the zebrafish system in the study of cancer. Surprisingly, all of the remaining tumor-prone lines identified in the screen were ones in which a ribosomal protein (*rp*) gene was mutated. In total, 17 of 28 *rp* heterozygous lines were found to be predisposed to rare tumors, especially zebrafish malignant peripheral nerve sheath tumors (zMPNST).

Though the precise molecular mechanism of tumorigenesis in *rp* heterozygous lines remains elusive, we have demonstrated that these rps are haploinsufficient tumor suppressors based on two lines of evidence: (1) The retroviral insertion causes a loss of function of the *rp* gene, as evidenced by a reduction in mRNA encoding the rp and a disruption of ribosomal integrity in the homozygous *rp* mutants; and (2) the wild-type allele is not lost or mutated in the tumors arising in *rp* heterozygotes. Haploinsufficiency of certain rps could lead to tumorigenesis either because a key extraribosomal function of these rps is compromised or global protein synthesis is impaired due to insufficient production of ribosomal subunits. We have found evidence in support of the latter hypothesis: Heterozygotes from all tumor-prone *rp* lines are growth-impaired, suggesting that a primary defect in global protein translation predisposes these fish to the development of tumors.

This work has succeeded in identifying a novel class of haploinsufficient tumor suppressors, the ribosomal proteins, whose role in human cancers is as yet unknown. This demonstrates the powerful utility of the zebrafish as an ideal organism for genetic screens as well as a model in which to study human diseases.

Thesis Supervisor: Jacqueline A. Lees
Title: Professor of Biology

Thesis Supervisor: Nancy Hopkins
Title: Professor of Biology

Acknowledgements

I thank my co-advisors Jackie Lees and Nancy Hopkins for giving me the opportunity to start a collaborative effort between their labs. Their unwavering enthusiasm for my project provided a great environment in which to complete my graduate degree.

I thank all the members past and present of both the Lees and Hopkins labs, especially those who were part of the fish cancer subgroup: Adam Amsterdam, Sarah Farrington, Kirsten Sadler Edepli, Chris Sansam, and Alyson Wilbanks. I would also especially like to acknowledge Ulrike Ziebold and Wenbiao Chen, who were instrumental in helping to set up my project during my first year in the lab. I thank all members of the fish technical staff, especially Kate Anderson, Sam Farrington, and Tim Angelini for their devotion to the maintenance and excellent health of our fish colony. I thank the CCR Histology Facility, especially Alicia Caron and Mike Brown, for sectioning many thousands of fish. I thank Manlin Luo and Charlie Whittaker for their help with microarrays and data analysis.

I thank members of my thesis committee, especially Terry Orr-Weaver and Angelika Amon, for many years of helpful advice to focus my efforts. I am much indebted to David Sabatini and Tom Look for their service on my thesis defense committee.

I thank my teachers at Brookline High School, Mrs. Nancy Howard and Dr. Wallace Gleekman, for introducing me to biology and scientific research, respectively. I thank Drs. Juliet Fuhrman and Ross Feldberg at Tufts University for providing invaluable experience in their labs and further encouraging my career in biological research.

I thank all my friends of Biograd2000 and associates for being a source of fun and great memories over the past 6 years. I thank my family, especially my parents Kin Lun and Susanna Lai, for their tremendous sacrifice in immigrating to the United States with the sole goal of providing the best education and means for their children. Finally, I thank Jenny Li for her enduring patience, understanding, and support.

Table of Contents

List of Tables and Figures

Chapter 2:

Chapter 3:

Appendix A:

Appendix B:

Chapter 1

Introduction

Preface

The work of this thesis represents the first demonstration of the use of adult zebrafish to screen for genes involved in cancer. This screen has led to the novel finding that many ribosomal proteins (rps) act as haploinsufficient tumor suppressors in the zebrafish. In the following introduction, I will delineate the advantages of using zebrafish as a model organism for cancer as well as review the known cellular roles of ribosomal proteins and their involvement in disease.

1. The Zebrafish: An Ideal Model Organism for Genetic Screening

1a. The Development of a Vertebrate Model System

The zebrafish has recently shown tremendous potential as a model for human disease (Penberthy et al., 2002) and continues to grow in stature among the standard model organisms of biology. In addition to the benefit of genetic tractability shared by lower model organisms such as *Drosophila* and *C. elegans*, the zebrafish, *Danio rerio*, has a significant relevance to humans claimed by other model vertebrates, such as the mouse. The rise of this small fish into this elite class was initiated by the seminal work of George Streisinger in the late 1970's, which laid the groundwork for genetic manipulation in the fish (Streisinger et al., 1981). The zebrafish was selected as an ideal model organism in which to study the embryonic development of a vertebrate, since the optical clarity of the embryos and the external fertilization of the eggs allow for easy observation of developing organs and body structures (Wixon, 2000). Development is relatively rapid, with embryos becoming free-swimming larvae only five days after fertilization (Kimmel et al., 1995). These characteristics, along with the ability of adult females to lay

several hundred eggs per mating, have made the zebrafish an ideal system in which to conduct large-scale phenotype-based mutagenesis screens.

Following the demonstration that ethyl nitrosourea (ENU) could be used to induce mutations (primarily point mutations) at specific loci in zebrafish (Grunwald and Streisinger, 1992), two large-scale chemical mutagenesis screens were performed in Boston, USA and Tübingen, Germany (Driever et al., 1996; Haffter et al., 1996). In these screens, males were mutagenized with ENU, and the mutations were transmitted to F1 fish. From these heterozygous F1 fish, F2 families were raised. F2 fish were intercrossed to bring mutations to homozygosity in the F3 progeny, which were visually screened for a multitude of phenotypic effects up to 6 days post-fertilization (dpf). The screens were extremely successful in identifying a large number of mutants with defects in the development of internal organs, such as the brain, heart, and gut; externally visible structures, such as the fins, eyes, and jaw; as well as behaviors, such as motility and touch responsiveness (Haffter et al., 1996).

Identification of the genes disrupted in a number of these mutants was achieved primarily by positional cloning. This has led to a growing catalog of genes that are essential for vertebrate development. Importantly, many of the same genes have been found to be mutated in human disease. For example, the *sauternes* mutation, which causes hypochromic anemia in zebrafish embryos, was mapped to a gene encoding a form of δ-aminolevulinate synthase (ALAS-2), which is also mutated in human patients with congenital sideroblastic anemia (Brownlie et al., 1998). Another human blood disease, hepatoerythropoietic porphyria, is well-modeled by the *yquem* mutant in zebrafish, in which a mutation in the gene encoding uroporphyrinogen decarboxylase (UROD) leads to a photosensitive porphyria syndrome in the embryos (Wang et al., 1998). More recently, it has been demonstrated that the *heartstrings* gene encodes the

transcription factor Tbx5, mutation of which causes cardiac and fin defects in zebrafish embryos, and Holt-Oram syndrome, a "heart-hand" disorder, in humans (Garrity et al., 2002).

1b. The Hopkins Insertional Mutagenesis Screen in Zebrafish

The laboratory of Nancy Hopkins has developed an alternative approach to the chemical mutagenesis strategy that uses pseudo-typed retroviruses as the mutagenic agent in zebrafish embryos (Gaiano et al., 1996). Blastula-stage embryos were injected with retroviruses, which integrate into generally random sites in the genome, creating founder fish. These founders were mated with one another to create F1 pools. To increase the number of insertions screened, F1s with multiple insertions were mated to produce F2 pools. Then, pairs of F2s from each pool were mated to homozygose the insertions, and it is these F3 embryos that were screened for mutant phenotypes up to day 5 of development (Amsterdam et al., 1999). Similar to the chemical mutagenesis screens, insertional mutagenesis led to a wide variety of phenotypes, including defects in specific organs, body shape, and swimming behavior (Golling et al., 2002). In addition, a large proportion of the mutants displayed "non-specific" phenotypes, such as widespread necrosis and other pleiotropic defects. Such mutants were systematically discarded in the chemical mutagenesis screens due to the difficulty of cloning the mutated genes, and the expectation that such genes would likely be "uninteresting" housekeeping genes. In the Hopkins screen, however, since the retrovirus acted both as a mutagen and as a molecular tag allowing relatively easy identification of the mutated gene by inverse PCR, many such non-specific mutants were retained.

To date, the Hopkins laboratory has generated a collection of 525 insertional mutants, nearly all of which are homozygous recessive lethal. The disrupted gene has been cloned for 463

of these, representing 335 distinct genes (Amsterdam et al., 2004 and A. Amsterdam, unpublished results). At the time of their identification in the screen, 20% of these genes were novel or poorly characterized; thus, this collection of mutants represented an ideal starting point for identifying novel components of known pathways or completely new pathways in the development of specific organs or structures of the zebrafish. To this end, members of the Hopkins laboratory as well as external collaborators have conducted "shelf screens" of the collection, using specific assays to re-screen for selected phenotypes of interest. One such shelf screen by Zhaoxia Sun identified a class of genes that, when mutated, are associated with the development of kidney cysts; many of the same genes are mutated in human patients with polycystic kidney disease (PKD) (Sun et al., 2004). Significantly, among this class of genes were a number of *novel* genes involved in cilia production; thus, the results of this shelf screen have contributed not only to our understanding of this biosynthetic pathway, but have also yielded clues as to the etiology of PKD.

Another shelf screen performed by Kirsten Sadler also yielded insights into human liver disorders that manifest as hepatomegaly (Sadler et al., 2005). Seven out of 297 lines screened had large livers by 5 dpf. Among these, the phenotype of a mutant in the *vps18* gene, encoding a class C vacuolar sorting protein, includes a large liver filled with vesicles. This resembles the human disease arthrogryposis-renal dysfunction-cholestasis (ARC), in which another class C *vps* gene is mutated (Gissen et al., 2004). Zebrafish mutants in the *nf2* gene were also found to have liver abnormalities, suggesting a previously unknown role for this tumor suppressor gene in liver disease (Sadler et al., 2005). Finally, a mutant in a novel gene, *foie gras*, developed fatty liver, in a manner similar to human patients with fatty liver disease (Sadler et al., 2005).

These examples from the Hopkins insertional mutagenesis screen and others from the chemical mutagenesis screens clearly demonstrate the utility of the zebrafish in identifying novel genes and pathways with relevance to human disease.

2. The Zebrafish as a Cancer Model System

2a. History

Prior to its emergence as a model organism for genetic screens, the zebrafish had already had a long history in cancer research. The first demonstration of experimental carcinogenesis in zebrafish was published in 1965 by Mearl Stanton, who showed that treatment of fish with diethylnitrosamine induced hepatic neoplasia (Stanton, 1965). Even prior to this, other fishes, most prominently those of the *Xiphophorus* genus, were used to study cancer (Schartl, 1995). In the late 1920's, certain hybrids of these fish were found to develop malignant melanomas, predisposition to which was determined to be heritable (Gordon, 1931). To this day, *Xiphophorus* remains an effective model in the elucidation of the molecular mechanisms of UV-induced melanoma formation (Wood et al., 2006).

The use of the zebrafish itself, formerly known as *Brachydanio rerio*, was documented in experimental studies in the 1950's. Embryos were used to test the developmental toxicity of compounds and identify environmental pollutants (Battle and Hisaoka, 1952; Hisaoka, 1958a; Hisaoka, 1958b; Hisaoka and Hopper, 1957). These studies have been extended further over the past several decades (Baumann and Sander, 1984; Ensenbach and Nagel, 1995). The fact that the embryos are permeable to many compounds that are simply added to the water in which they grow has proven to be useful not only in carcinogenesis studies but in performing small molecule screens as well (Hertog, 2005; Peterson et al., 2000).

2b. Emergence of a New Cancer Model

The long-known fact that zebrafish are susceptible to chemical carcinogens, coupled with the more recent advances in zebrafish genetics and genomics, make the zebrafish an attractive model for cancer. The work of this thesis and the growing body of literature in the past several years suggest that the zebrafish will be an informative tool, alongside the mouse, in discovering the underlying molecular mechanisms of tumorigenesis. Compared to the mouse, the relatively small size of the mature fish allows for large-scale screens for cancer and other adult phenotypes. The ability to generate and maintain large numbers of adults simultaneously with the same genotype allows for studies of tumor incidence and onset with great statistical power. Most importantly, the zebrafish cancer model has human relevance since the fish develop a wide variety of tumors that closely resemble their human counterparts, even at the histological level (Amatruda et al., 2002; Spitsbergen et al., 2000a; Spitsbergen et al., 2000b). Even more significant is the recent demonstration that the gene expression signature of zebrafish tumors resembles that of human tumors. Lam et al. showed that carcinogen-induced zebrafish liver tumors exhibited a gene expression pattern similar to that of human liver tumors. Moreover, the histologically most anaplastic zebrafish tumors had expression profiles most similar to the highest-grade human tumors (Lam et al., 2006).

Several zebrafish models of various cancers have been described since the work in this thesis was initiated. Langenau and colleagues showed that transgenes can be used to induce cancer in the zebrafish (Langenau et al., 2003). Specifically, murine *c-myc* was expressed under the control of the zebrafish *Rag2* promoter, inducing T-cell acute lymphoblastic leukemias with a very short latency of less than two months after germline transmission. Furthermore, the leukemic cells were demonstrated to be transplantable into sublethally irradiated hosts, thus

allowing for the possibility of studying cancer in adult zebrafish with this model. In a related study, targeted expression of the human *MYCN* gene under the control of the zebrafish *myod* promoter induced neuroendocrine tumors at 3 to 6 months of age (Yang et al., 2004). Patton et al. also recently described a model in which a human oncogene was introduced into zebrafish. The V600E mutation of BRAF is an activating mutation found in many human melanomas; when expressed under the control of the zebrafish *mitfa* promoter, the fish developed nevi (Patton and Zon, 2005). In a *p53* mutant background, integration of the transgene led to the development of malignant melanoma in the fish (Patton et al., 2005).

In addition to experimental demonstrations that transgenic zebrafish with human oncogenes develop the expected tumor types, there is reason to believe that the function of human tumor suppressor genes is also conserved in the zebrafish. For example, truncation of the tumor suppressor adenomatous polyposis coli (APC) leads to neoplasias of the digestive tract in 15 month-old zebrafish, similar to the effects of the same mutation in mice and humans (Haramis et al., 2006). Many other known tumor suppressor genes have been characterized in zebrafish in a developmental context (Bertrand et al., 2004; Bollig et al., 2006; Croushore et al., 2005; Imamura and Kishi, 2005). Likewise, a number of genes involved in the epithelial-mesenchymal transition (EMT) of cancer cells have also been studied in zebrafish embryos (Taylor et al., 2004; Wallace et al., 2005). The process of EMT in cancer has been shown to parallel key events in development (Thiery, 2003); that is, the process of epithelial cell migration in zebrafish embryos may involve the same genes as those important for tumor progression. It is unknown, however, whether activating oncogenic mutation of any of these EMT genes also leads to increased tumor susceptibility in adult zebrafish. Likewise, very little is currently known about the tumor phenotype of zebrafish with mutations in orthologs of many known human tumor suppressor

genes. The conservation of function among the cancer genes characterized in zebrafish thus far (Stern and Zon, 2003) suggests that these others may also be playing the same role as in mammals. As the BRAF melanoma model suggests (Patton et al., 2005), additional mutations, such as that of *p53*, may be required for tumorigenesis in fish.

The utility of the zebrafish as a cancer model system is enhanced by the ability to transplant tumor cells among fish, as described above and in other studies (Langenau et al., 2003; Mizgireuv and Revskoy, 2006). However, the zebrafish seems not to be as useful a model as the mouse with respect to performing direct transplants of human tumor cells. In at least one published case, human cancer cells transplanted into fish do not form tumors (Lee et al., 2005). However, in this study, the malignant human melanoma cells managed to survive and migrate in the fish, often to the skin, suggesting at least that homing cues may be conserved in the fish.

While it is clear that many aspects of human cancer are conserved in zebrafish, the true power of the zebrafish as a cancer model, and its prime advantage over the mouse, is its utility as a gene-finding tool. The forward genetic screen is an unbiased approach that can identify novel cancer genes or known genes with a previously uncharacterized role in cancer. Shepard et al. conducted an ENU-based screen in zebrafish embryos for mutants in cell proliferation (Shepard et al., 2005). Among these mutants, one was found to have a mutation in *bmyb*, a putative proto-oncogene in humans. Whereas homozygous mutant embryos display an increase in mitotic cells due to an inability to exit from mitosis, heterozygous adults have an enhanced susceptibility to chemical carcinogenesis compared to their wild-type siblings.

Since the Hopkins screen was not biased towards discarding mutants with non-specific phenotypes, the final collection contains mutants in genes, such as housekeeping genes, which, when mutated, lead to pleiotropic effects. In fact, many known cell-essential genes such as cell

cycle regulators, components of the DNA synthesis machinery, and translation factors are represented in the collection (Amsterdam et al., 2004). Since, with the exception of p53, the majority of known tumor suppressors are homozygous recessive lethal (Jacks, 1996), we reasoned that a re-screen of our collection of mainly embryonic lethal mutants would yield some previously uncharacterized tumor suppressors. As I will describe in Chapter 2, this hypothesis proved to be correct. A major finding of this thesis is that many ribosomal proteins comprise a novel class of haploinsufficient tumor suppressors in the zebrafish.

3. Ribosomal Proteins

3a. The Translation Machinery: Structure and Function

The ribosome is a macromolecular complex responsible for protein synthesis in the cell. It is composed of one large subunit (60*S* in Eukarya; 50*S* in Bacteria and Archaea) and one small subunit (40*S* in Eukarya; 30*S* in Bacteria and Archaea). Each subunit consists of one to three ribosomal RNAs (rRNAs) and numerous ribosomal proteins (rps). In eukaryotes, protein translation begins when the small subunit, along with associated initiation factors, binds to the 5' cap of mRNAs (cap-dependent translation) or to internal ribosome entry sites (IRES) on the mRNA (cap-independent translation) (Merrick, 2004). This complex scans the mRNA for an AUG start codon, upon which the large subunit binds, forming the complete, active ribosome. A tRNA bearing methionine enters the A (aminoacyl) site, and translocates into the P (peptidyl) site, allowing the subsequent aminoacyl-tRNA to bind in the A site. A peptide bond is formed between the amino acids, and the ribosome moves processively along the mRNA, with the nascent polypeptide chain emerging from the exit pore (Kapp and Lorsch, 2004). Ribosomes can

load and initiate translation before the previous ribosome reaches the end of the message, thereby forming polysomes.

While the basic mechanics of translation have been known since the 1960's (Watson, 1964), the structural basis for these actions has only been recently elucidated. The crystal structures of ribosomal subunits from several prokaryotic species, including *Haloarcula marismortui*, *Deinococcus radiodurans,* and *Thermus thermophilus* (Ban et al., 2000; Harms et al., 2001; Wimberly et al., 2000), have been solved at high resolution, thus revealing the spatial locations of the rps, as well their interactions with the rRNA and with one another.

In eukaryotes, ribosomal subunit assembly begins in the nucleolus, the site of most rRNA transcription. The genes encoding rRNAs occur as tandem repeats in the genome, often in stretches of hundreds of copies (Raška et al., 2004). The major rRNA is transcribed as a single unit by RNA polymerase III and undergoes extensive processing to become the individual rRNAs in both the small and large subunit. This process has been studied extensively, and many of the participating exonucleases, snoRNAs, and chaperone proteins have been characterized in detail (Fromont-Racine et al., 2003). Less is known about the role of the ribosomal proteins in this process. Since rps are produced in the cytoplasm, they must be transported into the nucleus. While interactions among the rps have been demonstrated by the crystal structures (Brodersen et al., 2002; Klein et al., 2004), the order of their loading onto the complex is only beginning to be understood (Talkington et al., 2005). The precise interactions of the rps with the non-ribosomal proteins that also participate in the assembly process (Alix, 1993) are also poorly characterized (Fromont-Racine et al., 2003).

3b. Properties of Ribosomal Proteins

There are approximately 79 ribosomal proteins in the mammalian ribosome, 68 in the archaeal ribosome, and 57 in the *E. coli* ribosome (Wilson and Nierhaus, 2005; Wool et al., 1995). Rps from *E. coli* were first purified biochemically in late 1960's (Kaltschmidt et al., 1967), and were named according to their position on two-dimensional gels, with the prefix S or L indicating whether they belong to the small or large ribosomal subunit, respectively (Kaltschmidt and Wittmann, 1970). Since they are so numerous and generally quite similar in size and other properties, several rps have been mistakenly labeled as distinct proteins when in fact they were identical to other previously identified rps; the names of such proteins have thus been dropped from the sequence. For example, L30 is L25; S22 is L32; L16 is L12; and L33 is L24 (Wool et al., 1995). In *E. coli*, L7 is actually the acetylated form of L12. In at least one case, a multimer of rps was mistaken as a single rp: The protein initially identified as L8 in *E. coli* is now known to consist of a tetramer of L7/L12 in complex with a single copy of L10 (Wilson and Nierhaus, 2005).

The ribosomal proteins are generally small, averaging about 164 amino acids in length (ranging from 25 to 421 amino acids) (Wool et al., 1995). They are nearly all very basic, as one might expect, due to the fact that most of them interact extensively with the phosphate backbone of rRNA. The notable exceptions are the P0, P1, and P2 proteins in the mammalian large subunit, the so-called acidic P-type rps whose name is derived from the fact that they are phosphorylated (Tsurugi et al., 1978). They form a complex with a stoichiometry of $P1_2$-$P2_2$-P0 and seem to be functionally analogous to the L10-$(L7/L12)_4$ complex of *E. coli* in that they both bind to initiation, elongation, and release factors (Brot and Weissbach, 1981; MacConnell and Kaplan, 1982).

There are a few structural domains common among ribosomal proteins that were deduced from the primary sequence of amino acids. Several rps have zinc finger domains, and a few others have leucine zipper motifs (Chan et al., 1993; Neumann et al., 1995; Soultanas et al., 1998; Wool et al., 1995). In addition, since the eukaryotic rps are transported into the nucleus, the rps generally have a nuclear localization signal. The sequence of this signal, however, is not conserved, and seems only to rely on certain stretches of basic residues that are common in rps (Wool et al., 1995). The recent crystal structures of the rps have revealed similarities among the three-dimensional topology of some rps. In the *H. marismortui* large subunit, 20 of the 27 rps can be placed into five groups by their topological similarities (Klein et al., 2004). Among these are a group of four rps (L24e, L37ae, L37e, and L44e) that have been found to bind zinc, thus confirming the predictions based on their amino acid sequence (Klein et al., 2004).

3c. Normal Roles of Ribosomal Proteins in Translation

The early efforts to purify and catalogue the individual rps were undertaken since it was assumed that the activity of the protein synthesis machinery depended upon the enzymatic activity of these proteins. In the 1980's, the discovery of catalytic RNAs (Cech et al., 1981; Guerrier-Takada et al., 1983) shifted the focus of the protein translation field away from the rps and towards the rRNA. Yet, it is clear that the ribosomal proteins are key components of the ribosome that are required for both its assembly and its function (Wilson and Nierhaus, 2005). Since it has now been accepted that the first ribosome consisted solely of RNA, the question of the origin of the ribosomal proteins remains unanswered. It is unknown whether the rps evolved to be part of the ribosome, or whether they were recruited there after having evolved to serve some other function in the cell. It has been proposed that the rps were added to improve the

fidelity of translation, or perhaps to aid in the folding and structural maintenance of the rRNA, which has evolved to be longer and more complex (Stern et al., 1989). Another proposed initial role for rps was to protect the rRNA from degradation upon the evolutionary development of nucleases (Wool et al., 1995).

It is currently clear that the ribosomal proteins act cooperatively within the ribosome; thus, it has been difficult to assign particular roles to individual rps. Among the known specific functions, as revealed by studies in *H. marismortui* and *T. thermophilus*, are the following (Wilson and Nierhaus, 2005): In the large subunit, L16/L27 are involved in tRNA binding whereas L1 is involved in tRNA release; L9 stabilizes tRNA at the P site; L22 interacts with particular nascent polypeptide chains; and L23 and L29 are located at the tunnel exit site, binding the chaperone trigger factor and the signal recognition particle, respectively. In the small subunit, S1 binds mRNA during initiation; S3, S4, and S5 form the entry pore for mRNA; and S4, S5, and S12 are involved in decoding and error checking.

3d. Evolutionary Conservation of Ribosomal Proteins

The mechanisms of translation are highly conserved among species (Ganoza et al., 2002). Although, to date, the crystal structures of the eukaryotic ribosomal subunits have not been reported, much can be inferred from the prokaryotic structures. A low-resolution analysis by cryo-electron microscopy revealed a great deal of similarity in three-dimensional morphology between the eukaryotic 80*S* and the *E. coli* 70*S* ribosomes (Verschoor et al., 1996). This is in spite of the fact that eukaryotic ribosomes are significantly larger than their *E. coli* counterparts (approximately 4 million Daltons versus 2.8 million Daltons, respectively) (Verschoor et al., 1996). At the level of the ribosomal proteins, the eukaryotic rps are also generally larger than

those in *E. coli*, but clear homologies exist in their amino acid sequences (Ramakrishnan and White, 1998). The most well-conserved rps are, not surprisingly, the ones found to have an important functional role (Müller and Wittmann-Liebold, 1997). For example, *E. coli* S12, a protein involved in maintaining accuracy of translation, is highly similar to the human and rat S23 (Müller and Wittmann-Liebold, 1997). Other *E. coli* rps with close homologs in mammals include S7, S11, S19, and L12 (Müller and Wittmann-Liebold, 1997). In total, of the ~79, 68, and 57 rps in the eukaryotic, archaeal, and bacterial ribosomes, respectively, 34 are common to all three, 15 of these in the large subunit and 19 in the small subunit (Lecompte et al., 2002). This leads to the question: what is the function of the rps that are unique to eukaryotes? As noted above, it has been proposed that many rps have been added to aid in the folding of the more complex rRNA (Wool et al., 1995). However, many of these additional rps have also been demonstrated to have functions in translation similar to the conserved rps, such as substrate binding, error checking, and translocation of the nascent polypeptide chain (Dresios et al., 2006).

3e. Extraribosomal Functions of Ribosomal Proteins

While the ribosomal proteins are considered key elements of the translation machinery, many of the individual proteins have also been demonstrated to have distinct functions outside of the ribosome (Wool, 1996). As noted above, it is unclear whether the rps already possessed these functions before their evolutionary incorporation into the ribosome, or whether they evolved these functions as a consequence of being part of the ribosome. The presence of leucine zipper motifs in some rps suggests that these rps may have had a former role as DNA binding proteins (Chan et al., 1993; Wool et al., 1995). The quality of experimental evidence for the existence of any particular one of these extraribosomal functions varies. In many cases,

investigators purified a protein possessing an activity of interest, and when the protein was sequenced, it was discovered to be a ribosomal protein. It is generally unclear whether each extraribosomal activity is a role that the rp plays in all normal cells, or whether it exhibits this activity only in response to some spatially- or temporally-dependent cue. Furthermore, it is unclear whether any of the extraribosomal roles assigned to the rps are independent of their role in translation; *i.e.*, these various functions may be activated as a consequence of a disruption of normal translation. Regardless, it is of interest to review some of these reports here, as they need to be taken into consideration in our analysis of the mechanism of tumorigenesis in *rp* mutant zebrafish.

In *E. coli*, S1 is required for initiation of replication of RNA phages as a subunit of the viral replicase (Kamen, 1975). Both L3 and L14 are also required in the replication of some bacteriophages as an activator of a DNA helicase (Soultanas et al., 1998; Yancey and Matson, 1991). In addition, lambda phage transcription is affected by the activity of S10, which stimulates antitermination via its interaction with NusB (Friedman et al., 1981; Mason et al., 1992). This latter activity seems to have been conserved in yeast, as the homologue, S20, participates in antitermination by RNA polymerase III (Denmat et al., 1994). Another *E. coli* rp, S9, seems to have a role in DNA repair, a process in which some mammalian rps also seem to be involved. *E. coli* S9 binds UmuC, which is induced in SOS repair (Woodgate et al., 1989); human S3 is the same as apurinic/apyramidinic endonuclease III (Kim et al., 1995), and human P0 has also been found to have apurinic/apyramidinic endonuclease activity (Grabowski et al., 1991). Furthermore, human L7a was found to be induced upon DNA damage by UV irradiation (Ben-Ishai et al., 1990), though a direct role for this rp in DNA repair remains to be found. Intriguingly, however, levels of L7a have been found to be upregulated in human colorectal

carcinomas and prostate cancers (Vaarala et al., 1998; Wang et al., 2000), and it is thought to activate the trk oncoprotein (Zhu et al., 2001).

Another function of various rps with implications for cancer is in the regulation of apoptosis and cell proliferation. For example, overexpression of human L13a has been demonstrated to arrest cells in G2/M phase of the cell cycle, leading to subsequent apoptosis. Knockdown of L13a also appears to sensitize cells to apoptosis induced by treatment with camptothecin, presumably because the inhibition of cell proliferation by L13a normally delays this effect (Chen and Ioannou, 1999). Human L7 is a homologous protein that also induces apoptosis, presumably by arresting cells in G1 (Neumann and Krawinkel, 1997). In addition, human L35a has been shown to be an inhibitor of apoptosis (Lopez et al., 2002).

Among the largest classes of rps with identified extraribosomal functions are, not surprisingly, those that are involved in the regulation of translation itself. This is accomplished by a variety of mechanisms including: (1) inhibiting the transcription of its own message, [e.g., human S14 (Tasheva and Roufa, 1995)] or inhibiting the transcription of another *rp* gene [e.g., *E. coli* L4 inhibits transcription of the S10 operon (Zengel and Lindahl, 1991)]; (2) inhibiting the splicing of its own message [e.g., yeast L32 (Vilardell and Warner, 1994) and *Xenopus* L1 (Bozzoni et al., 1984)]; and (3) inhibiting the translation of its own message and other messages [e.g., *E. coli* S4, S7, S8, L1, L4, and L10 (Nomura et al., 1984); yeast L32 (Vilardell and Warner, 1994); and human L7 (Neumann et al., 1995)].

In addition, S6 has recently been of interest in the context of the mammalian target of rapamycin (mTOR) pathway, a key regulatory pathway that controls cell growth (Inoki et al., 2005). mTOR has been shown to phosphorylate and activate S6 kinase (S6K), which in turn phosphorylates S6, among other substrates (Proud, 2002). The precise role of S6 and its

phosphorylation, however, remains unclear. This signaling is usually correlated with an upregulation of translation, especially of messages containing the 5'-terminal oligopyrimidine (TOP) motif, a sequence of 4 to 14 pyrimidines following a cytosine in the 5' untranslated region (Meyuhas, 2000). Since TOP messages often encode rps and other abundant translation factors (Meyuhas, 2000), the result is an increase in the translational capacity of the cell. Some researchers have found that the phosphorylation of S6 is unnecessary for the translation of TOP messages (Ruvinsky et al., 2005; Stolovich et al., 2002), yet this does not rule out the existence of a redundant mechanism. Thus, S6 might still be involved in recruiting specific messages for translation.

The misregulation of growth control has some obvious implications for the development of cancer. There is further evidence that some rps may be even more directly involved in malignant transformation. The mammalian ribosomal proteins L5, L11, and L23 have all been found to interact with MDM2 (or HDM2 in humans), the oncoprotein which targets the p53 tumor suppressor for destruction. The current model suggests that these rps prevent the interaction of MDM2 with p53, thus increasing the stability of p53 and inducing cell cycle arrest (Zhang and Zhang, 2005). The implications of these findings with respect to tumorigenesis in zebrafish *rp* mutants will be discussed in Chapter 4.

A number of other extraribosomal functions have not been mentioned here (Wool, 1996), though it seems likely that many of these are indirect consequences of their roles in translation. For example, many *Drosophila* rps have been found to play a role in the regulation of development, but these effects are probably a result of a general defect in translation, as discussed below.

3f. Ribosomal Protein Mutations in Model Organisms

Among the model organisms, *rp* mutations have been studied most extensively in *Drosophila.* The *Minute* phenotype of flies is a characteristic set of pleiotropic defects including delayed development, short thin bristles, and recessive lethality (Lambertsson, 1998). In all cases to date, *Minutes* have been mapped to ribosomal protein genes (Kongsuwan et al., 1985; Lambertsson, 1998). It is important to note, however, that not all *rp* mutations in *Drosophila* are associated with *Minutes* (Lambertsson, 1998). This may indicate that the gene dosage of each rp is an important factor in the phenotypic output of the mutation. Evidence in support of this idea was presented by Sæbøe-Larssen et al., who showed that among an allelic series of *S3* mutations, the stronger ones resulted in more pronounced morphological defects (Sæbøe-Larssen et al., 1998). Mutations in several specific *rp* genes in *Drosophila* have also been implicated in improper regulation of growth control, supporting the possibility that rps may be playing a role in tumorigenesis in higher organisms. For example, a P element insertion in the gene encoding the homolog of human S6 leads to an overgrowth of the hematopoietic organs and formation of melanotic tumors (Watson et al., 1992). Similarly, down-regulation of the *S21* gene leads to hyperplasia of the hematopoietic organs and overgrowth of the imaginal discs (Török et al., 1999).

Although the consequences of rp mutations in mammals have not been extensively characterized, there are several documented cases of *rp* mutations in the mouse. For example, Belly spot and tail (*Bst*) is a spontaneous semidominant mouse mutation that was recently found to be a deletion in the *L24* gene that impairs the splicing of the message (Oliver et al., 2004). The mutation is homozygous lethal, and heterozygotes have multiple defects, including a white ventral midline spot, white hind feet, a reduction in retinal ganglion cells of the eye, a kinked

tail, and other skeletal abnormalities. Significantly, the *Bst*/+ mice were also found to be 20% smaller than their wild-type littermates, leading the authors to conclude that *Bst* is a mouse *Minute*.

There are very few published reports of targeted knockouts of *rp* genes in the mouse, likely due to the dearth of data linking mutations of particular *rp* genes to disease states in humans. S19 is one exception: heterozygous mutation of the gene encoding this rp has been found in 25% of human patients with Diamond-Blackfan anemia (DBA) (Draptchinskaia et al., 1999) (See below). The *S19* mouse knockout is early embryonic lethal, as expected (Matsson et al., 2004). Unfortunately, the $S19^{+/-}$ mouse did not display any of the defects in the hematopoietic system that are characteristic of the human disease. The heterozygotes also appeared to develop normally otherwise, with no detectable difference in growth rate or weight, compared to littermate controls. The same group recently showed, however, that loss of one allele of *S19* appears to be compensated for completely at the transcriptional level (Matsson et al., 2006).

The only other published knockout of a ribosomal protein in the mouse is that of S6, the rp that has been implicated in control of cell growth, as described above. Volarević et al. generated a conditional *S6* knockout in which the gene was deleted in liver cells of adult mice (Volarević et al., 2000). Surprisingly, although the cells were impaired in 40*S* ribosome biogenesis, they were able to grow in size in response to nutrients. After partial hepatectomy, however, the *S6*-deficient liver cells displayed a defect in cell proliferation. The authors propose the existence of a checkpoint that arrests the cell cycle upon an acute impairment of ribosome biogenesis.

There is one additional study of *rp* mutations in mice that deserves mention, as it may be relevant in a discussion of the link between rps and tumorigenesis. Beck-Engeser et al. demonstrated that aggressively growing variants of two independent tumor cell lines expressed tumor antigens that were found to be rps (Beck-Engeser et al., 2001). In one line, one allele of *L9* had a point mutation and the other allele was lost; in another line, both copies of *L26* were mutant, suggesting that oncogenic activation can occur in *rp* genes. In the case of *L9*, reintroduction of a wild-type allele suppressed this putative oncogenic effect, as the tumor cells reverted to a slower-growing phenotype.

3g. Translation and Cancer

It has generally been accepted that cell growth and cell proliferation are intimately linked, but the direct causal links between protein translation and cancer are only beginning to be elucidated (Clemens, 2004). The aberrant activity of several translation factors has recently been associated with tumorigenesis. For example, levels of the initiation factor eIF2 have been found to be elevated in tumor cells (Lobo et al., 2000; Rosenwald, 1996). eIF2 normally forms a ternary complex with Met-tRNA$_f$ and GTP, and is important for binding to the 40*S* ribosomal subunit. eIF2 is regulated by phosphorylation of its smallest (α) subunit, the phosphorylated form being an inhibitor of the guanine nucleotide exchange factor eIF2B (Clemens, 2001). Since the unphosphorylated form of eIF2 is required for translation initiation, misregulation of its phosphorylation can lead to cancer. For example, a non-phosphorylatable form of eIF2α has been shown to induce malignant transformation of NIH 3T3 cells (Donzé et al., 1995).

Two other translation initiation factors, eIF4E and eIF4G have been implicated in tumorigenesis. Both factors have been found to be overexpressed in tumors (Bauer et al., 2001;

Zimmer et al., 2000). Moreover, experimental overexpression of both can induce cell transformation (Fukuchi-Shimogori et al., 1997; Lazaris-Karatzas et al., 1990). eIF4E normally binds to the 5' cap of mRNAs and also binds eIF4G, forming a complex along with eIF4A. Formation of this complex, which brings the mRNA to the ribosome, is a key step in translation initiation. The binding of eIF4E to eIF4G is inhibited by the eIF4E-binding proteins (4E-BPs). Phosphorylation of the 4E-BPs by mTOR has been shown to release them from eIF4E, thereby stimulating cap-dependent translation (Gingras et al., 1999).

At the level of the ribosome proper, there is limited data linking the rps to tumorigenesis. As hinted at above in the case of L7a (Vaarala et al., 1998; Wang et al., 2000), rps are generally overexpressed in cancer (Grabowski et al., 1992; Henry et al., 1993; Nadano et al., 2002). Of course, an increase in cell proliferation necessitates an increase in protein translation, so the elevated level of rps is likely to be an effect, rather than a cause, of cancer. Consistent with this notion is the finding that the transcription of some rps decreases upon apoptosis (Chen et al., 1998; Goldstone and Lavin, 1993; Lin et al., 1994). In contrast, a recent report has shown that L14 expression is reduced in some esophageal squamous cell carcinomas (Huang et al., 2006), though the functional significance of this is unknown.

There is some intriguing evidence to suggest that misregulation or mutation of some rps could lead to cancer. As mentioned above, some molecular data suggest that L5, L11, and L23 may be playing a role in the key p53 tumor suppressor pathway (Zhang and Zhang, 2005), though misregulation of these rps has not been demonstrated in human tumors *in vivo*. L10 has also been linked to cancer in that it was found to be identical to a putative Wilms' tumor suppressor protein (Chan et al., 1996), though any tumor suppressive activity of this protein has not been shown. In addition, as mentioned above, Diamond-Blackfan anemia is linked to a

mutation in S19 in 25% of patients. In addition to displaying the characteristic anemia caused by a deficiency of erythroid precursors, DBA patients are prone to the development of leukemias (Janov et al., 1996) and, at lower frequencies, solid tumors (Lipton et al., 2001). It is not known whether tumorigenesis is an indirect result of the anemia or whether S19 dosage plays a direct role. Furthermore, it is not clear whether there is a direct correlation between DBA patients who have an S19 mutation and those that develop leukemias. In short, causative links between *rp* mutations and cancer in humans are to date very weak, though a careful analysis has yet to be done.

4. Conclusion

The zebrafish has proven to be a valuable tool in gene discovery. Forward genetic screens in embryos have led to the elucidation of numerous genes and pathways involved in vertebrate development. We are now capitalizing on the strengths of the zebrafish model system to study diseases of adult humans, including cancer. As I will present in this thesis, a screen for cancer genes in zebrafish has led to the surprising discovery that many ribosomal protein genes are haploinsufficient tumor suppressors. While data in the literature supporting a role for *rp* mutations in cancer are sparse, there is considerable evidence to suggest that misregulation of protein translation can be an initiating event in tumorigenesis. The work of this thesis provides evidence that *rp* mutations, likely through an effect on translation, can cause cancer in the zebrafish. Since the rps and the translation machinery are extremely well-conserved throughout evolution, it is likely that we will find *rp* mutations in human cancers. This represents a novel mechanism of tumorigenesis that may inspire the development of new cancer therapies.

References

Alix, J.-H. (1993). Extrinsic Factors in Ribosome Assembly, In The Translational Apparatus: Structure, Function, Regulation, Evolution, K. H. Nierhaus, F. Franceschi, A. R. Subramanian, V. A. Erdmann, and B. Wittmann-Liebold, eds. (New York, NY: Plenum Press), pp. 173-184.

Amatruda, J. F., Shepard, J. L., Stern, H. M., and Zon, L. I. (2002). Zebrafish as a cancer model system. Cancer Cell *1*, 229-231.

Amsterdam, A., Burgess, S., Golling, G., Chen, W., Sun, Z., Townsend, K., Farrington, S., Haldi, M., and Hopkins, N. (1999). A large-scale insertional mutagenesis screen in zebrafish. Genes and Development *13*, 2713-2724.

Amsterdam, A., Nissen, R. M., Sun, Z., Swindell, E. C., Farrington, S., and Hopkins, N. (2004). Identification of 315 genes essential for early zebrafish development. Proceedings of the National Academy of Sciences *101*, 12792-12797.

Ban, N., Nissen, P., Hansen, J., Moore, P. B., and Steitz, T. A. (2000). The Complete Atomic Structure of the Large Ribosomal Subunit at 2.4 Å Resolution. Science *289*, 905-920.

Battle, H. I., and Hisaoka, K. K. (1952). Effects of ethyl carbamate (urethan) on the early development of the teleost *Brachydanio rerio*. Cancer Research *12*, 334-340.

Bauer, C., Diesinger, I., Brass, N., Steinhart, H., Iro, H., and Meese, E. U. (2001). Translation Initiation Factor eIF-4G Is Immunogenic, Overexpressed, and Amplified in Patients with Squamous Cell Lung Carcinoma. Cancer *92*, 822-829.

Baumann, M., and Sander, K. (1984). Bipartite axiation follows incomplete epiboly in zebrafish embryos treated with chemical teratogens. The Journal of Experimental Zoology *230*, 363-376.

Beck-Engeser, G. B., Monach, P. A., Mumberg, D., Yang, F., Wanderling, S., Schreiber, K., Espinosa, R., Beau, M. M. L., Meredith, S. C., and Schreiber, H. (2001). Point Mutation in Essential Genes with Loss or Mutation of the Second Allele: Relevance to the Retention of Tumor-specific Antigens. The Journal of Experimental Medicine *194*, 285-299.

Ben-Ishai, R., Scharf, R., Sharon, R., and Kapten, I. (1990). A human cellular sequence implicated in *trk* oncogene activation is DNA damage inducible. Proceedings of the National Academy of Sciences *87*, 6039-6043.

Bertrand, S., Pinte, S., Stankovic-Valentin, N., Deltour-Balerdi, S., Guérardel, C., Bégue, A., Laudet, V., and Leprince, D. (2004). Identification and developmental expression of the zebrafish orthologue of the tumor suppressor gene *HIC1*. Biochimica et Biophysica Acta *1678*, 57-66.

Bollig, F., Mehringer, R., Perner, B., Hartung, C., Schäfer, M., Schartl, M., Volff, J.-N., Winkler, C., and Englert, C. (2006). Identification and Comparative Expression Analysis of a Second *wt1* Gene in Zebrafish. Developmental Dynamics *235*, 554-561.

Bozzoni, I., Fragapane, P., Annesi, F., Pierandrei-Amaldi, P., Amaldi, F., and Beccari, E. (1984). Expression of two *Xenopus laevis* ribosomal protein genes in injected frog oocytes: A specific splicing block interferes with the L1 RNA maturation. Journal of Molecular Biology *180*, 987-1005.

Brodersen, D. E., Clemons, W. M., Carter, A. P., Wimberly, B. T., and Ramakrishnan, V. (2002). Crystal Structure of the 30 *S* Ribosomal Subunit from *Thermus thermophilus*: Structure of the Proteins and their Interactions with 16 *S* RNA. Journal of Molecular Biology *316*, 725-768.

Brot, N., and Weissbach, H. (1981). Chemistry and biology of *E. coli* ribosomal protein L12. Molecular and Cellular Biochemistry *36*, 47-63.

Brownlie, A., Donovan, A., Pratt, S. J., Paw, B. H., Oates, A. C., Brugnara, C., Witkowska, H. E., Sassa, S., and Zon, L. I. (1998). Positional cloning of the zebrafish *sauternes* gene: a model for congenital sideroblastic anaemia. Nature Genetics *20*, 244-250.

Cech, T. R., Zaug, A. J., and Grabowski, P. J. (1981). In Vitro Splicing of the Ribosomal RNA Precursor of Tetrahymena: Involvement of a Guanosine Nucleotide in the Excision of the Intervening Sequence. Cell *27*, 487-496.

Chan, Y.-L., Diaz, J.-J., Denoroy, L., Madjar, J.-J., and Wool, I. G. (1996). The Primary Structure of Rat Ribosomal Protein L10: Relationship to a Jun-Binding Protein and to a Putative Wilms' Tumor Suppressor. Biochemical and Biophysical Research Communications *225*, 952-956.

Chan, Y.-L., Suzuki, K., Olvera, J., and Wool, I. G. (1993). Zinc finger-like motifs in rat ribosomal proteins S27 and S29. Nucleic Acids Research *21*, 649-655.

Chen, F. W., Davies, J. P., and Ioannou, Y. A. (1998). Differential Gene Expression in Apoptosis: Identification of Ribosomal Protein 23K, a Cell Proliferation Inhibitor. Molecular Genetics and Metabolism *64*, 271-282.

Chen, F. W., and Ioannou, Y. A. (1999). Ribosomal Proteins in Cell Proliferation and Apoptosis. International Reviews of Immunology *18*, 429-448.

Clemens, M. J. (2001). Initiation factor eIF2 alpha phosphorylation in stress responses and apoptosis, In Progress in Molecular and Subcellular Biology, Vol. 27, R. E. Rhoads, ed. (Springer: Berlin), pp. 57-89.

Clemens, M. J. (2004). Targets and mechanisms for the regulation of translation in malignant transformation. Oncogene *23*, 3180-3188.

Croushore, J. A., Blasiole, B., Riddle, R. C., Thisse, C., Thisse, B., Canfield, V. A., Robertson, G. P., Cheng, K. C., and Levenson, R. (2005). *ptena* and *ptenb* Genes Play Distinct Roles in Zebrafish Embryogenesis. Developmental Dynamics *234*, 911-921.

Denmat, S. H.-L., Sipiczki, M., and Thuriaux, P. (1994). Suppression of Yeast RNA Polymerase III Mutations by the *URP2* Gene Encoding a Protein Homologous to the Mammalian Ribosomal Protein S20. Journal of Molecular Biology *240*, 1-7.

Donzé, O., Jagus, R., Koromilas, A. E., Hershey, J. W. B., and Sonenberg, N. (1995). Abrogation of translation initiation factor eIF-2 phosphorylation causes malignant transformation of NIH 3T3 cells. The EMBO Journal *14*, 3828-3834.

Draptchinskaia, N., Gustavsson, P., Andersson, B., Pettersson, M., Willig, T.-N., Dianzani, I., Ball, S., Tchernia, G., Klar, J., Matsson, H., *et al.* (1999). The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia. Nature Genetics *21*, 169-175.

Dresios, J., Panopoulos, P., and Synetos, D. (2006). Eukaryotic ribosomal proteins lacking a eubacterial counterpart: important players in ribosomal function. Molecular Microbiology *59*, 1651-1663.

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C. F., Malicki, J., Stemple, D. L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Rangini, Z., *et al.* (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. Development *123*, 37-46.

Ensenbach, U., and Nagel, R. (1995). Toxicity of Complex Chemical Mixtures: Acute and Long-Term Effects on Different Life Stages of Zebrafish (*Brachydanio rerio*). Ecotoxicology and Environmental Safety *30*, 151-157.

Friedman, D. I., Schauer, A. T., Baumann, M. R., Baron, L. S., and Adhya, S. L. (1981). Evidence that ribosomal protein S10 participates in control of transcription termination. Proceedings of the National Academy of Sciences *78*, 1115-1118.

Fromont-Racine, M., Sengerb, B., Saveanua, C., and Fasiolo, F. (2003). Ribosome assembly in eukaryotes. Gene *313*, 17-42.

Fukuchi-Shimogori, T., Ishii, I., Kashiwagi, K., Mashiba, H., Ekimoto, H., and Igarashi, K. (1997). Malignant transformation by overproduction of translation initiation factor eIF4G. Cancer Research *57*, 5041-5044.

Gaiano, N., Allende, M., Amsterdam, A., Kawakami, K., and Hopkins, N. (1996). Highly efficient germ-line transmission of proviral insertions in zebrafish. Proceedings of the National Academy of Sciences *93*, 7777-7782.

Ganoza, M. C., Kiel, M. C., and Aoki, H. (2002). Evolutionary Conservation of Reactions in Translation. Microbiology and Molecular Biology Reviews *66*, 460-485.

Garrity, D. M., Childs, S., and Fishman, M. C. (2002). The *heartstrings* mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. Development *129*, 4635-4645.

Gingras, A.-C., Raught, B., and Sonenberg, N. (1999). eIF4 Initiation Factors: Effectors of mRNA Recruitment to Ribosomes and Regulators of Translation. Annual Review of Biochemistry *68*, 913-963.

Gissen, P., Johnson, C. A., Morgan, N. V., Stapelbroek, J. M., Forshew, T., Cooper, W. N., McKiernan, P. J., Klomp, L. W., Morris, A. A. M., Wraith, J. E., *et al.* (2004). Mutations in *VPS33B*, encoding a regulator of SNARE-dependent membrane fusion, cause arthrogryposis-renal dysfunctioncholestasis (ARC) syndrome. Nature Genetics *36*, 400-404.

Goldstone, S. D., and Lavin, M. F. (1993). Isolation of a cDNA clone, encoding the ribosomal protein S20, downregulated during the onset of apoptosis in a human leukaemic cell line. Biochemical and Biophysical Research Communications *196*, 619-623.

Golling, G., Amsterdam, A., Sun, Z., Antonelli, M., Maldonado, E., Chen, W., Burgess, S., Haldi, M., Artzt, K., Farrington, S., *et al.* (2002). Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. Nature Genetics *31*, 135-140.

Gordon, M. (1931). Hereditary basis of melanosis in hybrid fishes. American Journal of Cancer *15*, 1495-1523.

Grabowski, D. T., Deutsch, W. A., Derda, D., and Kelley, M. R. (1991). Drosophila AP3, a presumptive DNA repair protein, is homologous to human ribosomal associated protein P0. Nucleic Acids Research *19*, 4297.

Grabowski, D. T., Pieper, R. O., Futscher, B. W., Deutsch, W. A., Erickson, L. C., and Kelley, M. R. (1992). Expression of ribosomal phosphoprotein P0 is induced by antitumor agents and increased in Mer- human tumor cell lines. Carcinogenesis *13*, 259-263.

Grunwald, D. J., and Streisinger, G. (1992). Induction of recessive lethal and specific locus mutations in the zebrafish with ethyl nitrosourea. Genetical Research *59*, 103-116.

Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., and Altman, S. (1983). The RNA Moiety of Ribonuclease P Is the Catalytic Subunit of the Enzyme. Cell *35*, 849-857.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Eeden, F. J. M. v., Jiang, Y.-J., Heisenberg, C.-P., *et al.* (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. Development *123*, 1-36.

Haramis, A.-P. G., Hurlstone, A., Velden, Y. v. d., Begthel, H., Born, M. v. d., Offerhaus, G. J. A., and Clevers, H. C. (2006). Adenomatous polyposis coli-deficient zebrafish are susceptible to digestive tract neoplasia. EMBO reports *27 January 2006 Online*, 1-6.

Harms, J., Schluenzen, F., Zarivach, R., Bashan, A., Gat, S., Agmon, I., Bartels, H., Franceschi, F., and Yonath, A. (2001). High Resolution Structure of the Large Ribosomal Subunit from a Mesophilic Eubacterium. Cell *107*, 679-688.

Henry, J. L., Coggin, D. L., and King, C. R. (1993). High-level expression of the ribosomal protein L19 in human breast tumors that overexpress erbB-2. Cancer Research *53*, 1403-1408.

Hertog, J. d. (2005). Chemical Genetics: Drug Screens in Zebrafish. Bioscience Reports *25*, 289-297.

Hisaoka, K. K. (1958a). The effects of 2-acetylaminofluorene on the embryonic development of the zebrafish: II. Histochemical studies. Cancer Research *18*, 664-667.

Hisaoka, K. K. (1958b). The effects of 4-acetylaminofluorene on the embryonic development of the zebrafish: I. Morphological studies. Cancer Research *18*, 527-535.

Hisaoka, K. K., and Hopper, A. F. (1957). Some effects of barbituric and diethylbarbituric acid on the development of the zebra fish, *Brachydanio rerio*. The Anatomical Record *129*, 297-307.

Huang, X.-P., Zhao, C.-X., Li, Q.-J., Cai, Y., Liu, F.-X., Hu, H., Xu, X., Han, Y.-L., Wu, M., Zhan, Q.-M., and Wang, M.-R. (2006). Alteration of *RPL14* in squamous cell carcinomas and preneoplastic lesions of the esophagus. Gene *366*, 161-168.

Imamura, S., and Kishi, S. (2005). Molecular cloning and functional characterization of zebrafish ATM. The International Journal of Biochemistry & Cell Biology *37*, 1105-1116.

Inoki, K., Ouyang, H., Li, Y., and Guan, K.-L. (2005). Signaling by Target of Rapamycin Proteins in Cell Growth Control. Microbiology and Molecular Biology Reviews *69*, 79-100.

Jacks, T. (1996). Tumor suppressor gene mutations in mice. Annual Review of Genetics *30*, 603-636.

Janov, A. J., Leong, T., Nathan, D. G., and Guinan, E. C. (1996). Diamond-Blackfan anemia. Natural history and sequelae of treatment. Medicine *75*, 77-78.

Kaltschmidt, E., Dzionara, M., Donner, D., and Wittmann, H. G. (1967). Ribosomal proteins, I. Isolation, amino acid composition, molecular weights and peptide mapping of proteins from *E. coli* ribosomes. Molecular and General Genetics *100*, 364-373.

Kaltschmidt, E., and Wittmann, H. G. (1970). Ribosomal Proteins, XII. Number of Proteins in Small and Large Ribosomal Subunits of *Escherichia coli* as Determined by Two-Dimensional Gel Electrophoresis. Proceedings of the National Academy of Sciences *67*, 1276-1282.

Kamen, R. I. (1975). Structure and function of the Qβ RNA replicase, In RNA Phages, N. D. Zinder, ed. (Cold Spring Harbor Press), pp. 203-234.

Kapp, L. D., and Lorsch, J. R. (2004). The Molecular Mechanics of Eukaryotic Translation. Annual Review of Biochemistry *73*, 657-704.

Kim, J., Chubatsu, L. S., Admon, A., Stahl, J., Fellous, R., and Linn, S. (1995). Implication of Mammalian Ribosomal Protein S3 in the Processing of DNA Damage. The Journal of Biological Chemistry *270*, 13620-13629.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. Developmental Dynamics *203*, 253-310.

Klein, D. J., Moore, P. B., and Steitz, T. A. (2004). The Roles of Ribosomal Proteins in the Structure Assembly, and Evolution of the Large Ribosomal Subunit. Journal of Molecular Biology *340*, 141-177.

Kongsuwan, K., Yu, Q., Vincent, A., Frisardi, M. C., Rosbash, M., Lengyel, J. A., and Merriam, J. (1985). A *Drosophila Minute* gene encodes a ribosomal protein. Nature *317*, 555-558.

Lam, S. H., Wu, Y. L., Vega, V. B., Miller, L. D., Spitsbergen, J., Tong, Y., Zhan, H., Govindarajan, K. R., Lee, S., Mathavan, S., *et al.* (2006). Conservation of gene expression signatures between zebrafish and human liver tumors and tumor progression. Nature Biotechnology *24*, 73-75.

Lambertsson, A. (1998). The *Minute* Genes in *Drosophila* and Their Molecular Functions. Advances in Genetics *38*, 69-134.

Langenau, D. M., Traver, D., Ferrando, A. A., Kutok, J. L., Aster, J. C., Kanki, J. P., Lin, S., Prochownik, E., Trede, N. S., Zon, L. I., and Look, A. T. (2003). Myc-Induced T Cell Leukemia in Transgenic Zebrafish. Science *299*, 887-890.

Lazaris-Karatzas, A., Montine, K. S., and Sonenberg, N. (1990). Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. Nature *345*, 544-547.

Lecompte, O., Ripp, R., Thierry, J.-C., Moras, D., and Poch, O. (2002). Comparative analysis of ribosomal proteins in complete genomes: an example of reductive evolution at the domain scale. Nucleic Acids Research *30*, 5382-5390.

Lee, L. M. J., Seftor, E. A., Bonde, G., Cornell, R. A., and Hendrix, M. J. C. (2005). The Fate of Human Malignant Melanoma Cells Transplanted Into Zebrafish Embryos: Assessment of Migration and Cell Division in the Absence of Tumor Formation. Developmental Dynamics *233*, 1560-1570.

Lin, C. H., Palma, J. F., and Solomon, W. B. (1994). Phorbol ester induction of differentiation and apoptosis in the K562 cell line is accompanied by marked decreases in the stability of globin mRNAs and decreases in the steady state level of mRNAs encoding for ribosomal proteins L35, L31, L27, and L21. Cellular and Molecular Biology Research *40*, 13-26.

Lipton, J. M., Federman, N., Khabbaze, Y., Schwartz, C. L., Hilliard, L. M., Clark, J. I., Vlachos, A., and Diamond-Blackfan Anemia Registry (2001). Osteogenic sarcoma associated with Diamond-Blackfan anemia: a report from the Diamond-Blackfan Anemia Registry. Journal of Pediatric Hematology/Oncology *23*, 39-44.

Lobo, M. V. T., Martín, M. E., Pérez, M. I., Alonso, F. J. M., Redondo, C., Álvarez, M. I., and Salinas, M. (2000). Levels, phosphorylation status and cellular localization of translational factor eIF2 in gastrointestinal carcinomas. The Histochemical Journal *32*, 139-150.

Lopez, C. D., Martinovsky, G., and Naumovski, L. (2002). Inhibition of cell death by ribosomal protein L35a. Cancer Letters *180*, 195-202.

MacConnell, W. P., and Kaplan, N. O. (1982). The Activity of the Acidic Phosphoproteins from the 80 S Rat Liver Ribosome. The Journal of Biological Chemistry *257*, 5359-5366.

Mason, S. W., Li, J., and Greenblatt, J. (1992). Direct interaction between two *Escherichia coli* transcription antitermination factors, NusB and ribosomal protein S10. Journal of Molecular Biology *223*, 55-66.

Matsson, H., Davey, E. J., Draptchinskaia, N., Hamaguchi, I., Ooka, A., Levéen, P., Forsberg, E., Karlsson, S., and Dahl, N. (2004). Targeted Disruption of the Ribosomal Protein S19 Gene Is Lethal Prior to Implantation. Molecular and Cellular Biology *24*, 4032-4037.

Matsson, H., Davey, E. J., Fröjmark, A. S., Miyake, K., Utsugisawa, T., Flygare, J., Zahou, E., Byman, I., Landin, B., Ronquist, G., *et al.* (2006). Erythropoiesis in the *Rps19* disrupted mouse: Analysis of erythropoietin response and biochemical markers for Diamond-Blackfan anemia. Blood Cells, Molecules, and Diseases *36*, 259-264.

Merrick, W. C. (2004). Cap-dependent and cap-independent translation in eukaryotic systems. Gene *332*, 1-11.

Meyuhas, O. (2000). Synthesis of the translational apparatus is regulated at the translational level. European Journal of Biochemistry *267*, 6321-6330.

Mizgireuv, I. V., and Revskoy, S. Y. (2006). Transplantable Tumor Lines Generated in Clonal Zebrafish. Cancer Research *66*, 3120-3125.

Müller, E.-C., and Wittmann-Liebold, B. (1997). Phylogenetic relationship of organisms obtained by ribosomal protein comparison. Cellular and Molecular Life Sciences *53*, 34-50.

Nadano, D., Notsu, T., Matsuda, T., and Sato, T.-A. (2002). A human gene encoding a protein homologous to ribosomal protein L39 is normally expressed in the testis and derepressed in multiple cancer cells. Biochimica et Biophysica Acta *1577*, 430-436.

Neumann, F., Hemmerich, P., Mikecz, A. v., Peter, H.-H., and Krawinkel, U. (1995). Human ribosomal protein L7 inhibits cell-free translation in reticulocyte lysates and affects the expression of nuclear proteins upon stable transfection into Jurkat T-lymphoma cells. Nucleic Acids Research *23*, 195-202.

Neumann, F., and Krawinkel, U. (1997). Constitutive Expression of Human Ribosomal Protein L7 Arrests the Cell Cycle in G_1 and Induces Apoptosis in Jurkat T-Lymphoma Cells. Experimental Cell Research *230*, 252-261.

Nomura, M., Gourse, R., and Baughman, G. (1984). Regulation of the synthesis of ribosomes and ribosomal components. Annual Review of Biochemistry *53*, 75-117.

Oliver, E. R., Saunders, T. L., Tarlé, S. A., and Glaser, T. (2004). Ribosomal protein L24 defect in Belly spot and tail (*Bst*), a mouse *Minute*. Development *131*, 3907-3920.

Patton, E. E., Widlund, H. R., Kutok, J. L., Kopani, K. R., Amatruda, J. F., Murphey, R. D., Berghmans, S., Mayhall, E. A., Traver, D., Fletcher, C. D. M., *et al.* (2005). BRAF Mutations Are Sufficient to Promote Nevi Formation and Cooperate with p53 in the Genesis of Melanoma. Current Biology *15*, 249-254.

Patton, E. E., and Zon, L. I. (2005). Taking Human Cancer Genes to the Fish: A Transgenic Model of Melanoma in Zebrafish. Zebrafish *1*, 363-368.

Penberthy, W. T., Shafizadeh, E., and Lin, S. (2002). The zebrafish as a model for human disease. Frontiers in Bioscience *7*, d1439-1453.

Peterson, R. T., Link, B. A., Dowling, J. E., and Schreiber, S. L. (2000). Small molecule developmental screens reveal the logic and timing of vertebrate development. Proceedings of the National Academy of Sciences *97*, 12965–12969.

Proud, C. G. (2002). Regulation of mammalian translation factors by nutrients. European Journal of Biochemistry *269*, 5338-5349.

Ramakrishnan, V., and White, S. W. (1998). Ribosomal protein structures: insights into the architecture, machinery and evolution of the ribosome. Trends in Biochemical Sciences *23*, 208-212.

Raška, I., Koberna, K., Malínský, J., Fidlerová, H., and Mašata, M. (2004). The nucleolus and transcription of ribosomal genes. Biology of the Cell *96*, 579-594.

Rosenwald, I. B. (1996). Upregulated expression of the genes encoding translation initiation factors eIF-4E and eIF-2α in transformed cells. Cancer Letters *102*, 113-123.

Ruvinsky, I., Sharon, N., Lerer, T., Cohen, H., Stolovich-Rain, M., Nir, T., Dor, Y., Zisman, P., and Meyuhas, O. (2005). Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. Genes and Development *19*, 2199-2211.

Sadler, K. C., Amsterdam, A., Soroka, C., Boyer, J., and Hopkins, N. (2005). A genetic screen in zebrafish identifies the mutants *vps18*, *nf2* and *foie gras* as models of liver disease. Development *132*, 3561-3572.

Sæbøe-Larssen, S., Lyamouri, M., Merriam, J., Oksvold, M. P., and Lambertsson, A. (1998). Ribosomal Protein Insufficiency and the Minute Syndrome in Drosophila: A Dose-Response Relationship. Genetics *148*, 1215-1224.

Schartl, M. (1995). Platyfish and swordtails: a genetic system for the analysis of molecular mechanisms in tumor formation. Trends in Genetics *11*, 185-189.

Shepard, J. L., Amatruda, J. F., Stern, H. M., Subramanian, A., Finkelstein, D., Ziai, J., Finley, K. R., Pfaff, K. L., Hersey, C., Zhou, Y., *et al.* (2005). A zebrafish *bmyb* mutation causes genome instability and increased cancer susceptibility. Proceedings of the National Academy of Sciences *102*, 13194-13199.

Soultanas, P., Dillingham, M. S., and Wigley, D. B. (1998). *Escherichia coli* ribosomal protein L3 stimulates the helicase activity of the *Bacillus stearothermophilus* PcrA helicase. Nucleic Acids Research *26*, 2374-2379.

Spitsbergen, J. M., Tsai, H. W., Reddy, A., Miller, T., Arbogast, D., Hendricks, J. D., and Bailey, G. S. (2000a). Neoplasia in zebrafish (*Danio rerio*) treated with 7,12-dimethylbenz[a]anthracene by two exposure routes at different developmental stages. Toxicologic Pathology *28*, 705-715.

Spitsbergen, J. M., Tsai, H. W., Reddy, A., Miller, T., Arbogast, D., Hendricks, J. D., and Bailey, G. S. (2000b). Neoplasia in zebrafish (*Danio rerio*) treated with N-methyl-N'-nitro-N-nitrosoguanidine by three exposure routes at different developmental stages. Toxicologic Pathology *28*, 716-725.

Stanton, M. F. (1965). Diethylnitrosamine-induced hepatic degeneration and neoplasia in the aquarium fish, *Brachydanio rerio*. Journal of the National Cancer Institute *34*, 117-130.

Stern, H. M., and Zon, L. I. (2003). Cancer genetics and drug discovery in the zebrafish. Nature Reviews Cancer *3*, 533-539.

Stern, S., Powers, T., Changchien, L.-M., and Noller, H. F. (1989). RNA-Protein Interactions in 30*S* Ribosomal Subunits: Folding and Function of 16*S* rRNA. Science *244*, 783-790.

Stolovich, M., Tang, H., Hornstein, E., Levy, G., Cohen, R., Bae, S. S., Birnbaum, M. J., and Meyuhas, O. (2002). Transduction of Growth or Mitogenic Signals into Translational Activation of TOP mRNAs Is Fully Reliant on the Phosphatidylinositol 3-Kinase-Mediated Pathway but Requires neither S6K1 nor rpS6 Phosphorylation. Molecular and Cellular Biology *22*, 8101-8113.

Streisinger, G., Walker, C., Dower, N., Knauber, D., and Singer, F. (1981). Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). Nature *291*, 293-296.

Sun, Z., Amsterdam, A., Pazour, G. J., Cole, D. G., Miller, M. S., and Hopkins, N. (2004). A genetic screen in zebrafish identifies cilia genes as a principal cause of cystic kidney. Development *131*, 4085-4093.

Talkington, M. W. T., Siuzdak, G., and Williamson, J. R. (2005). An assembly landscape for the 30S ribosomal subunit. Nature *438*, 628-632.

Tasheva, E. S., and Roufa, D. J. (1995). Regulation of human RPS14 transcription by intronic antisense RNAs and ribosomal protein S14. Genes and Development *9*, 304-316.

Taylor, K. M., Hiscox, S., and Nicholson, R. I. (2004). Zinc transporter LIV-1: a link between cellular development and cancer progression. Trends in Endocrinology and Metabolism *15*, 461-463.

Thiery, J. P. (2003). Epithelial–mesenchymal transitions in development and pathologies. Current Opinion in Cell Biology *15*, 740-746.

Török, I., Herrmann-Horle, D., Kiss, I., Tick, G., Speer, G., Schmitt, R., and Mechler, B. M. (1999). Down-Regulation of RpS21, a Putative Translation Initiation Factor Interacting with P40, Produces Viable Minute Imagos and Larval Lethality with Overgrown Hematopoietic Organs and Imaginal Discs. Molecular and Cellular Biology *19*, 2308-2321.

Tsurugi, K., Collatz, E., Todokoro, K., Ulbrich, N., Lightfoot, H. N., and Wool, I. G. (1978). Isolation of Eukaryotic Ribosomal Proteins: Purification and Characterization of the 60 S Ribosomal Subunit Proteins La, Lb, Lf, P1, P2, L13', L14, L18', L20, and L38. The Journal of Biological Chemistry *253*, 946-955.

Vaarala, M. H., Porvari, K. S., Kyllönen, A. P., Mustonen, M. V. J., Lukkarinen, O., and Vihko, P. T. (1998). Several genes encoding ribosomal proteins are over-expressed in prostate-

cancer cell lines: Confirmation of L7a and L37 over-expression in prostate cancer tissue samples. International Journal of Cancer *78*, 27-32.

Verschoor, A., Srivastava, S., Grassucci, R., and Frank, J. (1996). Native 3D Structure of Eukaryotic 80S Ribosome: Morphological Homology with the *E. coli* 70S Ribosome. The Journal of Cell Biology *133*, 495-505.

Vilardell, J., and Warner, J. R. (1994). Regulation of splicing at an intermediate step in the formation of the spliceosome. Genes and Development *8*, 211-220.

Volarević, S., Stewart, M. J., Ledermann, B., Zilberman, F., Terracciano, L., Montini, E., Grompe, M., Kozma, S. C., and Thomas, G. (2000). Proliferation, But Not Growth, Blocked by Conditional Deletion of 40S Ribosomal Protein S6. Science *288*, 2045-2047.

Wallace, K. N., Dolan, A. C., Seiler, C., Smith, E. M., Yusuff, S., Chaille-Arnold, L., Judson, B., Sierk, R., Yengo, C., Sweeney, H. L., and Pack, M. (2005). Mutation of Smooth Muscle Myosin Causes Epithelial Invasion and Cystic Expansion of the Zebrafish Intestine. Developmental Cell *8*, 717-726.

Wang, H., Long, Q., Marty, S. D., Sassa, S., and Lin, S. (1998). A zebrafish model for hepatoerythropoietic porphyria. Nature Genetics *20*, 239-243.

Wang, Y., Cheong, D., Chan, S., and Hooi, S. C. (2000). Ribosomal protein L7a gene is up-regulated but not fused to the tyrosine kinase receptor as chimeric *trk* oncogene in human colorectal carcinoma. International Journal of Oncology *16*, 757-762.

Watson, J. D. (1964). The synthesis of proteins upon ribosomes. Bulletin de la Société de Chimie Biologique *46*, 1399-1425.

Watson, K. L., Konrad, K. D., Woods, D. F., and Bryant, P. J. (1992). *Drosophila* homolog of the human S6 ribosomal protein is required for tumor suppression in the hematopoietic system. Proceedings of the National Academy of Sciences *89*, 11302-11306.

Wilson, D. N., and Nierhaus, K. H. (2005). Ribosomal Proteins in the Spotlight. Critical Reviews in Biochemistry and Molecular Biology *40*, 243-267.

Wimberly, B. T., Brodersen, D. E., Clemons, W. M., Morgan-Warren, R. J., Carter, A. P., Vonrhein, C., Hartschk, T., and Ramakrishnan, V. (2000). Structure of the 30S ribosomal subunit. Nature *407*, 327-339.

Wixon, J. (2000). *Danio rerio*, the zebrafish. Yeast *17*, 225-231.

Wood, S. R., Berwick, M., Ley, R. D., Walter, R. B., Setlow, R. B., and Timmins, G. S. (2006). UV causation of melanoma in *Xiphophorus* is dominated by melanin photosensitized oxidant production. Proceedings of the National Academy of Sciences *103*, 4111-4115.

Woodgate, R., Rajagopalan, M., Lu, C., and Echols, H. (1989). UmuC mutagenesis protein of *Escherichia coli*: Purification and interaction with UmuD and UmuD'. Proceedings of the National Academy of Sciences *86*, 7301-7305.

Wool, I. G. (1996). Extraribosomal functions of ribosomal proteins. Trends in Biochemical Sciences *21*, 164-165.

Wool, I. G., Chan, Y.-L., and Glück, A. (1995). Structure and evolution of mammalian ribosomal proteins. Biochemistry and Cell Biology *73*, 933-947.

Yancey, J. E., and Matson, S. W. (1991). The DNA unwinding reaction catalyzed by Rep protein is facilitated by an RHSP-DNA interaction. Nucleic Acids Research *19*, 3943-3951.

Yang, H. W., Kutok, J. L., Lee, N. H., Piao, H. Y., Fletcher, C. D. M., Kanki, J. P., and Look, A. T. (2004). Targeted Expression of Human *MYCN* Selectively Causes Pancreatic Neuroendocrine Tumors in Transgenic Zebrafish. Cancer Research *64*, 7256-7262.

Zengel, J. M., and Lindahl, L. (1991). Ribosomal protein L4 of *Escherichia coli*: in vitro analysis of L4-mediated attenuation control. Biochimie *73*, 719-727.

Zhang, Z., and Zhang, R. (2005). p53-Independent Activities of MDM2 and Their Relevance to Cancer Therapy. Current Cancer Drug Targets *5*, 9-20.

Zhu, Y., Lin, H., Li, Z., Wang, M., and Luo, J. (2001). Modulation of expression of ribosomal protein L7a (rpL7a) by ethanol in human breast cancer cells. Breast Cancer Research and Treatment *69*, 29-38.

Zimmer, S. G., DeBenedetti, A., and Graff, J. R. (2000). Translational control of malignancy: the mRNA cap-binding protein, eIF-4E, as a central regulator of tumor formation, growth, invasion and metastasis. Anticancer Research *20*, 1343-1351.

Chapter 2

Many Ribosomal Protein Genes Are Cancer Genes in Zebrafish

Adam Amsterdam, Kirsten C. Sadler*, Kevin Lai*, Sarah Farrington, Roderick T. Bronson
Jacqueline A. Lees, Nancy Hopkins

*Equal contributors
This chapter was published previously as PLoS Biology (2004), *2*: 690-698.

Author contributions:
Table 2.1: KCS & KL with assistance from SF & RB
Figure 2.1: KCS with assistance from RB
Figure 2.2: KCS with assistance from SF & RB
Figure 2.3: KL with assistance from SF & RB
Table 2.2: KL with assistance from RB
Figure 2.4: KL (panel A) & AA (panel B)
Figure 2.5: KL (top panel) & AA (bottom panel)
Figure 2.S1: AA

Abstract

We have generated several hundred lines of zebrafish, each heterozygous for a recessive embryonic lethal mutation. Since many tumor suppressor genes are recessive lethals, we screened our colony for lines that display early mortality and/or gross evidence of tumors. We identified 12 lines with elevated cancer incidence. Fish from these lines develop malignant peripheral nerve sheath tumors (zMPNSTs), and in some cases also other tumor types, with moderate to very high frequencies. Surprisingly, 11 of the 12 lines were each heterozygous for a mutation in a different ribosomal protein (*rp*) gene, while one line was heterozygous for a mutation in a zebrafish paralog of the human and mouse tumor suppressor gene, neurofibromatosis type 2 (*NF2*). Our findings suggest that many *rp* genes may act as haploinsufficient tumor suppressors in fish. Many *rp* genes might also be cancer genes in humans, where their role in tumorigenesis could easily have escaped detection up to now.

Introduction

The zebrafish has long been used as a model organism for the identification of genes required for early vertebrate development (Kimmel, 1989). There is reason to believe that the zebrafish can also be used in genetic screens to identify cancer genes. Zebrafish can live for 4-5 years (Gerhard et al., 2002) and like other fish species (Schmale et al., 1986; Wittbrodt et al., 1989) they develop tumors in a variety of tissues (Amatruda and Zon, 2002; Smolowitz et al., 2002). They are also susceptible to chemical carcinogens and to well known oncogenes, in a manner similar to the conventional mouse models (Beckwith et al., 2000; Spitsbergen et al., 2000a; Spitsbergen et al., 2000b; Langenau et al., 2003). Many of the spontaneous and

chemically- or oncogene-induced tumor types are histologically similar to their mammalian counterparts (Amatruda and Zon, 2002; Langenau et al., 2003).

The normal functions of many mammalian tumor suppressor genes are required for normal development (Jacks, 1996). In fact, non-essential tumor suppressors, such as p53 (Donehower et al., 1992), appear to be the exception rather than the rule. These findings raised the possibility that one could discover genes with a role in tumorigenesis among zebrafish genes identified initially for having essential roles during embryonic development. We have used retroviral vectors as a mutagen in a large-scale insertional mutagenesis screen and have isolated many zebrafish mutants with lesions in genes essential for embryogenesis (Amsterdam et al., 1999; Golling et al., 2002). We are maintaining approximately 500 lines, in most of which an embryonic lethal mutation is linked to a single proviral insert. We have identified the mutated genes in over 400 of the lines, and these include mutations in 300 distinct zebrafish genes. To maintain the lines, we identify approximately 15 heterozygous carriers and outcross these at 15-20 months of age to produce the subsequent generation. The maintenance of these mutations in adults provides a unique opportunity to ask whether heterozygosity in genes required for embryonic development predisposes the animals to cancer. Here we describe how such an analysis has identified genes that encode ribosomal proteins as cancer genes in zebrafish.

Results

Mutations in Many Ribosomal Protein Genes Predispose Zebrafish to Malignant Peripheral Nerve Sheath Tumors and Other Cancers

In the course of establishing and maintaining heterozygous mutant lines of fish, we noticed several lines that displayed early mortality by 2 years of age, and this phenotype was

seen in successive generations. Typically only about 10-15% of fish in a tank are lost by 2 years of age, but in these apparently high-mortality lines losses could exceed 50%. Furthermore, fish from these lines were often found to have gross lumps (Figures 2.1A and B). Histological analysis of step sections showed that the growths were predominantly large, malignant spindle cell tumors that were highly invasive, had a high mitotic index, and often exhibited focal necrosis (Figures 2.1C-H). The tumor cells were aligned into stacks and fascicles to form a whirling, storiform pattern (Figures 2.1E, F, H) that resembles malignant peripheral nerve sheath tumors (MPNSTs) seen in other species of fish (Schmale et al., 1983; Roberts, 2001) and in mammals (Woodruff, 1999; Cichowski et al., 1999; Jimenez-Heffernan et al., 1999). In keeping with the published work on fish tumors, while adhering to the caution suggested by the National Neurofibromatosis Foundation regarding animal models of MPNST (M. McLaughlin pers. comm.), we have designated these tumors zMPNSTs (zebrafish MPNSTs).

Although we had occasionally observed individual fish with lumps in our colony, it was unusual to find so many within a single line. Thus we reasoned that the lines with early mortality that also frequently displayed gross lumps by 2 years of age might be lines with elevated rates of lethal cancer. Surprisingly, we found that the several potentially high-tumor lines were all heterozygous for mutations in genes that encode different ribosomal proteins. This unexpected observation, combined with the knowledge that many tumor suppressors are recessive embryonic lethal genes, prompted us to survey our colony systematically to determine the incidence and spectrum of tumors arising in the colony as a whole, and to ask specifically whether genes that encode many different ribosomal proteins predispose to cancer.

To determine cancer incidence in the colony as a whole, we sectioned 152 "control" fish that were 20-26 months of age. Forty-nine of the control fish were non-transgenic while 103

were selected at random from 54 lines heterozygous for mutations in genes other than *rp* genes. The latter fish had been generated and maintained in a comparable manner to our *rp* mutant lines and thus were appropriate controls. The incidence of tumors detected by step sectioning in this control population was 11% (Table 2.1D). Although we observed a variety of different tumor types (most frequently seminomas and pancreatic islet cell adenomas), most of the tumors (15/17) were benign neoplasias and none were zMPNSTs. There was neither a significant difference in spectrum nor an increase in incidence of tumors in the non-*rp* heterozygous mutant fish relative to the wild-type fish, indicating that the presence of viral insertions, per se, does not have an obvious effect on tumorigenicity.

To compare the frequency and types of tumors arising in *rp* mutant lines to those of the control population, we established the fate of all heterozygotes in a single generation of each of 16 *rp* mutant lines. In each family, some fish were lost prior to any observation of external symptoms, precluding determination of the cause of death. The rest were sacrificed either when they developed visible masses or when they reached 18 to 26 months of age, and step sections were examined (Table 2.1). The 16 *rp* families fell into three groups with respect to tumor incidence: Six lines had high mortality (including both lost fish and those with external growths) and a high tumor incidence (≥60% including both fish with gross tumors and tumors detected only upon sectioning). Nearly all of the tumors observed by 22 months in these lines were zMPNSTs (Table 2.1A). These lines included those with mutations in *rp* genes *S8* (GenBank Acc. AY561509), *S15a* (GenBank Acc. AY561512), *L7* (GenBank Acc. AY561515), *L35* (GenBank Acc. AF506205), *L36* (GenBank Acc. AY561518), and *L36a* (GenBank Acc. AY099511). Five *rp* mutant lines made up a second group. These lines had either a moderate incidence of cancer, or had a low incidence but were unusual in having an apparently elevated

incidence of zMPNSTs. This group included lines with mutations in *L13* (GenBank Acc. AY561516), *L23a* (GenBank Acc. AY561517), *S7* (GenBank Acc. AY561508), *S18* (GenBank Acc. AY099517), and *S29* (GenBank Acc. AY561513). As in the high cancer lines, in most lines with moderate cancer incidence, most tumors observed in fish by 22-24 months of age were zMPNSTs (Table 2.1B). In one line, however (*hi1026*, with a mutation in *S18*), other tumor types predominated, suggesting that *rp* mutations can increase the frequency of tumor types besides zMPNSTs. The third group of *rp* mutant lines included 5 lines, none of which were tumor prone. These lines, with mutations in *L3* (GenBank Acc. AY561514), *L24* (GenBank Acc. AY099532), *LP1* (GenBank Acc. AY561519), *S12* (GenBank Acc. AY561510), and *S15* (GenBank Acc. AY561511), were indistinguishable from controls in tumor incidence and spectrum (Table 2.1C versus D). In summary, 11 of 16 *rp* mutant lines had an elevated incidence of cancer, and most of these 11 lines are predisposed to develop zMPNSTs.

Together these findings suggested that zMPNSTs are rare in our colony except in *rp* mutant lines. However, because the cancer incidence was low in the control fish, we observed only 17 tumors in this group of fish in the experiment described above. Furthermore, only 4 of these 17 tumors were grossly visible, with 13 being detected only after sectioning. To obtain more data on tumor spectrum in our colony, including the types of tumors that present as externally visible growths in non-*rp* mutant lines and wild-type, we sought out fish with externally visible tumors from throughout our colony, coded them to avoid bias, and identified the tumor types by histological analysis of step sections. In total, we analyzed gross tumors from 41 control fish (wild-type or non-*rp* mutant lines, including the 4 tumors found above). We also analyzed a total of 65 *rp* heterozygotes with grossly visible tumors (including the fish represented in Table 2.1A, B, and C). Figure 2.2 shows a comparison of the types of tumors in

control versus *rp* mutant lines that present as externally visible growths. In the control fish, seminomas accounted for 57% of these tumors, while a wide variety of other tumor types, including ultimobranchial gland tumors, neuroblastomas, islet cell adenomas, and lymphomas, each arose at low frequency. Overall, 69% of the grossly visible tumors observed in non-*rp* fish were benign. Only 10% of these externally visible tumors were zMPNSTs (see below). In contrast to the control fish, and as apparent from the data in Table 2.1, the majority of grossly visible tumors in the *rp* mutants were zMPNSTs (81%), greatly exceeding the number of seminomas (4%) or other (15%) tumor types (Figure 2.2). Since fish with external growths were found far more often within *rp* families than the colony at large, the dramatic shift in the spectrum of tumors in *rp* relative to non-*rp* mutant lines reflects the profound increase in incidence of zMPNSTs rather than any obvious reduction in the incidence of seminomas and other tumor types.

As noted above, we detected zMPNSTs in only 4 of 41 control fish with grossly visible tumors. Two of these fish, aged 15 and 24.5 months, were from the *hi3332* line, the only non-*rp* line in which more than a single zMPNST has been observed to date. Significantly, the viral insertion that is linked to the embryonic lethal phenotype of this line lies within one of two distinct zebrafish genes (*NF2a*, GenBank Acc. AY561520) that are highly homologous to the mammalian neurofibromatosis type 2 gene (*NF2*). The insertion abrogates expression of this gene in homozygous mutant embryos (Figure 2.S1 and data not shown). *NF2* was originally identified as a tumor suppressor gene that predisposes individuals to develop tumors of the nervous system (Trofatter et al., 1993; Ruttledge et al., 1994). Given this finding, we screened the remaining 53 fish in this family for tumors between 17.5 and 23 months of age by sectioning. Seven of these 53 fish had small spindle cell tumors. These tumors were not identical to typical

zMPNSTs found in *rp* families, but shared some key characteristics (data not shown). Given the elevated incidence of rare tumor types including zMPNSTs, we conclude that *NF2a* acts as a tumor suppressor gene in fish, as it does in mammals.

Early Mortality in an rp Mutant Line Results from Multiple Types of Cancer

The experiment described above identified 6 *rp* mutant lines with high mortality. While some of the mortality could be accounted for by fish that displayed gross tumors and therefore were removed from the tanks before they died, many fish simply disappeared or were found dead and were too deteriorated to be analyzed histologically. To determine whether early mortality in these lines was entirely due to lethal cancers, and if so, whether it was due to zMPNSTs or to other tumor types, we performed two experiments using fish from the early-mortality, high tumor *hi10* line. In one experiment we screened *hi10* heterozygotes and their wild-type siblings weekly for evidence of ill health or externally visible growths in an effort to catch all sick fish before they died or were lost. Sickly fish were sacrificed and subjected to histological examination, as were all of the fish that still appeared healthy at 22 months of age. The results are shown in Figure 2.3. Only the *rp* heterozygous carrier fish displayed early mortality, and, as anticipated, this was due to cancers. Strikingly, among tumors found by 15 months of age, while two were zMPNSTs, one was a retinoblastoma and three were lymphomas, tumor types that, like zMPNSTs, arise infrequently in our control populations. The tumors detected in the older fish were predominantly zMPNSTs. By the endpoint of the experiment (22 months) all of the non-carrier sibling controls appeared healthy, and step sectioning detected only one tumor-bearing fish among 13, a frequency comparable to the control population. These results support the

conclusion that the early mortality observed in the *hi10* line is the result of lethal tumors, and reveal that these include zMPNSTs but also other tumor types.

Further evidence that fish from the *hi10* line are predisposed to multiple tumor types was obtained in the second experiment, in which we sectioned *hi10* heterozygotes and their non-carrier sibling controls (specifically including any sick or growth-bearing fish along with apparently healthy fish) at approximately six-week intervals between 8 and 14 months of age. As shown in Table 2.2, we found both grossly visible and occult zMPNSTs and other tumor types in the *hi10* carrier fish. Thus the *hi10* line (and presumably other high mortality *rp* lines) is predisposed to multiple tumor types, though particularly strongly predisposed to develop zMPNSTs, especially at later time points.

rp Genes May Be Haploinsufficient Tumor Suppressors

Dominant mutations that predispose vertebrates to cancer can be activated oncogenes, recessive tumor suppressors, or haploinsufficient tumor suppressors (Largaespada, 2001). Several lines of evidence suggest that *rp* mutant genes may be acting as haploinsufficient tumor suppressors in zebrafish. The mutagenic inserts in all of our *rp* mutant lines reduced or eliminated expression of the *rp* gene, as determined by RT-PCR and, in some cases, Northern blotting (Figure 2.4A and data not shown). Thus, most if not all of these viral insertions appear to be loss of function mutations. This suggests that the *rp* genes are not mutated to form activated oncogenes, but rather may act as tumor suppressors. In mammals, the most frequent mechanism of inactivation of recessive tumor suppressor genes is the acquisition of a mutation (either germline or somatic) in one allele and subsequent loss of the wild-type allele through loss of heterozygosity (LOH) (Haber and Harlow, 1997). Thus, we investigated whether the wild-

type *rp* gene had been lost in the zebrafish tumors. We isolated both normal and tumor tissue from three *rp* heterozygous mutant lines, *hi10*, *hi258*, and *hi1974*, each of which shows a reduction in expression of its respective *rp* mutant gene of ≥10-fold (Figure 2.4A) and examined DNA from these samples for the presence of the mutant and wild-type *rp* alleles by PCR (Figure 2.4B). In every case we detected the wild-type allele, arguing against loss of heterozygosity in these tumors. A concern is that tissue contamination can yield misleading LOH results, particularly because the red blood cells of fish are nucleated. Thus control PCR experiments were performed in which DNA samples from heterozygous and homozygous embryos were mixed at different ratios. The results show that our assay was sensitive to as small as a 3-fold decrease in the relative amount of the wild-type allele (data not shown). Thus, unless the tumor samples contained more than 33% non-tumor cells, we can conclude that the wild-type *rp* alleles were not lost in these tumors, and thus the *rp* genes are probably not recessive tumor suppressors. In one of the tumor samples shown in Figure 2.4, tumor *hi10*-1, the wild-type allele appears not only to be present but possibly at higher concentration than the mutant allele, and Southern analysis of this same DNA sample supported this observation (data not shown). Thus, in this particular tumor the mutant allele may have been lost and only the wild-type allele retained.

In mice, a tumor cell line has been described in which one copy of an *rp* gene is deleted and the other copy has suffered a mutation that may contribute to tumorigenesis (Beck-Engeser et al., 2001). To rule out the acquisition of a point mutation in the wild-type allele in *rp* mutant tumors, all of the coding exons of the appropriate *rp* gene and at least 50 bp of intronic sequence flanking them were sequenced from each normal and tumor DNA sample. There was no indication of any point mutations in any of the tumors. The apparent retention of the wild-type

allele in the tumor cells in these samples and the fact that no point mutations were observed in the wild-type *rp* genes in the tumor cell DNA suggests that it is not a second hit in these loci that leads to tumorigenesis. Rather, the data obtained suggest that these genes function as haploinsufficient tumor suppressors in zebrafish.

rp Mutations Alter the Relative Amounts of 18S and 28S rRNAs

In yeast, a decrease in the amount of at least some *rp* genes results in a reduction in the amount of the corresponding ribosomal subunit and a reduction in the number of assembled ribosomes (Moritz et al., 1990). To determine if this is also true in fish, we examined the relative amounts of 18*S* and 28*S* rRNA in homozygous mutant embryos compared to sibling controls. Embryos from heterozygote crosses of lines *hi10*, *hi1974*, and *hi2649* were sorted by phenotype at three days post-fertilization, and total RNA was prepared from pools of mutant or phenotypically wild-type sibling embryos. Electrophoresis and ethidium bromide staining was used to determine the amounts of 18*S* and 28*S* RNA, which we assume reflect the amounts of 40*S* and 60*S* ribosomal subunits, respectively (Figure 2.5). As a loading control, the same RNA samples were subjected to Northern analysis and probed for beta actin (Figure 2.5). In each case we observed a decrease in the overall amount of rRNA, and significantly, a preferential loss of the rRNA found in the ribosomal subunit with which the mutated *rp* was associated. Thus in *hi10*, where a component of the large ribosomal subunit was mutated, while both 18*S* and 28*S* RNA levels were decreased, the level of 28*S* RNA was affected more than the 18*S*. Conversely, in *hi1974* and *hi2649*, where components of the small ribosomal subunit were mutated, the 28*S* RNA levels were mildly reduced, but 18*S* RNA was sharply decreased. In none of these cases was the actin level reduced, so the effect was not simply a result of a reduction of cell number,

RNA degradation, or cell death. Thus, as in yeast, *rp* mutations in fish that result in reduced gene expression lead to a relative decrease in the amount of the subunit to which they belong as measured by a decrease in rRNA.

Discussion

In this study, we have found that heterozygous mutations in 11 different ribosomal protein genes predispose zebrafish to cancer, predominantly to zMPNSTs, but also to other rare tumor types. All of these mutations reduce *rp* gene expression, indicating that these 11 genes are not oncogenes. Moreover, in the tumors we examined, the wild-type allele appeared to be present and did not contain point mutations; thus these genes are not recessive tumor suppressors. Rather, our findings suggest that these 11 genes are haploinsufficient tumor suppressor genes; that is, reducing their activities by about a factor of two increases the likelihood of cancer. These findings raise two important, unanswered, questions: first, how do these mutations lead to cancer, and second, do similar mutations cause cancer in humans?

How Do These Mutations Cause Cancer?

The finding that mutations in so many different *rp* genes, including *S7, S8, S15a, S18, S29, L7, L13, L23a, L35, L36, and L36a,* predispose to cancer suggests that a function shared by ribosomal proteins underlies their role in this phenotype. However, not all *rp* genes were cancer genes: *S12, S15, L3, L24,* and *LP1* heterozygotes appeared normal. This raises the possibility that the oncogenic *rp* genes could conceivably share some novel biological function independent of their role in the ribosome and that inhibition of this function leads to tumor formation. Individual ribosomal proteins have been implicated in a wide variety of biological functions,

including cell cycle progression, apoptosis, and DNA damage responses (Ben-Ishai et al., 1990; Sonenberg, 1993; Chen et al., 1998; Chen and Ioannou, 1999; Hershey and Miyamoto, 2000; Volarević et al., 2000; Volarević and Thomas, 2001; Lohrum et al., 2003), and it has been suggested that their role in these processes may arise independently of their role in the ribosome itself (Wool, 1996; Wool et al., 1996; Soulet et al., 2001). However, it seems somewhat unlikely to us that there could be such an important, yet still undetected function involving so many different ribosomal proteins. Thus we favor the possibility that it is a shared, ribosome-associated function that allows them to be tumor suppressors. If so, then why were not all *rp* genes cancer genes in this study? At present we can only speculate. We have not found any correlation that distinguishes the *rp* genes that predispose to cancer from those that do not. Both can belong to either the large or the small ribosomal subunit, and all the mutants show reduced gene expression. Possibly some *rp* genes are normally expressed at higher levels than others, so that a 50% reduction in their expression does not reduce their protein level below some critical, hypothetical threshold required for tumor suppression.

The best-known function shared by ribosomal proteins is their role in the assembly of ribosomal subunits, and as a result, their role in translation. In homozygous mutant fish embryos, the *rp* mutations reduce the amount of the rRNA of the subunit to which they belong, and hence almost certainly reduce the amount of the corresponding ribosomal subunit relative to the remaining subunit. In yeast this is known to reduce the number of ribosomes, and thus also to reduce the amount of protein synthesis. How might this predispose to cancer? In truth, we do not know, and suspect that understanding the mechanism that explains these findings will lead to new insights into growth control. At present we can only list our speculations and several relevant observations.

Reduced protein synthesis could lead to a reduction in the level of a critical tumor suppressor protein, or of a positive regulator of apoptosis or differentiation, either of which could favor growth. A reduction in ribosome number might signal the cell to try to overcome the deficit by making more of the components required for ribosome biogenesis, and this in turn might promote cell growth. Alternatively, a reduction in the number of ribosomes might alter the identity of the messages recruited to ribosomes, similar to the way that modulation of the translational capacity of mammalian cells by oncogenes such as *Ras* or *Akt* is known to alter the identity of mRNAs recruited to polysomes, changing the translation rate of growth-promoting genes (Rajasekhar et al., 2003). Finally, and most speculative of these possibilities, reduced ability of a ribosomal subunit to assemble properly might generate a signal that cells interpret as growth-promoting. For example, degradation of excess rRNA, a molecule with many hairpins, might generate such a signal in the form of RNAi.

Are rp Genes Cancer Genes in Other Vertebrates?

Given that so many different *rp* genes can be cancer genes in fish, it seems surprising that they are not already a well-known class of cancer genes in vertebrates. Only two examples are known that suggest a role for *rp* mutations in mammalian tumor susceptibility, one in mice and one in humans. In the mouse study, two independent murine tumor cell lines were found to express tumor antigens which were mutated ribosomal proteins (Beck-Engeser et al., 2001). In both cases, the tumors were found to become more aggressive upon either loss or mutation of the wild-type allele of the *rp* gene. It was postulated that the mutant ribosomal proteins might have an oncogenic activity that was suppressed by the wild-type protein. Such a mechanism does not

seem to be involved in the tumors that develop in the *rp* mutant fish described here, since we failed to detect evidence of oncogenic activation of *rp* genes.

In humans, there is a possible association of mutations in one particular *rp* gene with cancer: approximately 25% of both sporadic and familial cases of Diamond-Blackfan anemia (DBA) are associated with a mutation of *rpS19* (Draptchinskaia et al., 1999), and this syndrome includes an increased risk of developing leukemia (Wassler et al., 1978). It has been demonstrated that the anemia is likely due to a block in erythroid differentiation (Hamaguchi et al., 2002), but it is currently unclear if the leukemia is an indirect result of the anemia, caused by a stimulation in the production of hematopoietic precursors, or whether the *rpS19* gene dosage plays a direct role in tumorigenesis. It is important to note that DBA is a multigenic disease with very heterogeneous clinical presentation. While DBA patients in general have an increased predisposition to certain cancers, it is not yet clear whether this is true of the subset whose DBA is caused by *rpS19* mutation.

While these examples from mouse and human are consistent with the idea that mutations in individual *rp* genes might contribute to tumorigenesis in mammals, they have seemed to be unusual examples, rather than suggesting that *rp* genes in general might be potential cancer genes. Our study suggests for the first time, we believe, that this is a general property of many *rp* genes. The possibility that a reduction in ribosome levels might be oncogenic in mammals is further supported by the fact that mutations in DKC1, a pseudouridine synthase that is required for rRNA processing and for properly functioning ribosomes, cause dyskeratosis congenita, a disease characterized by both premature aging and increased tumor susceptibility (Ruggero et al., 2003).

If *rp* genes frequently cause human cancers it is not at all certain that their role would have been detected. Even a deliberate search for their involvement in human cancers would be difficult because there are so many (80) *rp* genes. This plethora of genes, the fact that it is hard to know which tumor type(s) to examine for *rp* mutations, and the fact that the mutations might lie in regulatory elements rather than protein coding regions of the genes, would make such a search difficult. Nonetheless, given the high degree of conservation of biological mechanisms among vertebrates, it seems likely that *rp* mutations will prove to increase the incidence of tumors in humans as they do in zebrafish. If so, it may be advantageous to devise diagnostic strategies based on ribosomal protein levels or on a function that these proteins share; for example, in translation, rather than on the analysis of such a large number of individual genes.

In summary, by examining aging populations of mutant lines of fish with defects in embryonic essential genes, we identified a novel group of cancer genes. The ability to identify cancer genes by screening populations of fish heterozyogous for recessive embryonic mutations and the reassuring finding that *NF2a* is a tumor suppressor gene in this system demonstrate the power of large-scale, forward-genetic screens in the zebrafish to identify new disease susceptibility genes.

Materials and Methods

Mutagenesis and Maintenance of Mutants Lines

The insertional mutagenesis screen was carried out as previously described (Amsterdam et al., 1999). Stocks of all lines were maintained by outcrossing heterozygotes to non-transgenic fish, preparing DNA from tail fin biopsies of 8-18 week old fish, and performing PCR with insert-specific primers for each line to identify heterozygotes.

Fixation and Histology

Adult fish were euthanized in ice water and fixed within thirty minutes in Bouin's solution, embedded in paraffin, and sectioned as described (Moore et al., 2002).

Loss of Heterozygosity Analysis

DNA was prepared from tumor tissue or tail tissue isolated from fish prior to fixation for histology. PCR was conducted with one primer complementary to proviral sequence and two primers complementary to sequences on either side of the insertion for the appropriate mutation. Primer sequences were as follows:

hi10: 10gen5 (5'-CAGCACAGATTCTTGAAAGCGCC-3')

10gen3 (5'-GCATATGTAGCATCTCGAAGGTCC-3')

NU3X (5'- TGATCTCGAGCCAAACCTACAGGTGGGGTC-3')

hi258: 258A5a (5'-GGTACGTCTGTGCTTATGTTTGTGTC-3')

258A3a (5'-TCTCAAGACTTCATCCATTCATAATTCTGC-3')

NU3X (see above)

hi1974:1974c1 (5'-CTACACCACAGGTATCTCAAGGG-3')

1974c1est3 (5'-CCACCACGGACTCTTATTGTGTG-3')

IPL3 (5'-TGATCTCGAGTTCCTTGGGAGGGTCTCCTC-3')

RNA Analysis

RNA was prepared from mutant and wild-type embryos using Trizol reagent (Invitrogen, Carlsbad, CA). For RT-PCR, serial dilutions of 1st strand cDNA were amplified for 30 (*hi1974*) or 35 (*hi10* and *hi258*) cycles using the following primers for the genes indicated:

rpL36a: 10rt5 (5'-CAACCATGGTAAACGTACCGAAG-3')

10RTR (5'-CACAAAAGAAGCACTTGGCCCAGC-3')

rpL35: 258RTF2 (5'-GCTGCTTCCAAGCTCTCAAAAATCC-3')

258RTR (5'-TGCCTTGACGGCGAACTTGCGAATG-3')

rpS8: 1974RTF1 (5'-TCTCAAGGGATAACTGGCACA-3')

1974RTR1 (5'-GAACTCCAGTTCTTTGCCCTC-3')

beta-actin: actinF (5'-CATCAGCATGGCTTCTGCTCTGTATGG-3')

actinR (5'-GACTTGTCAGTGTACAGAGACACCCT-3').

For visualization of 18*S* and 28*S* RNA, 2 embryo equivalents of RNA were electrophoresed through a non-denaturing agarose gel containing 0.5μg/ml ethidium bromide. For detection of beta-actin RNA, 4 embryo equivalents of RNA were electrophoresed through a 7.5% formaldehyde/MOPS-buffered agarose gel, blotted to Hybond N+ (Amersham/Pharmacia, Piscataway, NJ), and hybridized with a random-primed beta-actin probe.

Acknowledgements

We thank Meg Cunningham and Kate Anderson for maintenance of the mutant lines of fish and Tom Such, Sam Farrington, Chris Doller, and Tim Angelini for maintenance of the zebrafish colony. We thank the CCR Histology Facility, especially Alicia Caron, for sample processing and sectioning, and Jan Spitsbergen, Michael Schmale, and Margaret McLaughlin for their advice on the analysis of the histology. We thank Tyler Jacks for valuable comments on the manuscript and Philip Sharp for useful discussions. We thank Joan Ruderman for her support of K. C. S. This work was supported by grants from the National Center for Research Resources of

the National Institutes of Health (to N. H.), Amgen (to N. H.), the David Koch Research Fund (to J. A. L. and N. H.) and the NIH (to the Center for Cancer Research at MIT). A. A. was supported by a fellowship from the Ford Foundation, K. C. S. was supported by a fellowship from the NIH, and K. L. was supported by a predoctoral training grant from the NIH.

Abbreviations: DBA, Diamond-Blackfan anemia; LOH, loss of heterozygosity; PCR, polymerase chain reaction; rp, ribosomal protein; RT-PCR, reverse transcription – polymerase chain reaction; zMPNST, zebrafish malignant peripheral nerve sheath tumor

Figure 2.1: Spindle cell tumors resembling MPNSTs in zebrafish heterozygous for mutations in *rp* genes.

(A and B) Fish with apparent masses, as indicated by the arrows, or other evident pathology, were selected for histological analysis: (A) a *hi2582* fish, (B) a *hi1034B* fish.

(C-H) Histopathology of representative tumors stained with hematoxylin and eosin reveals patterns consistent with the diagnosis of MPNST. (C and D) *hi10* fish, (E-G) *hi1974* fish, (H) *hi1807* fish. (C) Tumors typically filled the entire abdomen; sb-swim bladder, br-brain (80X). (D) A large tumor with central necrosis is seen emanating from the optic nerve (n); e-eye (20X). (E) Tumors consist of spindle cells that stack into short fascicles, typically organizing into whorls (400X). (F) Tumor is aggressively invading muscle (m) and gill (g); br-brain (100X). (G) Mitotic figures (arrows) are evident (1000X). (H) Areas of focal necrosis are frequently seen (arrows; 200X).

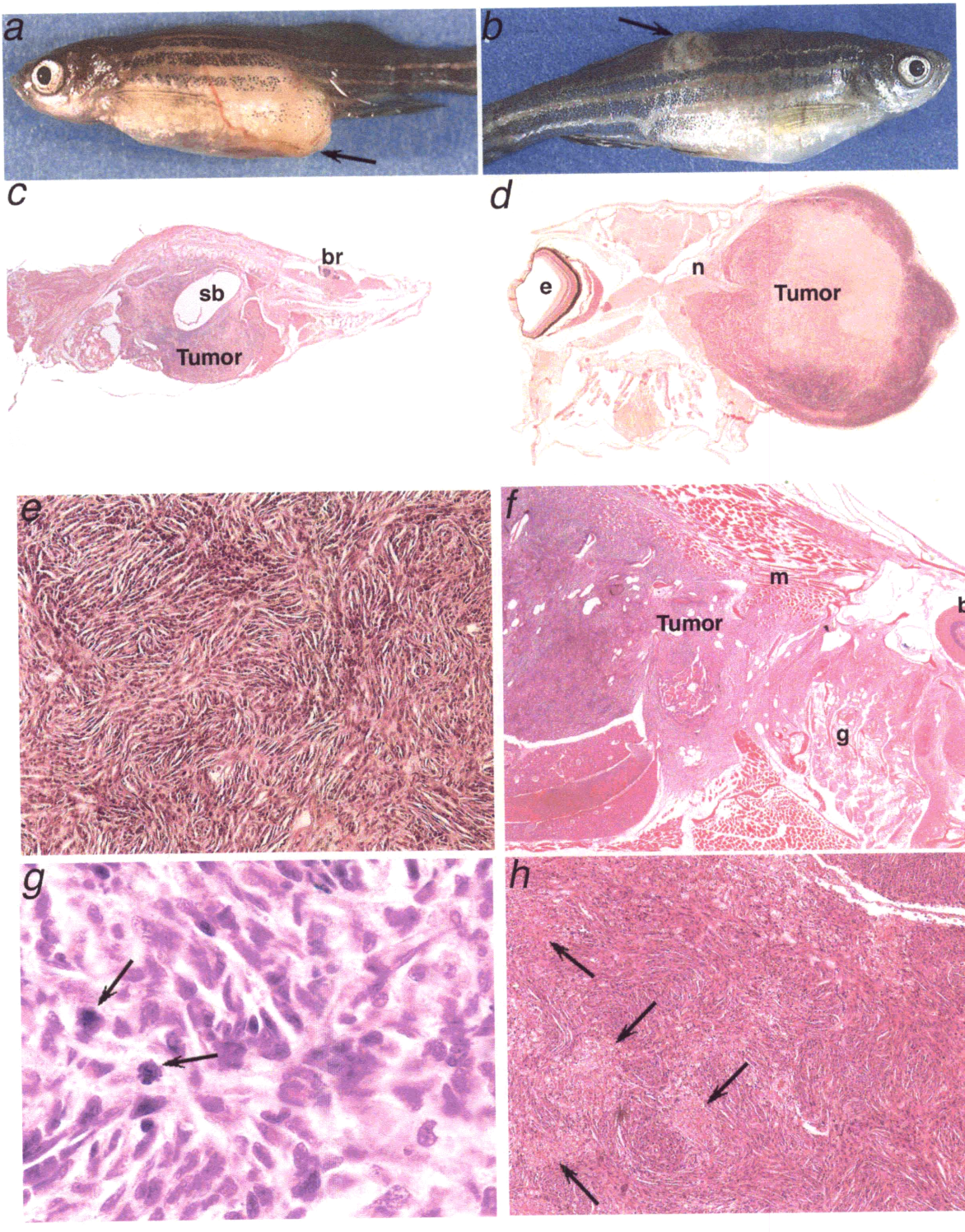

a
b
c
br
sb
Tumor
d
e
n
Tumor
e
f
m
Tumor
br
g
g
h

Table 2.1: Tumor incidence in zebrafish *rp* heterozygous lines and in the colony.
rp animals were collected as tumors became apparent, or as healthy animals at the maximum age specified (age range). # lost indicates those that either died before the appearance of external symptoms or were lost from their tanks. Control animals from the colony were selected without regard to gross appearance. Incidence rates are based on the number of fish examined histologically (i.e., excluding lost fish).

	Gene	Line	Age Range	Initial # fish	# Lost	# Grossly-Apparent Tumors / # Fish Examined	# Tumor-Bearing Fish / # Fish Examined	# zMPNST / # Fish Examined	Tumor Incidence
A	**L35**	**hi258**	up to 21.5 months	13	7	5/6	6/6	6/6	100%
	S15a	**hi2649**	up to 18 months	9	2	4/7	6*/7	5/7	86%
	S8	**hi1974**	up to 22 months	19	6	6/13	9/13	8/13	69%
	L36a	**hi10**	up to 22 months	14	6	4/8	5/8	5/8	63%
	L36	**hi1807**	up to 21.5 months	14	6	4/8	5/8	4/8	63%
	L7	**hi1061**	up to 22 months	14	4	6/10	6*/10	5/10	60%
B	**S7**	**hi1034B**	up to 22 months	19	4	3/15	7/15	5/15	47%
	L13	**hi1016**	up to 23 months	15	4	2/11	5/11	3/11	45%
	S18	**hi1026**	up to 24 months	23	9	1/14	6/14	1/14	43%
	S29	**hi2903**	up to 22 months	18	3	4/15	4/15	4/15	27%
	L23a	**hi2582**	up to 22 months	40	5	3/35	5/35	4/35	14%
C	**S12**	**hi1227**	all at 22 months	14	1	0/13	1/13	0/13	8%
	acidic LP1	**hi1444**	up to 22.5 months	18	0	1/18	1/18	0/18	6%
	L3	**hi2437**	all at 23 months	19	2	0/17	1/17	0/17	6%
	L24	**hi1284**	all at 26 months	18	0	0/18	0/18	0/18	0%
	S15	**hi2430**	all at 23 months	16	0	0/16	0/16	0/16	0%
D	NA	**Colony**	20-26 months	152	ND	4/152	17/152	0/152	11%

*one individual had two tumors, each of which was malignant

Figure 2.2: The tumor spectrum in fish heterozygous for mutations in *rp* genes shows an increased proportion of MPNSTs.

Fish with apparent masses were selected and processed for histological analysis. Numbers are shown as percent of the total number of diagnosed tumors from either population. The control group includes 42 tumors from 41 fish, including both wild-type and non-*rp* family transgenics. The *rp* group includes 68 tumors from 65 *rp* heterozygotes from 18 different lines representing mutations in 16 different genes. The "other" tumor category includes pancreatic islet adenomas, ultimobranchial gland tumors, neuroblastomas, retinoblastomas, lymphomas, ganglioneuromas, ductal carcinomas, gastrointestinal adenocarcinomas, hepatocellular carcinomas, leukemias, meningiomas, and histiocytic sarcomas.

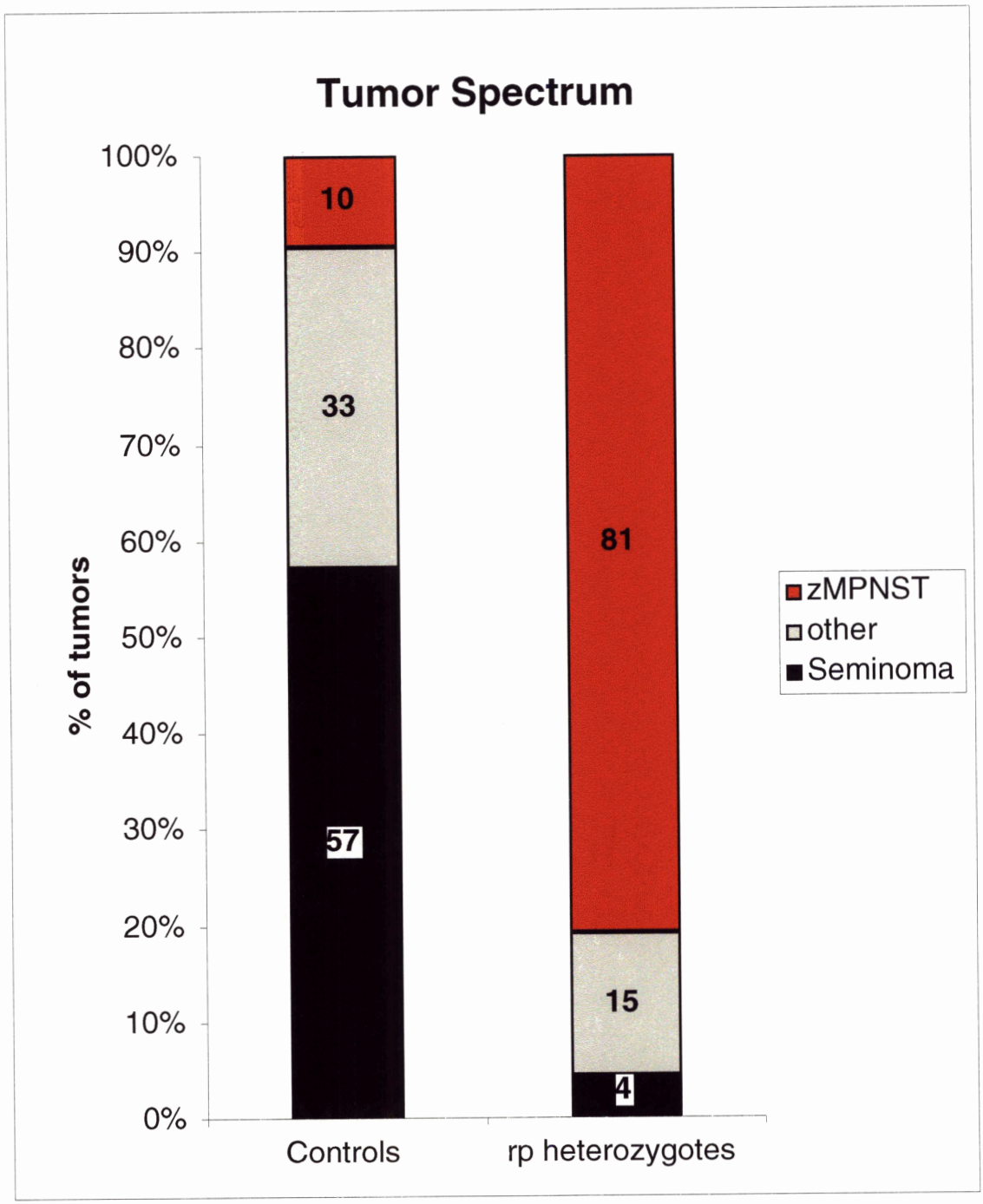

Tumor Spectrum
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of tumors
10
33
57
81
15
4
Controls
rp heterozygotes
zMPNST
other
Seminoma

Figure 2.3: Rate of tumor appearance in *hi10* heterozygotes.

A cohort of 28 *hi10* fish (red) and 13 of their non-carrier siblings (blue) were observed over 22 months for the appearance of ill health or externally visible tumors. Symptomatic individuals were sacrificed, fixed, and sectioned for histological analysis. The graph represents the percentage of fish remaining over time, with the diagnosis of each removed fish. Three fish labeled "dead" died before fixation and had too much tissue damage to establish a diagnosis. Also, seven of the carrier fish (though none of the non-carriers) were lost to unknown causes over the course of the experiment; while they most likely died, to be conservative these were removed from the total number of fish charted. At 22 months, the remaining externally healthy fish (4/21 carriers, 13/13 non-carriers) were also histologically examined, and the status of these fish is indicated.

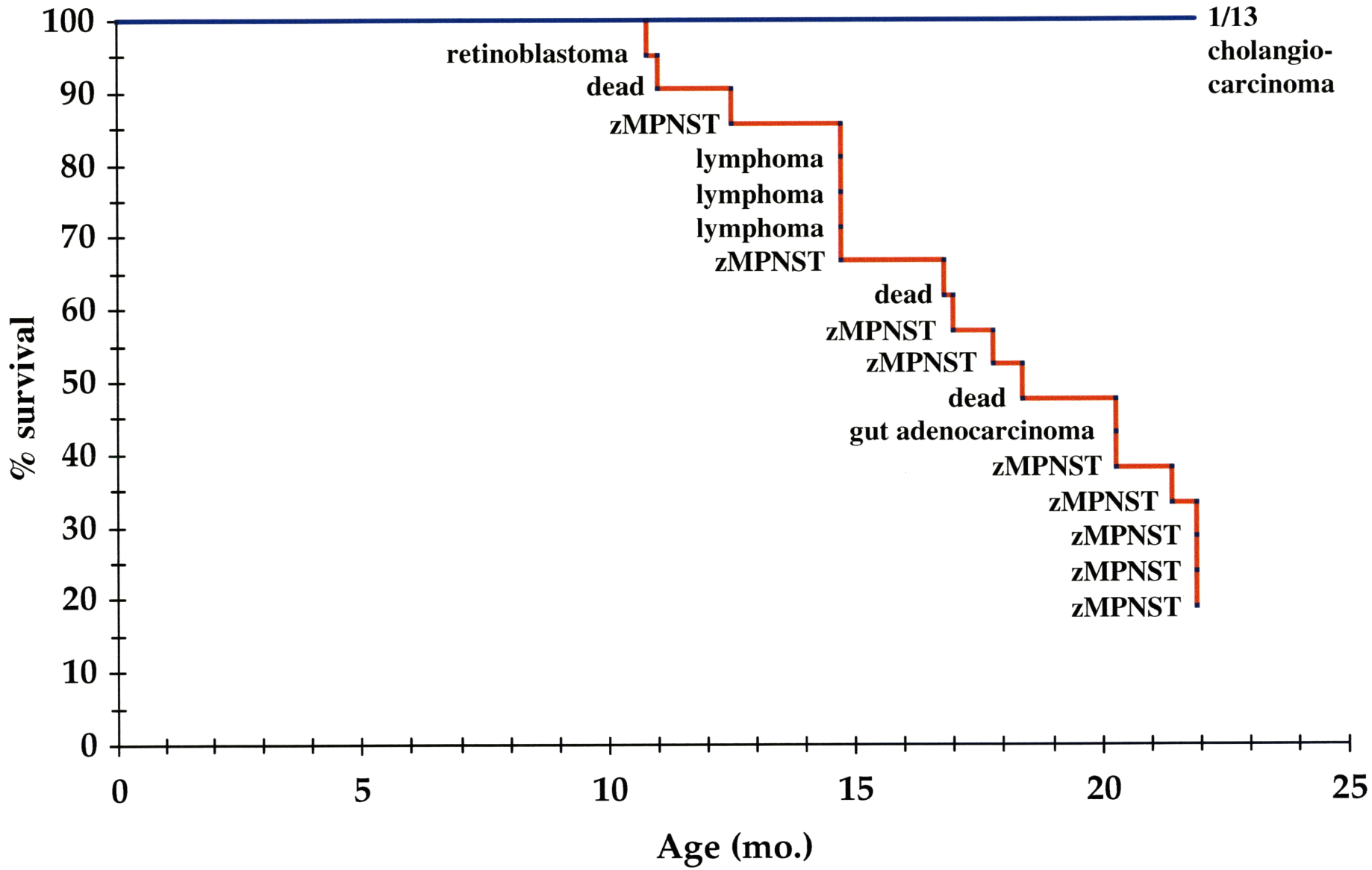

100
90
80
70
60
50
40
30
20
10
0
% survival
0
5
10
15
20
25
Age (mo.)
retinoblastoma
dead
zMPNST
lymphoma
lymphoma
lymphoma
zMPNST
dead
zMPNST
zMPNST
dead
gut adenocarcinoma
zMPNST
zMPNST
zMPNST
zMPNST
zMPNST
1/13
cholangio-
carcinoma

Table 2.2: Onset of tumor development in *hi10* fish and non-carrier siblings

	Carriers (70*)				Non-carriers (92**)			
Age (in months)	Fish with external growth or sick	Fish collected for histology	Total tumor-bearing fish	Tumor types	Fish with external growth or sick	Fish collected for histology	Total tumor-bearing fish	Tumor types
7.4-8.3	2	19	4***	1 lymphoma, 3 zMPNST (2 occult) 1 occult histiocytic sarcoma	1	19	1	Tumor of uncertain origin
9.2	1	10	2	1 zMPNST 1 occult gut adenocarcinoma	0	10	0	
10-10.6	4	9	5	4 zMPNST 1 occult renal cell carcinoma	0	8	0	
11.9-12.5	5	12	8***	7 zMPNST (3 occult) 1 occult gut adenocarcinoma 1 uncertain origin	0	10	0	
14	4	13	8	1 lymphoma 5 zMPNST (2 occult) 1 occult pancreatic ductal carcinoma 1 occult uncertain origin	0	10	0	

* Starting population was 70 fish; 7 fish were lost over the course of the experiment.
** Starting population was 92 fish; 3 fish were lost over the course of the experiment, 32 externally healthy fish at the end of the study (14 months) were not histologically examined.
*** One fish at each of these time points had two tumors.

Figure 2.4: *rp* genes appear to be haploinsufficient tumor suppressors.

(A) *rp* mutations decrease the amount of *rp* gene expression. RNA was prepared from 3 day old homozygous mutant embryos and their wild-type siblings, and serial dilutions of 1st strand cDNA were used as templates for PCR. The decrease in expression in the mutants can be determined by the difference in the dilution between wild-type and mutant where the PCR product amount diminishes. The actin control shows that the total amount of mRNA was the same between samples.

(B) Loss of heterozygosity is not observed in *rp* mutant tumors. DNA was prepared from tumors (T) and normal tissue (N) from the same fish and PCR was conducted with three primers that will show the presence or absence of both the insert-bearing (mutant) and wild-type chromosomes. In each case, the upper band is the wild-type chromosome and the lower band is the insert-bearing one. *hi10* fish #1 normal (lane 1), tumor (lane 2); *hi10* fish #2 normal (lane 3), tumor (lane 4); *hi258* fish normal (lane 5), tumor (lane 6); *hi1974* fish normal (lane 7), tumor (lane 8).

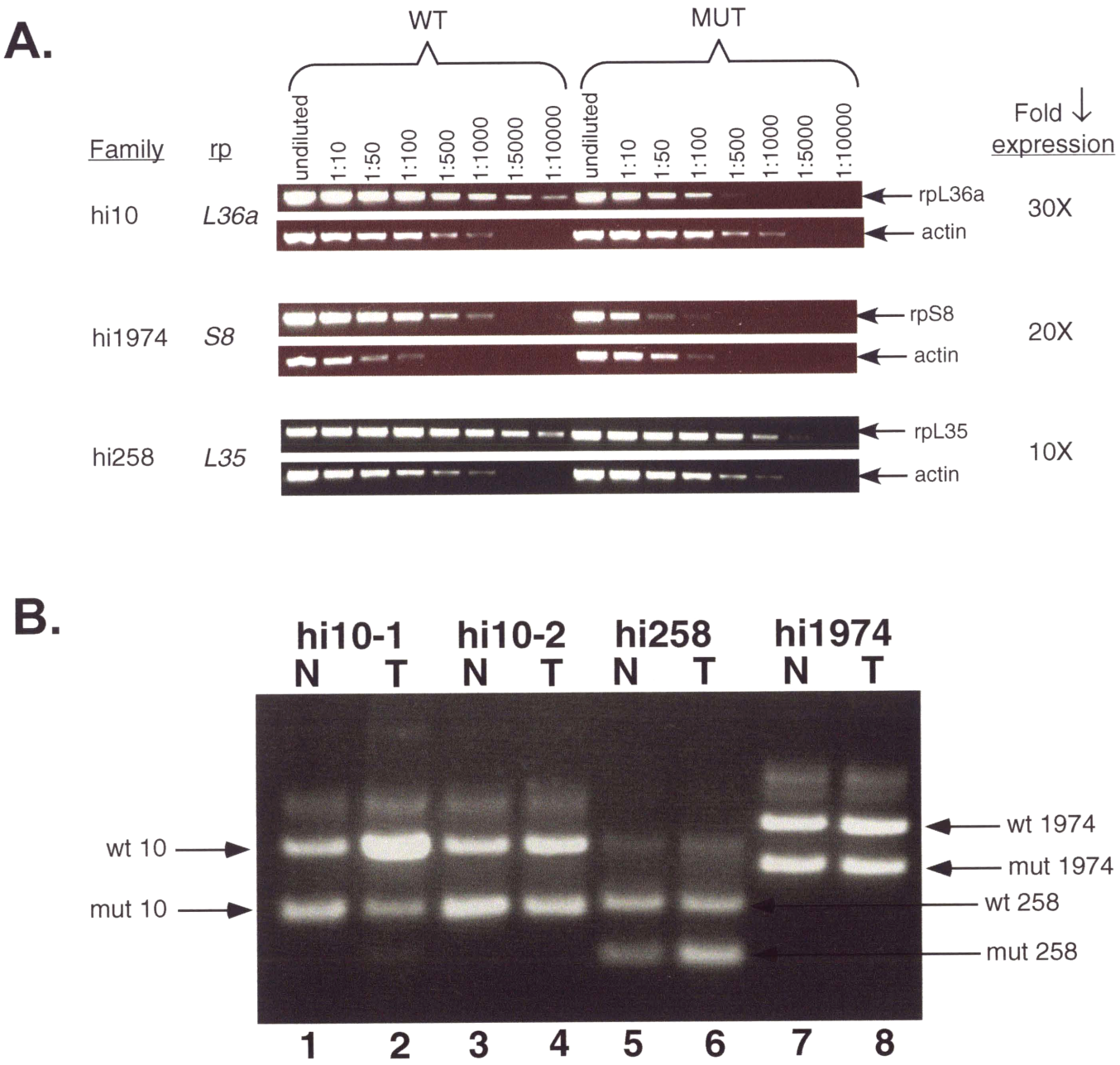

A.
WT
MUT
undiluted
1:10
1:50
1:100
1:500
1:1000
1:5000
1:10000
Family
rp
Fold ↓ expression
hi10
L36a
rpL36a
actin
30X
hi1974
S8
rpS8
actin
20X
hi258
L35
rpL35
actin
10X
B.
hi10-1
hi10-2
hi258
hi1974
N T N T N T N T
wt 10
mut 10
wt 1974
mut 1974
wt 258
mut 258
1 2 3 4 5 6 7 8

Figure 2.5: Ribosomal RNA levels are reduced in *rp* mutants.

RNA was prepared from 3-day-old homozygous mutant embryos (M) or their wild-type siblings (W) from lines *hi10* (*L36a*), *hi1974* (*S8*), and *hi2649* (*S15a*), and RNA content was visualized by electrophoresis and ethidium bromide staining. The ratio of 28*S*/18*S* as determined by densitometry is shown below each lane. Note that *L36a* mutants show a preferential loss of the 28*S* band by 1.5-fold, while *S8* and *S15a* mutants show a preferential loss of the 18*S* band by 1.9- and 1.8-fold, respectively. These RNAs were also northern blotted and probed for beta actin as an mRNA content control.

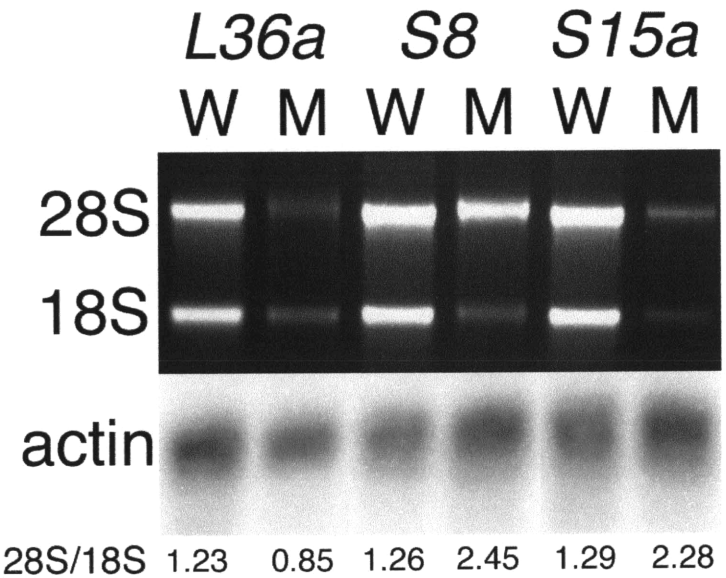
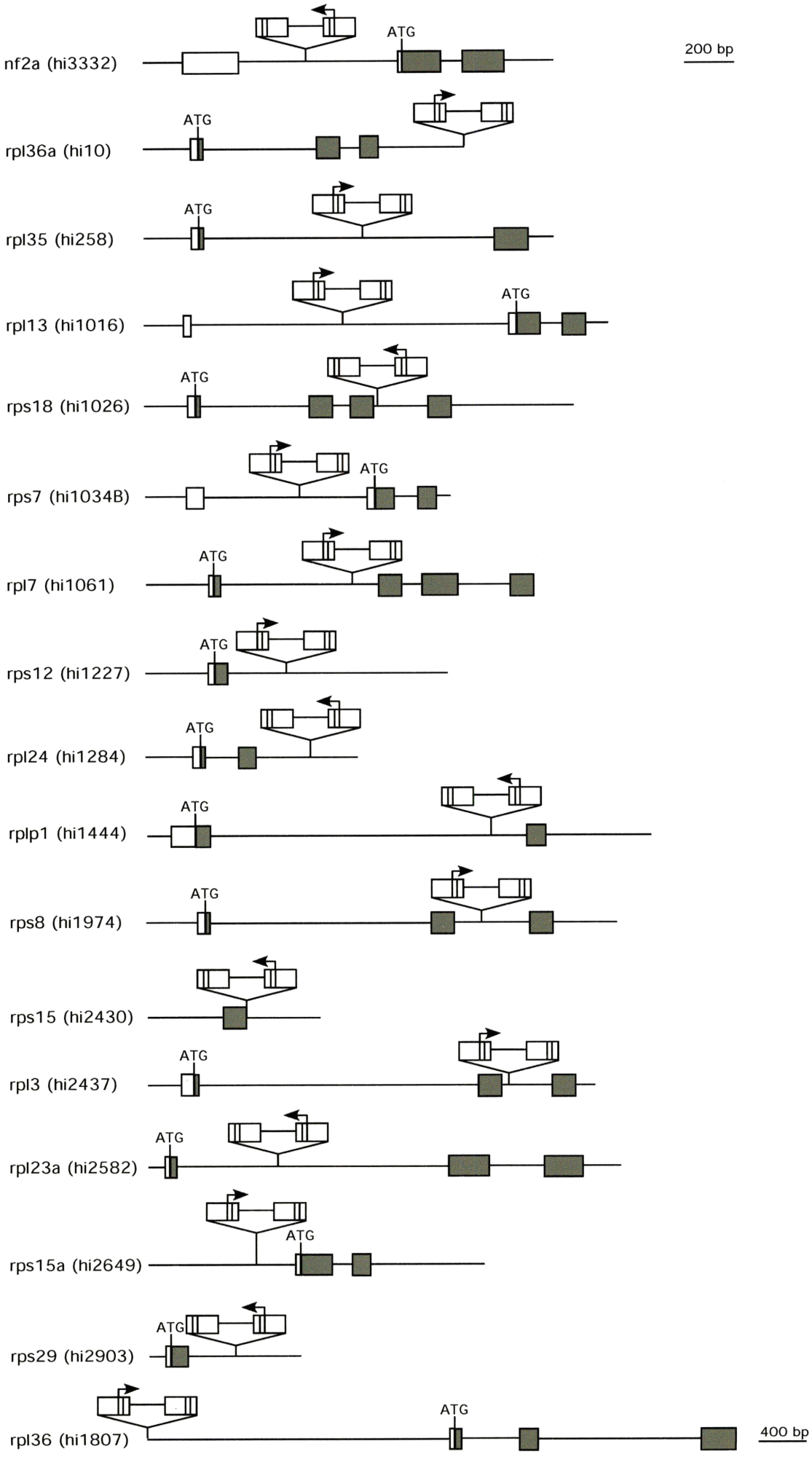

L36a
S8
S15a
W M W M W M
28S
18S
actin
28S/18S 1.23 0.85 1.26 2.45 1.29 2.28

Figure 2.S1: Position of mutagenic insertions.

The genomic sequence of part of each of these genes is represented as exonic (boxed) and promoter or intronic (line). White boxes represent 5' UTR while shaded boxes represent coding exons. Where no white boxes are shown, the location of the 5' UTR and beginning of the coding region has not been determined relative to the part of the locus shown here. In all cases, at least one coding exon (and all of the 3' UTR) is downstream of the region of the gene represented here. The position and orientation of the proviruses is shown above each genomic sequence. All drawings are to scale of the top scale bar, except the *rpl36* locus, which has its own scale bar.


nf2a (hi3332)
ATG
200 bp
rpl36a (hi10)
ATG
rpl35 (hi258)
ATG
rpl13 (hi1016)
ATG
rps18 (hi1026)
ATG
rps7 (hi1034B)
ATG
rpl7 (hi1061)
ATG
rps12 (hi1227)
ATG
rpl24 (hi1284)
ATG
rplp1 (hi1444)
ATG
rps8 (hi1974)
ATG
rps15 (hi2430)
rpl3 (hi2437)
ATG
rpl23a (hi2582)
ATG
rps15a (hi2649)
ATG
rps29 (hi2903)
ATG
rpl36 (hi1807)
ATG
400 bp

References

Amatruda, J. F., and Zon, L. I. (2002). Zebrafish as a cancer model system. Cancer Cell *1*, 229-231.

Amsterdam, A., Burgess, S. B., Golling, G., Chen, W., Sun, Z., Townsend, K., Farrington, S., Haldi, M., and Hopkins, N. (1999). A large-scale insertional mutagenesis screen in zebrafish. Genes and Development *13*, 2713-2724.

Beck-Engeser, G. B., Monach, P. A., Mumberg, D., Yang, F., Wanderling, S., Schreiber, K., Espinosa, R., Beau, M. M. L., Meredith, S. C., and Schreiber, H. (2001). Point Mutation in Essential Genes with Loss or Mutation of the Second Allele: Relevance to the Retention of Tumor-specific Antigens. The Journal of Experimental Medicine *194*, 285-299.

Beckwith, L. G., Moore, J. L., Tsao-Wu, G. S., Harshbarger, J. C., and Cheng, K. C. (2000). Ethylnitrosourea induces neoplasia in zebrafish (*Danio rerio*). Laboratory Investigation *80*, 379-385.

Ben-Ishai, R., Scharf, R., Sharon, R., and Kapten, I. (1990). A human cellular sequence implicated in *trk* oncogene activation is DNA damage inducible. Proceedings of the National Academy of Sciences *87*, 6039-6043.

Chen, F. W., Davies, J. P., and Ioannou, Y. A. (1998). Differential Gene Expression in Apoptosis: Identification of Ribosomal Protein 23K, a Cell Proliferation Inhibitor. Molecular Genetics and Metabolism *64*, 271-282.

Chen, F. W., and Ioannou, Y. A. (1999). Ribosomal Proteins in Cell Proliferation and Apoptosis. International Reviews of Immunology *18*, 429-448.

Cichowski, K., Shih, T. S., Schmitt, E., Santiago, S., Reilly, K., McLaughlin, M. E., Bronson, R. T., and Jacks, T. (1999). Mouse Models of Tumor Development in Neurofibromatosis Type 1. Science *286*, 2172-2176.

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Butel, J. S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. Nature *356*, 215-221.

Draptchinskaia, N., Gustavsson, P., Andersson, B., Pettersson, M., Willig, T.-N., Dianzani, I., Ball, S., Tchernia, G., Klar, J., Matsson, H., *et al.* (1999). The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia. Nature Genetics *21*, 169-175.

Gerhard, G. S., Kauffman, E. J., Wang, X., Stewart, R., Moore, J. L., Kasales, C. J., Demidenko, E., and Cheng, K. C. (2002). Life spans and senescent phenotypes in two strains of Zebrafish (*Danio rerio*). Experimental Gerontology *37*, 1055-1068.

Golling, G., Amsterdam, A., Sun, Z., Antonelli, M., Maldonado, E., Chen, W., Burgess, S., Haldi, M., Artzt, K., Farrington, S., *et al.* (2002). Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. Nature Genetics *31*, 135-140.

Haber, D., and Harlow, E. (1997). Tumor-suppressor genes: evolving definitions in the genomic age. Nature Genetics *16*, 320-322.

Hamaguchi, I., Ooka, A., Brun, A., Richter, J., Dahl, N., and Karlsson, S. (2002). Gene transfer improves erythroid development in ribosomal protein S19-deficient Diamond-Blackfan anemia. Blood *100*, 2724-2731.

Hershey, J. W. B., and Miyamoto, S. (2000). Translational Control and Cancer. In Translational Control of Gene Expression, N. Sonenberg, J. W. B. Hershey, M. B. Mathews, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Press), chap. 20.

Jacks, T. (1996). Tumor suppressor gene mutations in mice. Annual Review of Genetics *30*, 603-636.

Jimenez-Heffernan, J. A., Lopez-Ferrer, P., Vicandi, B., Hardisson, D., Gamallo, C., and Viguer, J. M. (1999). Cytologic features of malignant peripheral nerve sheath tumor. Acta Cytologica *43*, 175-183.

Kimmel, C. B. (1989). Genetics and early development of zebrafish. Trends in Genetics *5*, 283-288.

Langenau, D. M., Traver, D., Ferrando, A. A., Kutok, J. L., Aster, J. C., Kanki, J. P., Lin, S., Prochownik, E., Trede, N. S., Zon, L. I., and Look, A. T. (2003). Myc-Induced T Cell Leukemia in Transgenic Zebrafish. Science *299*, 887-890.

Largaespada, D. A. (2001). Haploinsufficiency for tumor suppression: the hazards of being single and living a long time. The Journal of Experimental Medicine *193*, F15-F18.

Lohrum, M. A. E., Ludwig, R. L., Kubbutat, M. H. G., Hanlon, M., and Vousden, K. H. (2003). Regulation of HDM2 activity by the ribosomal protein L11. Cancer Cell *3*, 577-587.

Moore, J. L., Aros, M., Steudel, K. G., and Cheng, K. C. (2002). Fixation and decalcification of adult zebrafish for histological, immunocytochemical, and genotypic analysis. Biotechniques *32*, 296-298.

Moritz, M., Paulovich, A. G., Tsay, Y. F., and Woolford, J. L. (1990) Depletion of yeast ribosomal proteins L16 or rp59 disrupts ribosome assembly. The Journal of Cell Biology *111*, 2261-2274.

Rajasekhar, V. K., Viale, A., Socci, N. D., Wiedmann, M., Hu, X., and Holland, E. C. (2003). Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. Molecular Cell *12*, 889-901.

Roberts, R. J. (2001). Neoplasia of Teleosts, In Fish Pathology, R. J. Roberts, ed. (London: W. B. Saunders), chap 5.

Ruggero, D., Grisendi, S., Piazza, F., Rego, E., Mari, F., Rao, P. H., Cordon-Cardo, C., and Pandolfi, P. P. (2003). Dyskeratosis Congenita and cancer in mice deficient in ribosomal RNA modification. Science *299*, 259-262.

Ruttledge, M. H., Sarrazin, J., Rangaratnam, S., Phelan, C. M., Twist, E., Merel, P., Delattre, O., Thomas, G., Nordenskjold, M., Collins, V. P., *et al.* (1994). Evidence for the complete inactivation of the NF2 gene in the majority of sporadic meningiomas. Nature Genetics *6*, 180-184.

Schmale, M. C., Hensley, G., and Udey, L. R. (1983). Neurofibromatosis, von Recklinghausen's disease, multiple schwannomas, malignant schwannomas. Multiple schwannomas in the bicolor damselfish, *Pomacentrus partitus* (pisces, pomacentridae). The American Journal of Pathology *112*, 238-241.

Schmale, M. C., Hensley, G. T., and Udey, L. R. (1986). Neurofibromatosis in the bicolor damselfish (*Pomacentrus partitus*) as a model of von Recklinghausen neurofibromatosis. Annals of the New York Academy of Sciences *486*, 386-402.

Smolowitz, R., Hanley, J., and Richmond, H. (2002). A three-year retrospective study of abdominal tumors in zebrafish maintained in an aquatic laboratory animal facility. The Biological Bulletin *203*, 265-266.

Sonenberg, N. (1993) Translation factors as effectors of cell growth and tumorigenesis. Current Opinion in Cell Biology *5*, 955-960.

Soulet, F., Al Saati, T., Roga, S., Amalric, F., Bouche, G. (2001). Fibroblast growth factor-2 interacts with free ribosomal protein S19. Biochemical and Biophysical Research Communications *289*, 591-596.

Spitsbergen, J. M., Tsai, H. W., Reddy, A., Miller, T., Arbogast, D., Hendricks, J. D., and Bailey, G. S. (2000a). Neoplasia in zebrafish (*Danio rerio*) treated with 7,12-dimethylbenz[a]anthracene by two exposure routes at different developmental stages. Toxicologic Pathology *28*, 705-715.

Spitsbergen, J. M., Tsai, H. W., Reddy, A., Miller, T., Arbogast, D., Hendricks, J. D., and Bailey, G. S. (2000b). Neoplasia in zebrafish (*Danio rerio*) treated with N-methyl-N'-nitro-N-nitrosoguanidine by three exposure routes at different developmental stages. Toxicologic Pathology *28*, 716-725.

Trofatter, J. A., MacCollin, M. M., Rutter, J. L., Murrell, J. R., Duyao, M. P., Parry, D. M., Eldridge, R., Kley, N., Menon, A. G., Pulaski, K., *et al.* (1993). A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. Cell *72*, 791-800.

Volarević, S., Stewart, M. J., Ledermann, B., Zilberman, F., Terracciano, L., Montini, E., Grompe, M., Kozma, S. C., and Thomas, G. (2000). Proliferation, But Not Growth, Blocked by Conditional Deletion of 40S Ribosomal Protein S6. Science *288*, 2045-2047.

Volarević, S., and Thomas, G. (2001). Role of S6 phosphorylation and S6 kinase in cell growth. Progress in Nucleic Acid Research and Molecular Biology *65*, 101-127.

Wasser, J. S., Yolken, R., Miller, D. R., and Diamond, L. (1978). Congenital hypoplastic anemia (Diamond-Blackfan syndrome) terminating in acute myelogenous leukemia. Blood *51*, 991-995.

Wittbrodt J., Adam, D., Malitschek, B., Maueler, W., Raulf, F., Telling, A., Robertson, S. M., and Schartl, M. (1989). Novel putative receptor tyrosine kinase encoded by the melanoma-inducing Tu locus in Xiphophorus. Nature *341*, 415-421.

Woodruff, J. M. (1999). Pathology of tumors of the peripheral nerve sheath in type 1 neurofibromatosis. American Journal of Medical Genetics *89*, 23-30.

Wool, I. G. (1996). Extraribosomal functions of ribosomal proteins. Trends in Biochemical Sciences *21*, 164-165.

Wool, I. G., Chan, Y.-L., and Gluck, A. (1996). Mammalian Ribosomes: The structure and the evolution of the proteins, In Translational Control of Gene Expression, N. Sonenberg, J. W. B. Hershey, M. B. Mathews, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Press), chap. 24.

Chapter 3

Ribosomal Protein Mutations Lead to Growth Impairment and Tumor Predisposition in Zebrafish

Kevin Lai, Adam Amsterdam, Sarah Farrington, Roderick T. Bronson,
Nancy Hopkins, and Jacqueline A. Lees

Author contributions:
Table 3.1: KL, Kirsten Sadler (previously published data), & AA with assistance from SF & RB
Figures 3.1, 3.2, 3.3, 3.4: KL
Figure 3.5: AA
Table 3.S1: KL

Abstract

We have characterized 28 zebrafish lines with heterozygous mutations in ribosomal protein (*rp*) genes, and found that 17 of these are prone to develop zebrafish malignant peripheral nerve sheath tumors (zMPNST) and other rare tumors. Heterozygotes from the most highly tumor-prone *rp* lines were found to be growth-impaired. In addition, heterozygous cells from one such line were also growth-impaired relative to wild-type cells in chimeric embryos. These findings suggest that the primary effect of heterozygosity for many *rp* genes is a global defect in protein translation, which leads to growth impairment. This raises the possibility that a deficiency of translational capacity can promote the development of cancer.

Introduction

Control of cell growth and cell proliferation are intimately linked. While it has long been known that protein translation is upregulated in many tumors, this has been assumed to be a consequence of increased cell proliferation. However, a direct causal role for aberrant translation in cancer is just beginning to be elucidated (Clemens, 2004). Recent evidence suggests that many translation factors may be proto-oncogenes, most notably eIF2, eIF4E, and eIF4G. Overactivity or overexpression of these proteins have been demonstrated to cause malignant transformation (Donzé et al., 1995; Fukuchi-Shimogori et al., 1997; Lazaris-Karatzas et al., 1990). It has also been shown that oncogenic signaling by *Ras* and *Akt* leads directly to a preferential recruitment of certain mRNAs to the ribosome (Rajasekhar et al., 2003), thus suggesting a possible role for translation in mediating tumorigenesis. Furthermore, we previously reported the surprising finding that numerous ribosomal proteins (rps) are haploinsufficient tumor suppressors in the zebrafish (Amsterdam et al., 2004). Although several

rp mutant lines were not tumor prone, the large number of lines that were suggested that the mechanism of tumorigenesis involved the role of the rps in translation.

Ribosomal protein mutations have been studied in other metazoan species, most extensively in *Drosophila* where a number of *Minute* mutants have been mapped to ribosomal protein genes (Lambertsson, 1998). The *Minutes* have characteristic pleiotropic defects, including recessive lethality, short thin bristles, and often a developmental delay, resulting in smaller flies. Heterozygous mutation of *rpS6* and *rpS21* also causes an overgrowth of the hematopoietic organs (Török et al., 1999; Watson et al., 1992), suggesting that at least some rps may play a role in suppressing a tumor-like phenotype in flies. Similar to the *Drosophila Minutes*, a mouse heterozygous for *rpL24* displays a growth impairment, among other developmental abnormalities (Oliver et al., 2004); however, an increased predisposition to cancer has not yet been demonstrated. In humans, heterozygous mutation of *rpS19* is found in 25% of cases of Diamond-Blackfan anemia (DBA) (Draptchinskaia et al., 1999). DBA patients suffer not only from anemia caused by a deficiency of erythroid precursors, but are also prone to the development of leukemias (Wasser et al., 1978). It is currently unknown whether rpS19 plays a direct role in leukemogenesis.

Here, we present evidence that many *rp* mutations in zebrafish lead to a growth impairment, *i.e.*, that we have identified the first examples of fish *Minutes*. While these fish have no other detectable developmental abnormalities, they are prone to tumor development later in life. Furthermore, we show that the growth defect is manifested at the level of *rp* heterozygous cells. Since growth defects are often associated with defects in translation in other species, the correlation between growth impairment and cancer suggests that an impairment in translation may play a role in driving tumorigenesis in *rp* heterozygous fish.

Results

Heterozygous Mutation of Many rp Genes Predisposes Zebrafish to Malignant Peripheral Nerve Sheath Tumors and Other Rare Tumor Types

We previously reported that 11 of 16 lines bearing mutations in *rp* genes are predisposed to zebrafish malignant peripheral nerve sheath tumors (zMPNST) (Amsterdam et al., 2004). We have since extended this analysis and found that, in total, 17 of 28 *rp* mutant lines are tumor-prone (Table 3.1). The previous data have also been re-evaluated with more stringent criteria for the diagnosis of tumors. For example, many of the tumors that we had previously identified as pancreatic islet adenomas have been re-classified as non-cancerous due to their common occurrence in the control population. Moreover, removing some of these "tumors" from the original analysis is appropriate since, even though the islets appear large upon sectioning, they are otherwise well-differentiated and therefore not indicative of true dysplastic disease.

Additionally, in the course of evaluating more control fish, *i.e.*, heterozygous mutant fish from non-*rp* and other non-tumor-prone transgenic lines, we found that the basal tumor incidence is at most 5%, which is less than the 11% we originally reported (Table 3.1C and data not shown). In large part, the discrepancy is accounted for by the re-classification of many "pancreatic islet adenomas" as non-cancerous. Among the new set of over 5800 control fish from 265 transgenic lines analyzed, 2% had seminomas, 1.4% had bile duct adenomas / adenocarcinomas, 0.5% had lymphomas, 0.3% had pancreatic duct hyperplasias / carcinomas, and 0.3% had true pancreatic islet adenomas / adenocarcinomas. In addition, 0.4% or 22 of the 5800 fish had other rare tumor types that were each observed in at most 4 fish. Among these 22 fish were 4 with zMPNSTs; thus the new data reinforce the notion that zMPNSTs are extremely rare within the control population (less than 1 in 1000).

With this new analysis of controls, the tumor susceptibility of the *rp* lines now appears even more significant. Since the incidence of zMPNSTs among the control population is so rare, the finding that in some *rp* lines, such as *hi1987*, only 3 of 39 fish develop zMPNSTs (1 in 13) is still highly significant (Table 3.1A). Thus, we have now classified the *rp* lines in our collection into only two categories: (1) "tumor-prone" lines, which develop zMPNSTs and other rare tumor types (these include lines heterozygous for *S3a*, *S5*, *S7*, *S8*, *S11*, *S15a*, *S18*, *S28*, *S29*, *L7*, *L13*, *L14*, *L19*, *L23a*, *L35*, *L36*, and *L36a*) (Table 3.1A); and (2) "non-tumor-prone" lines, which have a tumor incidence and spectrum that is indistinguishable from the controls (these include lines heterozygous for *Sa*, *S12*, *S15*, *L3*, *L6*, *L9*, *L11*, *L12*, *L24*, *L28*, and *LP1*) (Table 3.1B). Based on this distinction, the lines *hi2582* (*rpL23a*) and *hi1987* (*rpL19*) with only 11% and 10% tumor incidence, respectively, are classified as tumor-prone due to the appearance of zMPNSTs (Table 3.1A). On the other hand, the *hi3076* (*L12*) line is considered non-tumor-prone because, even though it had a higher tumor incidence of 14%, only one of the three tumors detected was malignant (a lymphoma) and the other two were more common benign seminomas (Table 3.1B and data not shown). As reported previously, there are some *rp* lines that are extremely tumor-prone, in which the incidence of zMPNSTs and other tumor types is greater than 60%. The previous distinction between "high-tumor" and "medium-tumor" lines, however, is somewhat arbitrary since the tumor incidence among the *rp* lines spans the full spectrum between 100% and control incidence (Table 3.1).

Degree of rp Message Knockdown in rp Homozygous Mutant Embryos Does Not Correlate with Tumor Incidence in rp Heterozygous Adults

Upon finding that some *rp* mutant lines are tumor-prone and some are not, we wanted to determine the molecular basis for this difference in tumor susceptibility. One possible explanation is that the retroviral insertion in the tumor-prone lines causes a severe reduction of the *rp* message level, leading to a greater loss of function of the rp in these lines than in the non-tumor-prone lines. Many of the mutations are predicted to be hypomorphs since, in most cases, the retrovirus has inserted into an intron, which can be spliced out (Figure 2.S1 and data not shown). We previously determined that the rp message level is knocked down to varying extents in homozygous *rp* mutant embryos from three tumor-prone lines (Figure 2.4). Analysis of additional lines, including non-tumor-prone lines, revealed that while the degree of rp message knockdown generally correlated with the severity of the homozygous mutant phenotype, as expected, it did not correlate with the tumor incidence in the adult heterozygotes (Table 3.S1). Thus, it appears that merely a reduction in expression of any *rp* does not account for the cancer phenotype.

Homozygous Mutant rp Embryos Have Severe Defects in Ribosome Integrity

While it is formally possible that some extraribosomal function of many of the rps is disrupted in the cancer-prone lines, a more likely explanation is that the mechanism of tumorigenesis involves the role of these proteins in their known common function, translation. In order to test whether certain *rp* lines have a defect in translation, we examined homozygous mutant *rp* embryos, in which the defect, if present, was expected to be most severe. Polysome fractionation analysis was performed to determine the proportion of ribosomal subunits that are free or bound in monosomes (80*S*) or polysomes. In wild-type embryos, distinct small and large ribosomal subunit peaks (40*S* and 60*S*, respectively) are detected (Figure 3.1A). In addition to

the predominant monosome peak, the presence of higher-order polysome peaks indicates a high rate of translation. In embryos that are homozygous mutant for a small subunit *rp*, *rpS8* (*hi1974*), there appears to be a reduction in the amount of the 40*S* subunit and a relative increase in the 60*S* subunit, as though the 60*S* subunit were in excess and accumulating (Figure 3.1B). Accordingly, there is a relative reduction in the monosome and polysome peaks. In contrast, mutants for a large subunit rp, *rpL35* (*hi258*), appear to have a reduction in both the 40*S* and 60*S* peaks, as well as a reduction in the number of monosomes and polysomes (Figure 3.1C). But, as one might expect, the reduction in the 60*S* peak is relatively more severe. The ribosome integrity defects observed in the homozygous *rp* mutants are specific and not merely a consequence of the general necrotic phenotype of these embryos, since mutants in a DNA polymerase subunit (*hi1703*) with a similar morphological phenotype have a polysome profile that is very similar to that of wild-type embryos (Figure 3.1D). The ribosome integrity defect was detected among homozygous mutant embryos from all *rp* lines analyzed (data not shown), including lines in which the heterozygous adults are not tumor prone. There was not a strong correlation between the severity of the defect and the tumor susceptibility of the adult heterozygotes (data not shown).

Heterozygous Embryos from a Tumor-Prone rp Line Are Developmentally Delayed, but Are Not Overtly Impaired in Translation

In the course of sorting out the homozygous *rp* mutant embryos for polysome analysis from a mating of heterozygous carriers, we noticed a variation in size among the phenotypically wild-type siblings, which was expected to consist of a mixture of two-thirds *rp* heterozygous and one-third non-transgenic embryos. This raised the possibility that a heterozygous effect was

causing a developmental delay. In order to test this possibility in a controlled manner, embryos produced from an outcross of a *hi258* (*rpL35*) heterozygote and a T-AB wild type fish were sorted at 4 days post-fertilization (dpf) for the presence of a swim bladder. This structure is a suitable reporter for a developmental delay since it appears suddenly on day 4 and is highly visible. Therefore, it allowed for easy sorting of the embryos into two groups. These groups were then subsequently sorted on the basis of the size of the embryos, by lining up the embryos and visually inspecting them. Among the swim bladder-containing embryos, the largest fish were predicted to be non-transgenic and the smallest of embryos lacking a swim bladder were predicted to be *hi258* heterozygotes. Genotyping of individual embryos revealed that the correct assignment was made in 88% of the cases (Figure 3.2A). Since these embryos were the offspring of an outcross of an *rp* heterozygote, a random assignment of genotypes would have been correct only about 50% of the time. In a similar experiment, embryos from a *hi258* outcross were sorted as above, but the embryos in each of the two groups, labeled "predicted heterozygotes" and "predicted wild-type," were pooled and genotyped. By performing PCR on serial dilutions of DNA from each pooled sample, the *hi258* transgenic band was found to be approximately 10 times more abundant in the predicted heterozygote sample than in the predicted wild-type sample (Figure 3.2B), suggesting that the mixtures were nearly 90% pure, which is consistent with the previous result. Thus, the heterozygous embryos have a developmental delay that can be detected visually.

Since the heterozygous embryos are otherwise phenotypically wild-type, this developmental delay may be indicative of a general impairment in growth. Since a decrease in global protein synthesis can lead to growth impairment, we analyzed polysome profiles to assess the level of translational capacity in the *rp* heterozygous embryos. Due to the difficulty of

keeping polysomes intact during the course of their isolation, time could not be taken to genotype individual embryos before performing the polysome fractionation. Since at least 100 embryos were required to generate an adequate polysome profile, we decided to sort the embryos into wild-type-enriched and *hi258* heterozygote-enriched samples as in the experiment above. When the mixtures were analyzed by polysome fractionation, the shapes of the profiles of the wild-type- and heterozygote-enriched samples were very similar, including the number of higher-order polysomes observed (See Appendix A, Figure A.1). While this result shows no overt translation defect in the *hi258* heterozygous embryos, it does not rule out the possibility that it may be too subtle to be detected by this method, especially since the sample populations were likely not completely pure.

Tumor-Prone rp Lines Are Growth-Impaired

In the absence of conclusive evidence of a translation defect in the *hi258* heterozygous embryos, it was still important to determine whether the growth impairment phenotype persisted to adulthood, which might hint at a causal link to tumorigenesis. We found that 28-day-old offspring generated from an outcross mating of a *hi258* heterozygote to a wild-type fish still varied greatly in size (Figure 3.3A), with the largest fish being over 10 times more massive than the smallest fish (Figure 3.3B). Again, upon genotyping the fish individually, the *rp* heterozygotes were found to be generally smaller than their wild-type siblings (Figure 3.3B). We then conducted a carefully controlled experiment with the *hi258* line, in which the offspring of a *hi258* outcross, *i.e.*, both heterozygous and wild-type siblings, were housed together in the same tank. After weighing the fish blindly at selected time points, then genotyping the fish, it was clear that the *hi258* heterozygous fish were smaller than their wild-type siblings at every

time point (Figure 3.3C). The *hi258* heterozygous fish are growth-impaired early on and never seem to catch up in size to their wild-type siblings. The growth *rate* of the heterozygotes, however, does appear to be comparable to that of the wild-type siblings after 67 days of age.

Next, we wanted to determine whether all of our *rp* mutant lines displayed a growth impairment. Since the growth defect was evident by day 25 in the *hi258* line, we repeated the experiment at this time point for a number of *rp* lines, both tumor-prone and non-tumor-prone. Strikingly, when the *rp* lines are ordered by tumor incidence, it is evident that the most tumor-prone lines (>33% tumor incidence) are all significantly growth-impaired (Figure 3.4A). Normalizing the data with respect to the average weight of each corresponding set of wild-type siblings makes this point even clearer (Figure 3.4B). Most of the non-tumor-prone lines show no significant growth impairment, similar to the control non-*rp* line, *hi1703*, a mutant for a DNA polymerase subunit. There were, however, several exceptions: *hi1479*, (*rpSa*), *hi2430*, (*rpS15*), and *hi3893* (*rpL28*) were growth-impaired, but not tumor-prone.

rp Mutants Are Growth-Impaired at the Cellular Level

The finding that tumor susceptibility is generally correlated with growth impairment suggests that the growth defect may play a role in tumorigenesis. There are two possible models to explain how this might occur: the formation of tumors could either be cell-autonomous or non-cell autonomous; that is, the tumors could arise as a result of an intrinsic defect of the *rp* heterozygous cells, or they could arise from cells that gain a proliferative advantage in the background of a growth-impaired *rp* heterozygous fish. To begin to address these models, we wanted to determine whether *rp* heterozygosity leads to a growth defect at the cellular level. We generated chimeric fish and analyzed the ability of *rp* heterozygous or wild-type cells to compete

in hosts of each genotype. Real-time quantitative PCR was used to measure the relative contribution of cells of each genotype. In order to track the wild-type cells, a line bearing a non-mutagenic insertion, termed *hiX*, was identified. Heterozygotes from a high-tumor line (and therefore a line that displays a growth defect at the organismal level), *hi10* (*rpL36a*), were used for the chimera analysis.

In the first experiment, cells were harvested from embryos upon a cross of a *hi10* heterozygote and a *hiX* heterozygote. The expected ratio of genotypes amongst the cells was 25% *hi10* only, 25% *hi10* and *hiX*, 25% *hiX* only, and 25% non-transgenic. This mixture of cells was injected into wild-type host embryos, which were allowed to grow until the fish were homogenized for quantitative PCR analysis. Five days after injection, the contribution of cells bearing the *hi10* insertion was significantly less than that of cells bearing the non-mutagenic *hiX* insertion (Figure 3.5A). At 4 to 6 weeks after injection, the relative contribution of *hi10* cells is still significantly less than that of *hiX* cells (Figure 3.5A).

In order to determine whether wild-type cells can compete better in an *rp* heterozygous background than in a wild-type background, the converse experiment was performed. Cells were harvested from embryos upon a mating of a *hiX* heterozygote and a non-transgenic fish. Thus, all of the cells were expected to be phenotypically wild-type, and 50% of them should have carried the *hiX* insertion that was being tracked. At five days after injection, the contribution of wild-type (*hiX*) cells was significantly higher in *hi10* heterozygous hosts than in wild-type hosts (Figure 3.5B). This held true when the fish were analyzed at 2 to 3 weeks post injection (Figure 3.5B). The data presented thus strongly suggest that the *rp* mutations cause a defect in growth, both at the cellular and organismal levels.

Discussion

In this study, we have completely characterized the tumor phenotype of 28 zebrafish lines with heterozygous mutations in ribosomal protein genes and found that 17 of these are tumor-prone. As noted previously (Amsterdam et al., 2004), these tumor-prone lines include mutants for proteins in both the large and small ribosomal subunits. We did not find a correlation between the severity of the rp message reduction in the homozygous *rp* mutants and the tumor incidence of the adult heterozygotes, which suggests that it is not simply a drastic reduction in the level of *any* ribosomal protein that leads to cancer. Intriguingly, we did find that the most highly tumor-prone lines have a significant impairment of growth that is detected as early as the embryonic stages. Furthermore, this growth impairment is detected at the cellular level, as *rp* heterozygous cells are out-competed by wild-type cells in chimeric embryos. The observed defect in growth, coupled with the fact that a ribosomal protein gene is mutated, strongly suggests that the underlying defect is an impairment in protein translation. Though a dramatic defect in ribosome integrity was detected in the polysome profiles of homozygous *rp* mutants, a mixture of embryos enriched for *rp* heterozygotes showed no apparent defect.

The lack of a detectable abnormality in the polysome profile does not rule out the possibility that a translation defect exists in the *rp* heterozygotes. Published evidence for defects in global translation demonstrates that the effect on the polysome profile is often quite subtle, but such minor shifts can have tremendous consequences. For example, tumor cells treated with the rapamycin analog RAD001 are impaired slightly in global translation, but this is enough to reduce the translation of p21 such that its induction is inhibited after DNA damage (Beuvink et al., 2005). In addition, it is possible that a translation defect could be manifested in the *rp* heterozygotes by a selective translation of mRNAs; *i.e.*, under conditions of impaired

translational capacity, certain messages may be translated at the expense of others that would normally also be translated (Rajasekhar et al., 2003). This possibility is currently being explored with the use of polysome microarrays (See Appendix A). Furthermore, certain cell types, for example, the nerve sheath cells that give rise to zMPNSTs may be more susceptible to a translation defect than other cell types. It is important to note that many of the tumor-prone lines also develop other rare tumors, which supports the notion that a translation defect could predispose all of the cells of the *rp* heterozygous fish to transformation, but certain cell types are especially vulnerable. This may account for the observed tumor spectrum among the *rp* heterozygotes.

Cell-type specific sensitivity to a translation defect may also account for the observation that the correlation between growth impairment and tumor susceptibility is not absolute. As noted, three lines displayed a growth impairment, but were not tumor-prone. In addition, there were several other lines that were prone to develop zMPNSTs and had relatively low overall tumor incidence between 10% and 26% [*hi1987* (*rpL19*), *hi2582* (*rpL23a*), and *hi1026* (*rpS18*)], but were not significantly growth-impaired (Figure 3.4). This again supports a model in which a general growth impairment predisposes to tumor development, but tumorigenesis also depends on some unknown modifying factors that may be cell-type specific. For example, in non-tumor-prone lines with a growth impairment, it is possible that reduced levels of the ribosomal protein can lead to a growth defect of the organism, but nerve sheath cells can withstand such a challenge of reduced translational capacity to remain untransformed. It is important to note that the severity of the growth defect is greatest among the extremely tumor-prone lines (>50% tumor incidence), which suggests that if the reduction in translational capacity is sufficiently severe, tumorigenesis is highly favored. That is, that there may be a threshold of translational capacity

below which cells become susceptible to transformation. This is consistent with the observation that some lines that were not significantly growth-impaired had a few fish with tumors because, here, it is important to realize that the average weight of a population of *rp* heterozygotes was used to determine whether the line had an overall growth impairment. There may be stochastic variation in the severity of the translation defect among individual fish. As shown for the *hi258* line, there certainly exists a large variation in size among the heterozygotes (Figure 3.3B). It is currently not known, and will be important to determine, if the fish that are smallest at a young age are the ones that will eventually develop tumors. It will also be interesting to determine whether the size of the fish correlates with the time to onset of cancer. Thus, there may exist an even finer correlation between growth and tumorigenicity than we have discovered thus far.

The preceding discussion of tumor susceptibility can also be framed in the context of the two models of tumorigenesis raised above: Tumor formation can be cell-autonomous, whereby an *rp* heterozygous cell has an intrinsic defect that predisposes it to transformation, or non-cell-autonomous, whereby the background of many growth-impaired cells in an *rp* heterozygous fish allows for the rapid growth of a cell that sustains a mutation imparting a growth advantage. The results presented clearly demonstrate that the *rp* heterozygous cells are growth-impaired compared to wild-type, which suggests that the cells have an intrinsic defect in translation. Yet, this does not preclude the possibility of the non-cell-autonomous model being correct, since a mutation that has the effect of increasing translation in a cell may give it a growth advantage in the background of an *rp* heterozygous fish that it would not normally have in an environment of wild-type cells. There is precedent to suggest that tumorigenesis can be highly context-dependent. In populations of hematopoietic progenitor cells of mice, an impairment in DNA replication allows certain cells that have acquired oncogenic mutations to out-compete other cells

within the population, leading to the development of leukemias (Bilousova et al., 2005). Among a cell population with healthy DNA replication, however, the same mutations provide no proliferative advantage (Bilousova et al., 2005). Thus, we should consider that the analogous situation, in which a general impairment of protein translation allows for the growth of cells that have sustained an oncogenic mutation, might be possible.

The cell-autonomous versus non-cell-autonomous debate should be resolved upon the demonstration of the origin of the tumors in the chimera models we have described. The chimera experiments have been repeated, and we are now aging the fish until they develop tumors. In the case where wild-type cells are injected into an *rp* heterozygous host, the fish should develop tumors since most of the cells of the fish are still *rp* heterozygous even if the small percentage of injected wild-type cells competes extremely well. If the tumor tissue is derived primarily from the wild-type cells, this would argue strongly in favor of the non-cell-autonomous model. If it is composed mainly of *rp* heterozygous cells, then we cannot conclude that the cell-autonomous model is true since the wild-type cells may not have contributed to the tissues that are most prone to tumor development (*e.g.*, nerve sheath). The converse experiment, in which *rp* heterozygous cells are injected into a wild-type host, should provide a more definitive answer on this point: If tumors arise, and they are composed primarily of *rp* heterozygous cells, then this would strongly support the cell-autonomous model.

In summary, we have identified a growth defect that is common to zebrafish lines that are heterozygous for mutations in ribosomal protein genes and prone to the development of rare cancers. The *rp* mutations impair translation in the homozygous state, though an overt translation defect has not been detected in the *rp* heterozygotes by the methods we have employed thus far. The observed correlation between growth impairment and tumor

susceptibility, however, raises the possibility that a defect in translation could promote tumorigenesis.

Materials and Methods

Fixation and Histology

Adult fish were euthanized in ice water or 250mg/L Tricaine and fixed in 10% neutral buffered formalin or Bouin's fixative. Embedding in paraffin and sectioning were performed as previously described (Amsterdam et al., 2004; Moore et al., 2002).

Polysome Fractionation

Fifty to 200 embryos at 3 or 4 dpf were washed in PBS and Dounce-homogenized in ice-cold lysis buffer containing 110mM potassium acetate, 2mM magnesium acetate, 10mM HEPES pH 7.6, 100mM potassium chloride, 10mM magnesium chloride, 0.1% NP-40, 2mM dithiothreitol, and 40U/mL RNase inhibitor. The dithiothreitol and RNase inhibitor were added immediately prior to use. The lysate was spun down at 1000×g for 10 min. at 4°C to pellet nuclei and cellular debris. The cytoplasmic extract was layered onto sucrose gradients prepared by layering 17% sucrose solution over 50% sucrose solution in a 13.2 mL Beckmann ultracentrifuge tube (Cat. No. 344059) and laying the Parafilm-sealed tube on its side overnight. The sucrose solutions also contained 110mM potassium acetate, 2mM magnesium acetate, and 10mM HEPES pH 7.6. The extract was fractionated by centrifuging at 40,000×g for 2 hrs. at 4°C in an Sw41Ti rotor. Fractions were collected by injecting 60% sucrose into the bottom of the tube on an ISCO Model 640 Density Gradient Fractionator, and absorbance at 280nm was

measured using a Pharmacia LKB Uvicord SII detector. Profiles were generated with a Pharmacia LKB 19-8003-01 chart recorder.

RNA Analysis

Degree of rp message reduction in the *rp* homozygous mutants was determined by PCR on serial dilutions of cDNA prepared from *rp* homozygous mutant embryos and their wild-type siblings, as previously described (Amsterdam et al., 2004).

Analysis of Growth of rp Heterozygous Mutants

rp heterozygous mutant fish were outcrossed to T-AB wild-type fish. Randomly selected, un-genotyped fish were housed in tanks at an average density of 20 fish per tank. At selected time points, fish were euthanized in ice water, blotted dry, and weighed on a Mettler AE50 balance. Genotyping PCRs were performed on DNA isolated from tail clippings or the entire fish, with insert specific primers for each line to detect heterozygotes. Primers for *Wnt* were used concurrently to control for the efficiency of the PCR.

Generation and Real-Time PCR Analysis of Chimeras

Chimeras were generated basically as previously described (Lin et al., 1992). Briefly, a pool of 100-200 embryos was dissociated at the 1000-cell stage with a Pasteur pipette in 1 mL Holtfreter's solution. Cells were spun down and washed in 10mL Holtfreter's solution followed by 10mL 1:1 (v:v) PBS:Holtfreter's solution. The final cell pellet was resuspended in 50-100μL 2:1 (v:v) PBS:Holtfreter's solution. Approximately 50 cells (range: 30-100 cells) were injected into 1000-stage embryo hosts.

Real-time PCR analysis was performed as previously described (Amsterdam et al., 1999). In order to determine the percentage contribution of the *hi10* and *hiX* alleles, a standard curve was generated by mixing known quantities of pure DNA samples from *hi10* or *hiX* embryos, and analyzing the samples by real-time PCR.

Acknowledgements

We thank Kirsten Sadler for use of her previously published data, included in Table 3.1. We thank Kate Anderson for maintenance of the mutant lines of fish, and Sam Farrington and Tim Angelini for maintenance of the zebrafish colony. We thank the CCR Histology Facility, especially Alicia Caron and Mike Brown, for sample processing and sectioning. We thank Christian Petersen and Philip Sharp for helpful advice and equipment for polysome fractionation analysis.

Table 3.1: Tumor Incidence in zebrafish *rp* heterozygous lines and in the colony.

rp animals were collected as tumors became apparent, or as healthy animals at the maximum age specified (Age Range). Number Lost indicates those that either died before the appearance of external symptoms or were lost from their tanks. Control animals from the colony were selected without regard to gross appearance. Incidence rates are based on the number of fish examined histologically (i.e., excluding lost fish). (A) Tumor-prone lines; (B) Non-tumor-prone lines; (C) Various lines from the colony.

	Gene	Line	Age Range (in months)	Initial Number of Fish	Number Lost	Number of Fish Examined	Number of Grossly-Apparent Tumors	Number of Tumor-Bearing Fish	Number of zMPNSTs	Tumor Incidence (%)
A	L35	hi258	up to 21.5	13	7	6	5	6	6	100
	L14	hi823	up to 24.5	26	4	22	20	21	16	95
	S15a	hi2649	up to 18	9	2	7	4	6	5	86
	S3a	hi1290	up to 23.5	41	10	31	24	25	20	81
	L36	hi1807	up to 23	62	11	51	34	40	33	78
	S8	hi1974	up to 22	19	6	13	6	9	8	69
	L36a	hi10	up to 22	14	6	8	4	5	5	63
	S11	hi2799	up to 23.5	23	8	15	6	9	7	60
	S5	hi577B	up to 23	27	4	23	7	12	10	52
	L7	hi1061	up to 22.5	28	6	22	11	11	10	50
	L13	hi1016	up to 23	15	4	11	2	5	3	45
	S7	hi1034B	up to 22	19	4	15	3	6	5	40
	S28	hi3883	up to 23	25	7	18	6	6	6	33
	S29	hi2903	up to 22	18	3	15	4	4	4	27
	S18	hi1026	up to 24	59	13	46	9	12	8	26
	L23a	hi2582	up to 22.5	71	8	63	5	7	6	11
	L19	hi1987	up to 23	55	16	39	2	4	3	10
B	L12	hi3076	up to 23.5	24	2	22	2	3	0	14
	S12	hi1227	all at 22	14	1	13	0	1	0	8
	L9	hi1422	all at 22.5	23	8	15	0	1	0	7
	L3	hi2437	all at 23	19	2	17	0	1	0	6
	LP1	hi1444	up to 22.5	18	0	18	1	1	0	6
	L6	hi3655B	up to 24.5	42	1	41	0	2	0	5
	Sa	hi1479	up to 24.5	29	7	22	0	1	0	5
	L28	hi3893	up to 24.5	34	8	26	0	1	0	4
	L11	hi3820B	up to 23.5	29	2	27	1	1	0	4
	L24	hi1284	all at 26	18	0	18	0	0	0	0
	S15	hi2430	all at 23	16	0	16	0	0	0	0
C	NA	Colony	20 to 26	ND	ND	>5800	ND	~300	4	5

Figure 3.1: Polysome profiles reveal a ribosome integrity defect in *rp* homozygous mutant embryos

Cytoplasmic extracts from 3-day-old embryos were fractionated on sucrose gradients, and profiles were generated by measuring UV absorption at 280nm.

(A) Wild-type embryos display distinct peaks representing the small ribosomal subunit (40*S*), the large ribosomal subunit (60*S*), the monosome (80*S*) and multiple polysomes. The presence of higher-order polysomes indicates a high rate of translation.

(B) Homozygous mutants for *hi1974* (*rpS8*) display a loss of the 40*S* peak, a relative increase in the 60*S* peak, and a reduction in the 80*S* and polysome peaks.

(C) Homozygous mutants for *hi258* (*rpL35*) display a general reduction in all peaks. The decrease of the 60*S* peak is relatively most severe.

(D) Homozygous mutants for *hi1703* (*DNA polymerase ε, subunit B*) have a polysome profile similar to that of wild-type embryos.

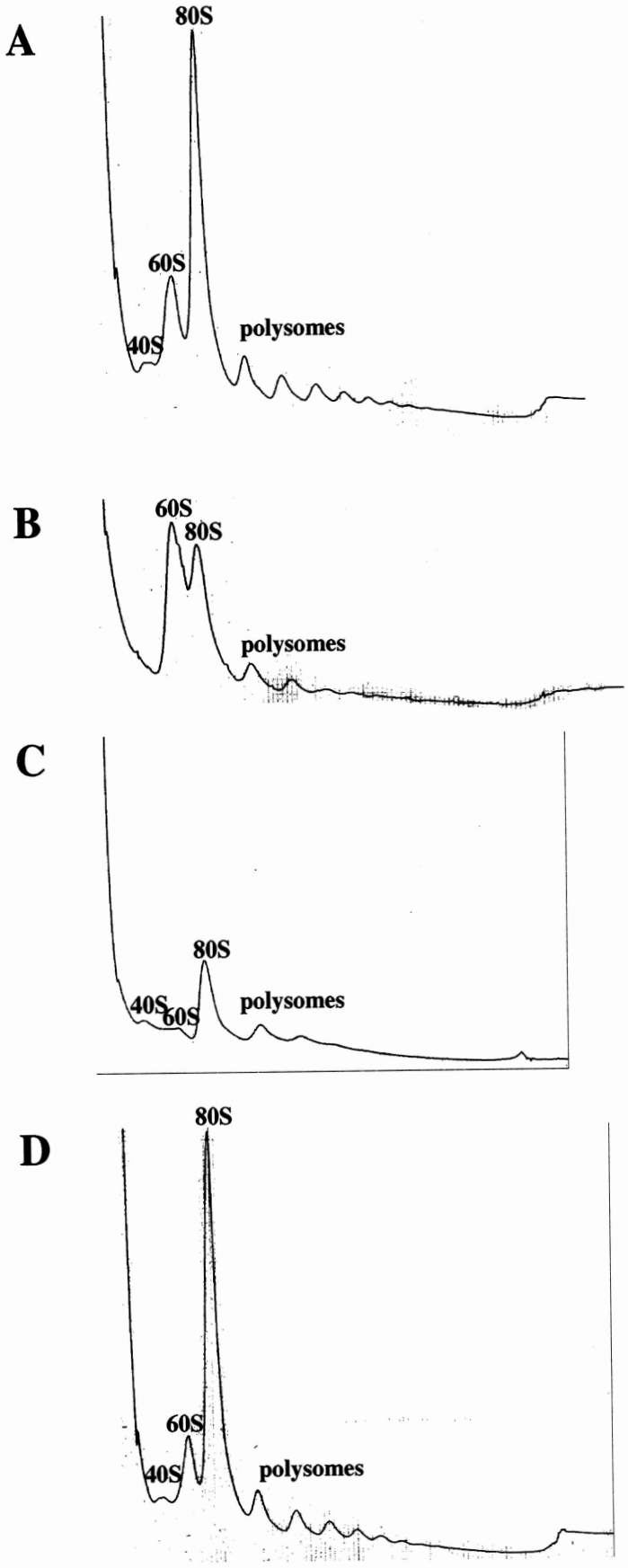

A
80S
60S
40S
polysomes
B
60S
80S
polysomes
C
80S
40S
60S
polysomes
D
80S
60S
40S
polysomes

Figure 3.2: Heterozygous mutant embryos from the tumor-prone *hi258* (*rpL35*) mutant line are growth-impaired.

(A) 122 embryos from an outcross of a *hi258* heterozygote were sorted into two groups by the presence or absence of a swim bladder at 4 days of age. The groups were further sorted into two groups on the basis of the size of the embryos. The embryos from the group of large, swim bladder-positive embryos were predicted to be wild-types ("W") and embryos from the group of small, swim bladder-negative embryos were predicted to be *hi258* heterozygotes ("H"). Individual embryos were genotyped by PCR revealing the presence or absence of the *hi258* transgenic (258 Tg) band. Primers for *Wnt* were used concurrently to control for the efficiency of the PCR. Genotyping ("Actual") revealed that the correct prediction was made in 56 of 64 cases (88%).

(B) Embryos from an outcross of a *hi258* heterozygote were sorted on the basis of swim bladder appearance and embryo size, as in (A), and pooled into groups of 100 embryos each. Genotyping PCR performed on a serial dilution of DNA from the predicted *hi258* heterozygotes ("Predicted HET") confirmed that this mixture of embryos was about 10 times more enriched for the *hi258* transgenic (258 Tg) band than the embryo mixture predicted to be comprised of mostly wild-type embryos ("Predicted WT"). Primers for *Wnt* were used concurrently to control for the efficiency of the PCR.

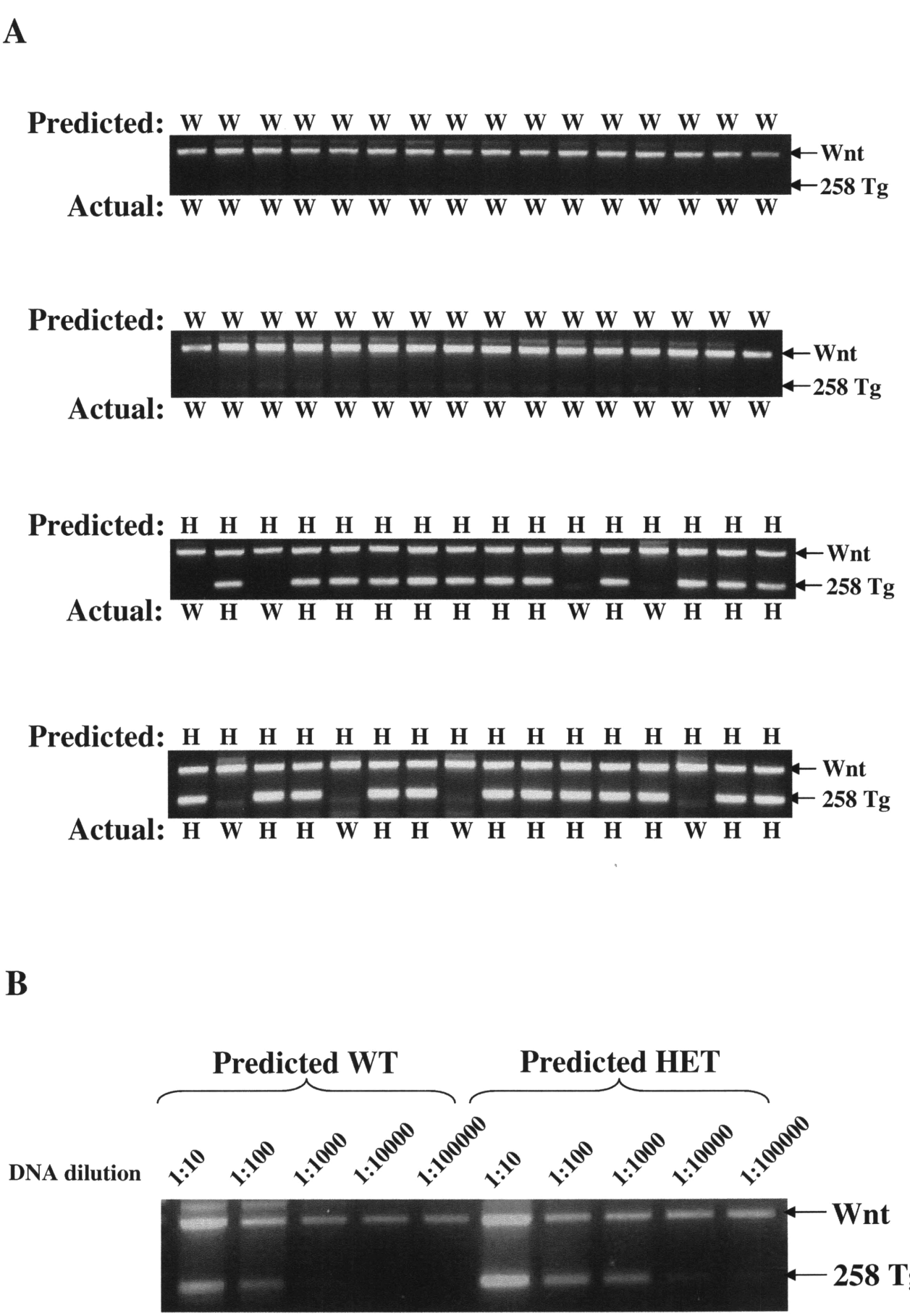

A
Predicted: W W W W W W W W W W W W W W W W
Wnt
258 Tg
Actual: W W W W W W W W W W W W W W W W
Predicted: W W W W W W W W W W W W W W W W
Wnt
258 Tg
Actual: W W W W W W W W W W W W W W W W
Predicted: H H H H H H H H H H H H H H H H
Wnt
258 Tg
Actual: W H W H H H H H H H W H W H H H
Predicted: H H H H H H H H H H H H H H H H
Wnt
258 Tg
Actual: H W H H W H H W H H H H H W H H
B
Predicted WT
Predicted HET
DNA dilution
1:10
1:100
1:1000
1:10000
1:100000
1:10
1:100
1:1000
1:10000
1:100000
Wnt
258 Tg

Figure 3.3: Heterozygous fish from the tumor-prone *hi258* (*rpL35*) mutant line are growth-impaired.

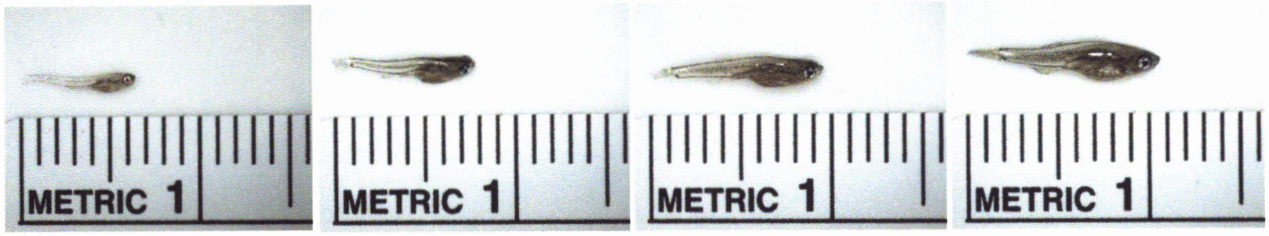
(A) The offspring of an outcross of a *hi258* (*rpL35*) heterozygote, expected to be 50% *hi258* heterzygous and 50% wild-type, are widely variable in size.

(B) *hi258* heterozygotes are generally smaller than their wild-type siblings.

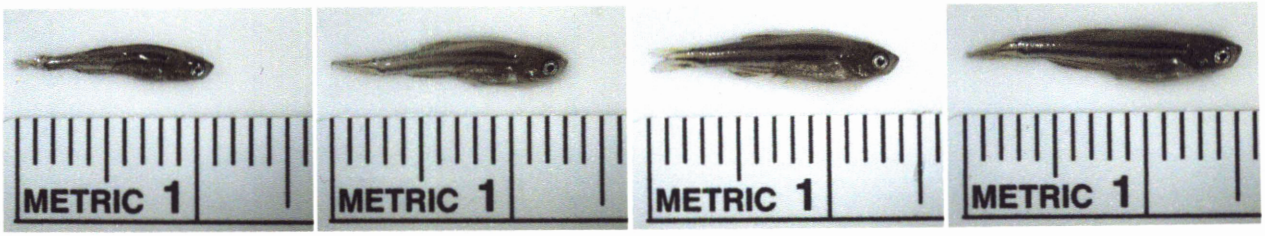
(C) *hi258* heterozygotes were outcrossed to T-AB wild-type fish, and their progeny were housed in the same tank. At selected time points, fish were weighed and genotyped. *hi258* heterozygotes are significantly smaller than their wild-type siblings at every age examined. Error bars are ±2 SEM.

A

B



0.0021g HET | 0.0034g HET | 0.0062g HET | 0.0087g WT



0.0118g HET | 0.0153g WT | 0.0205g WT | 0.0271g WT

C

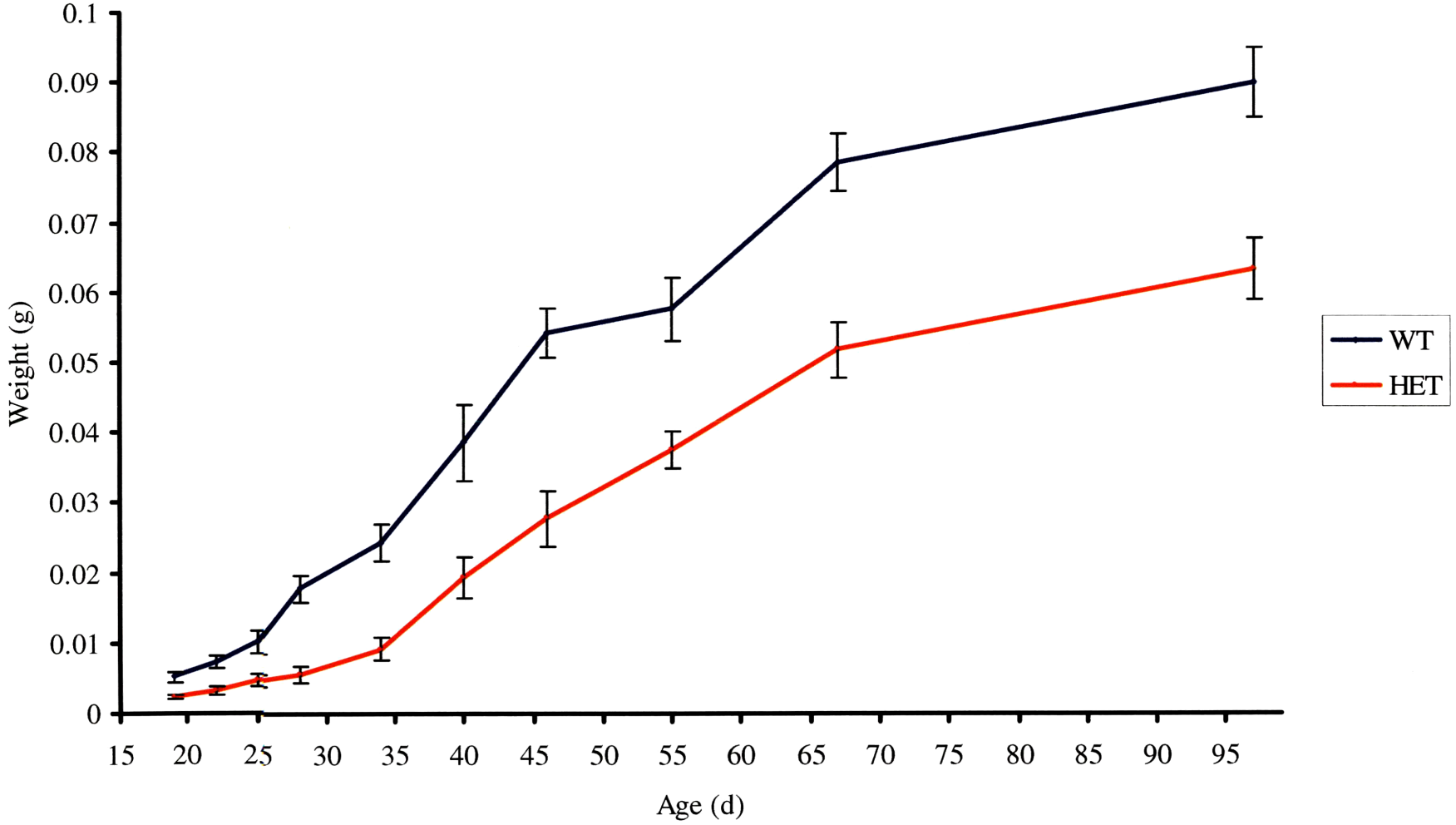
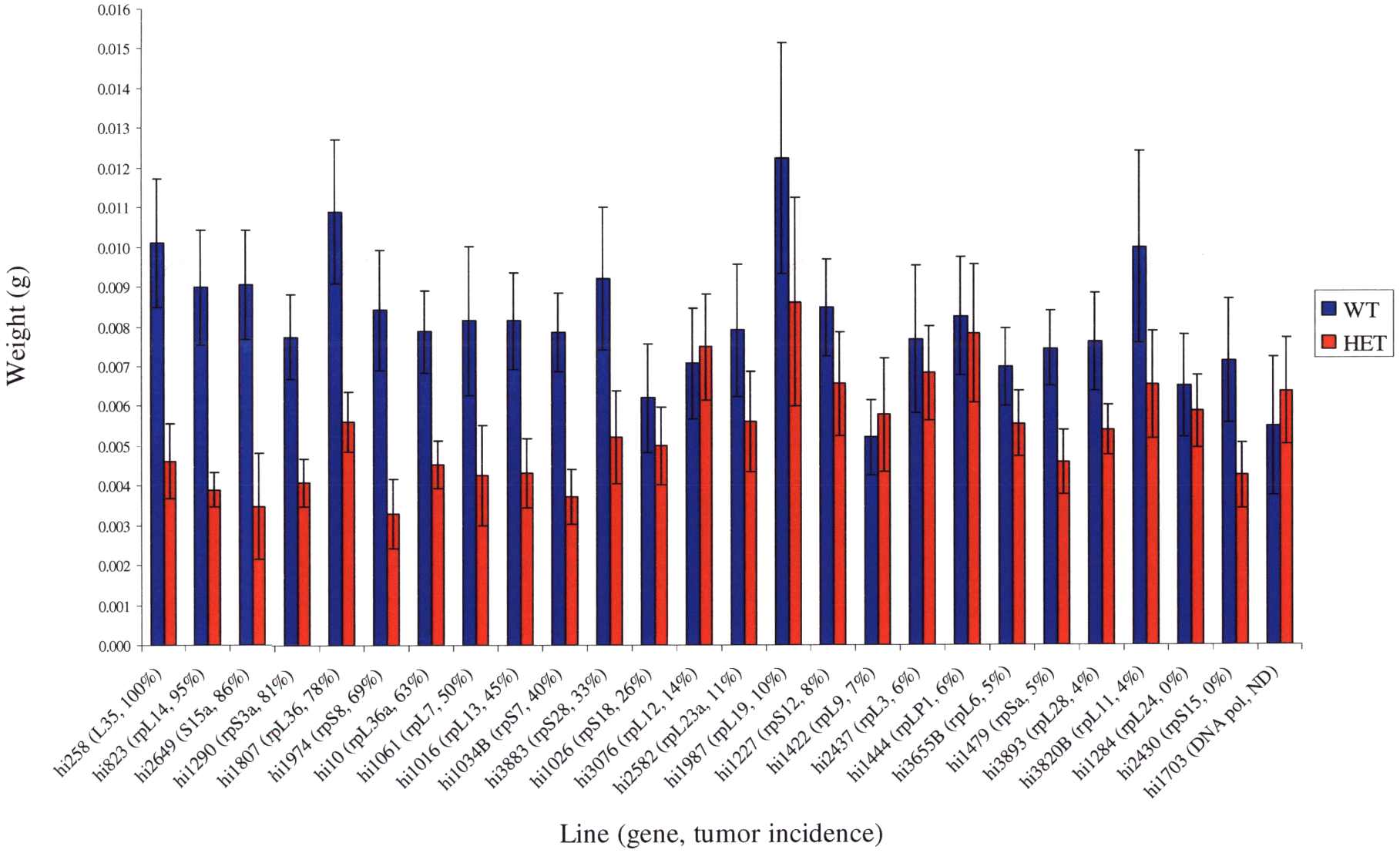

0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Weight (g)
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
Age (d)
WT
HET

Figure 3.4: Heterozygous fish from high-tumor *rp* mutant lines are growth-impaired

(A) Outcrosses of each *rp* line were performed, and their progeny were weighed and genotyped at day 25. Lines are ordered by decreasing tumor incidence, as determined in Table 3.1. The most highly tumor-prone lines appear to be the most severely growth-impaired. Error bars are ±2 SEM.

(B) The same data as (A), plotted with the average weights of the heterozygotes normalized to the average weight of their corresponding wild-type siblings. * indicates a significant difference, as shown in (A).

A


Weight (g)
0.016
0.015
0.014
0.013
0.012
0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
WT
HET
hi258 (L35, 100%)
hi823 (rpL14, 95%)
hi2649 (S15a, 86%)
hi1290 (rpS3a, 81%)
hi1807 (rpL36, 78%)
hi1974 (rpS8, 69%)
hi10 (rpL36a, 63%)
hi1061 (rpL7, 50%)
hi1016 (rpL13, 45%)
hi1034B (rpS7, 40%)
hi3883 (rpS28, 33%)
hi1026 (rpS18, 26%)
hi3076 (rpL12, 14%)
hi2582 (rpL23a, 11%)
hi1987 (rpL19, 10%)
hi1227 (rpS12, 8%)
hi1422 (rpL9, 7%)
hi2437 (rpL3, 6%)
hi1444 (rpLP1, 6%)
hi3655B (rpL6, 5%)
hi1479 (rpSa, 5%)
hi3893 (rpL28, 4%)
hi3820B (rpL11, 4%)
hi1284 (rpL24, 0%)
hi2430 (rpS15, 0%)
hi1703 (DNA pol, ND)
Line (gene, tumor incidence)

B

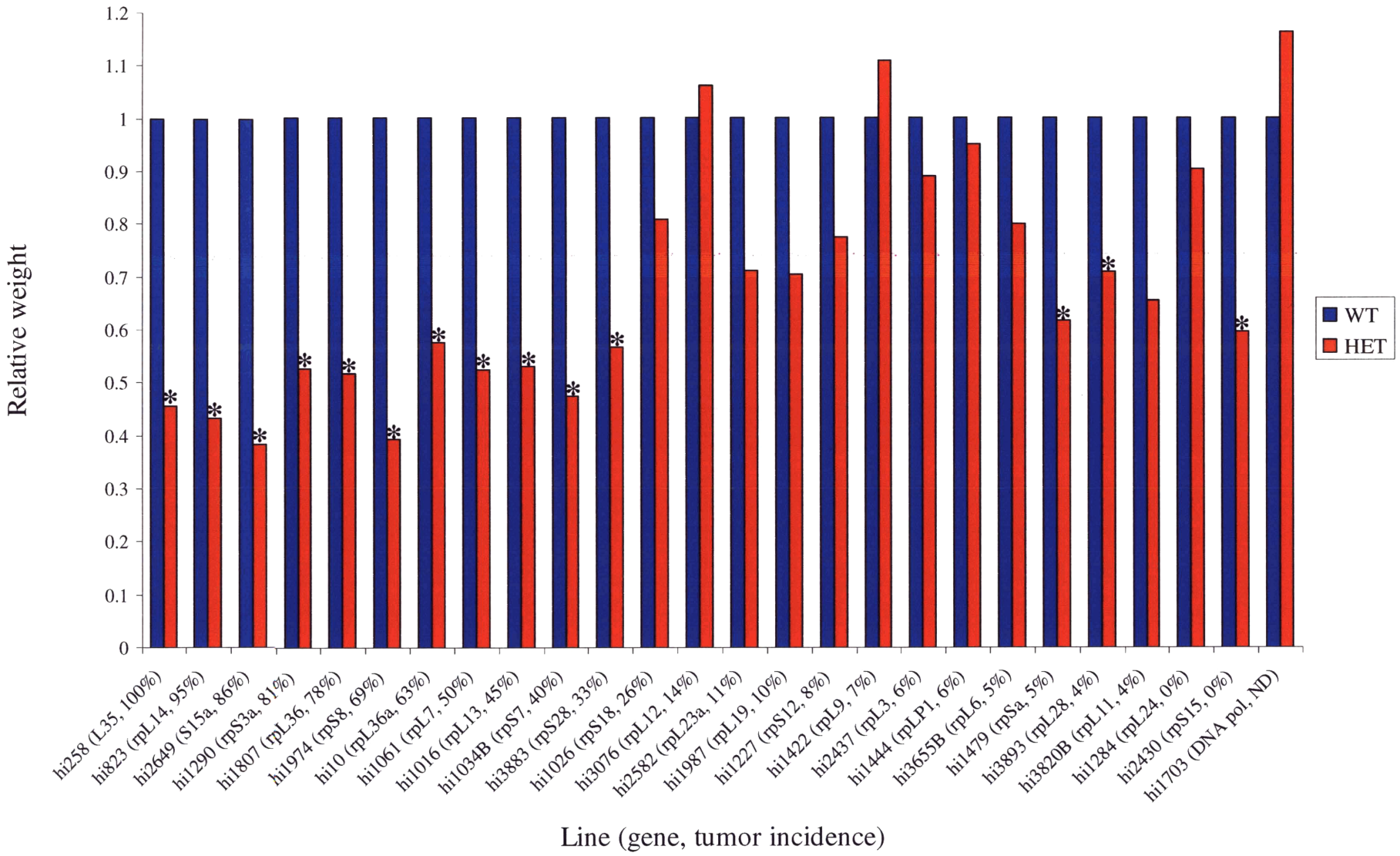

Relative weight
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
HET
hi258 (L35, 100%)
hi823 (rpL14, 95%)
hi2649 (S15a, 86%)
hi1290 (rpS3a, 81%)
hi1807 (rpL36, 78%)
hi1974 (rpS8, 69%)
hi10 (rpL36a, 63%)
hi1061 (rpL7, 50%)
hi1016 (rpL13, 45%)
hi1034B (rpS7, 40%)
hi3883 (rpS28, 33%)
hi1026 (rpS18, 26%)
hi3076 (rpL12, 14%)
hi2582 (rpL23a, 11%)
hi1987 (rpL19, 10%)
hi1227 (rpS12, 8%)
hi1422 (rpL9, 7%)
hi2437 (rpL3, 6%)
hi1444 (rpLP1, 6%)
hi3655B (rpL6, 5%)
hi1479 (rpSa, 5%)
hi3893 (rpL28, 4%)
hi3820B (rpL11, 4%)
hi1284 (rpL24, 0%)
hi2430 (rpS15, 0%)
hi1703 (DNA pol, ND)
Line (gene, tumor incidence)

Figure 3.5: Heterozygous mutation of an *rp* gene impairs growth at the cellular level

(A) Chimeric embryos were generated by injecting into wild-type hosts a mixture of cells isolated from the progeny of an intercross between *hi10* (*rpL36a*) and *hiX* heterozgyotes. *hi10* is a high-tumor *rp* line, and *hiX* is a line that bears a non-mutagenic insertion. The relative contribution of cells carrying each insertion was determined by real-time PCR at 5 days and 1 to 1.5 months post injection. In both cases, the cells bearing the *hiX* insertion comprise a greater portion of the embryo than cells bearing the *hi10* insertion.

(B) Cells from the progeny of a *hiX* outcross were injected into *hi10* (*rpL36a*) heterozygous or non-transgenic (ntg) host embryos. Contribution of cells carrying the *hiX* insertion was determined by real-time PCR at 5 days and 2 to 3 weeks post injection. The phenotypically wild-type *hiX* cells appear to be able to compete better in a *hi10* heterozygous host than a wild-type host.

A


3.00
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
hirpL36a | hiX
hirpL36a | hiX
5 days (n=38)
1-1.5 months (n=10)
donor cell genotype / age

B

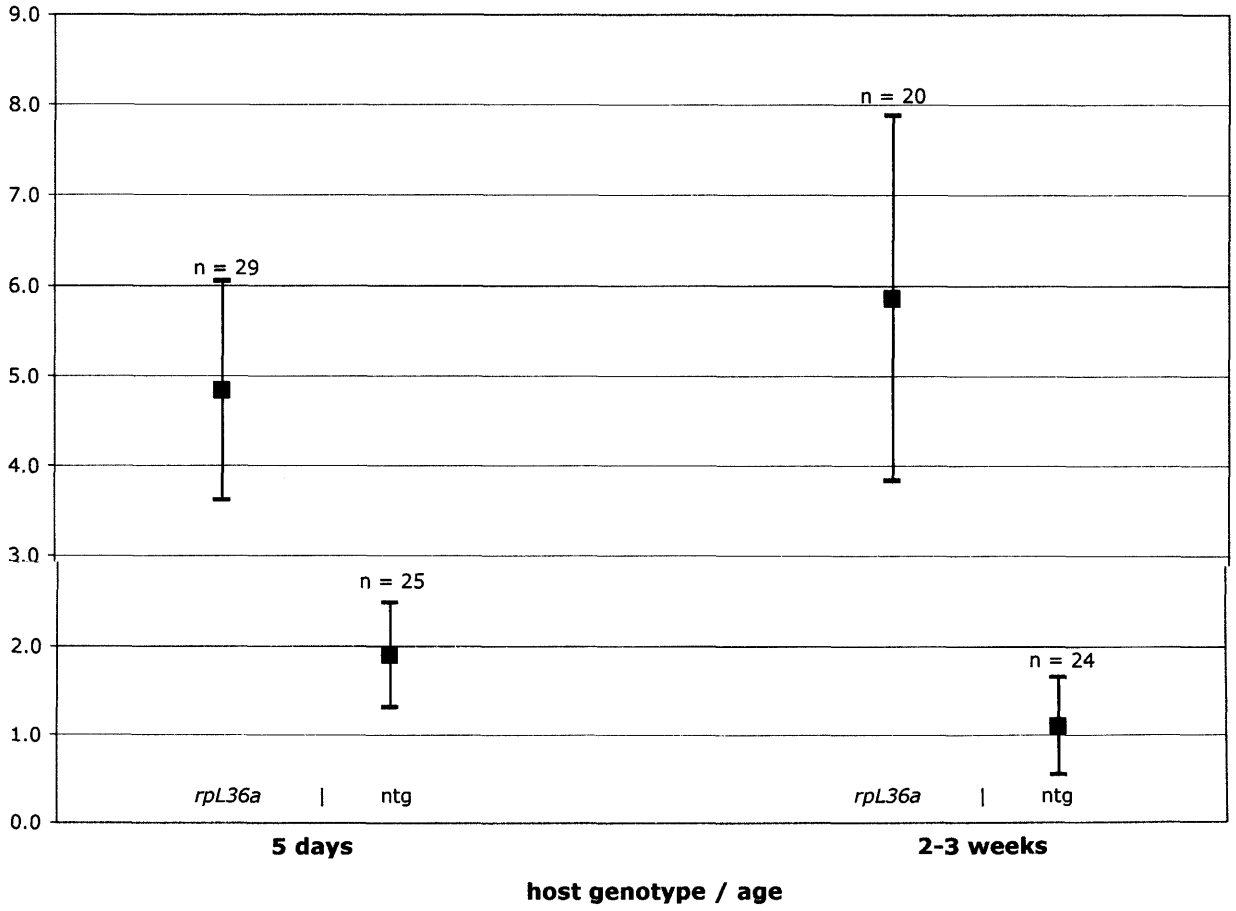

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
n = 29
n = 20
n = 25
n = 24
rpL36a | ntg
rpL36a | ntg
5 days
2-3 weeks
host genotype / age

Table 3.S1: Degree of *rp* message reduction in *rp* homozygous mutant embryos does not correlate with tumor incidence in adult *rp* heterozygotes.

Tumor incidence of adult *rp* heterozygotes was determined in Table 3.1. RNA was isolated from homozygous *rp* mutant embryos and their wild-type siblings. PCR was performed using serial dilutions of cDNA to detect the rp message and estimate the level of knockdown in the homozygous mutants compared to their wild-type siblings. The severity of the homozygous mutant phenotype was rated on a scale as follows:

+++ = severe defects, including very obvious brain necrosis on 1 dpf
++ = moderate defects, including some brain necrosis on 1 dpf
+ = mild defects: no visible necrosis on 1 dpf; mild necrosis and body curvature on 3 dpf
ND = not determined

Note that while the fold rp message reduction in *rp* homozygotes generally correlates with the severity of the homozygous mutant phenotype, it does not correlate with the tumor incidence of the adult *rp* heterozygotes.

Line	rp	Tumor incidence of adult heterozygotes (%)	Fold rp message reduction in *rp* homozygotes	Severity of homozygous mutant phenotype
hi2649	S15a	86	200	+++
hi1807	L36	78	10	+
hi1974	S8	69	20	++
hi10	L36a	63	30	++
hi577B	S5	52	50	ND
hi1061	L7	50	5	++
hi1034B	S7	40	10	++
hi2903	S29	27	200	+++
hi1444	LP1	6	3	+
hi1284	L24	0	5	+
hi2430	S15	0	2000	+++

References

Amsterdam, A., Burgess, S., Golling, G., Chen, W., Sun, Z., Townsend, K., Farrington, S., Haldi, M., and Hopkins, N. (1999). A large-scale insertional mutagenesis screen in zebrafish. Genes and Development *13*, 2713-2724.

Amsterdam, A., Sadler, K. C., Lai, K., Farrington, S., Bronson, R. T., Lees, J. A., and Hopkins, N. (2004). Many Ribosomal Protein Genes Are Cancer Genes in Zebrafish. Public Library of Science Biology *2*, 690-698.

Beuvink, I., Boulay, A., Fumagalli, S., Zilbermann, F., Ruetz, S., O'Reilly, T., Natt, F., Hall, J., Lane, H. A., and Thomas, G. (2005). The mTOR Inhibitor RAD001 Sensitizes Tumor Cells to DNA-Damaged Induced Apoptosis through Inhibition of p21 Translation. Cell *120*, 747-759.

Bilousova, G., Marusyk, A., Porter, C. C., Cardiff, R. D., and DeGregori, J. (2005). Impaired DNA Replication within Progenitor Cell Pools Promotes Leukemogenesis. PLoS Biology *3*, 2135-2147.

Clemens, M. J. (2004). Targets and mechanisms for the regulation of translation in malignant transformation. Oncogene *23*, 3180-3188.

Donzé, O., Jagus, R., Koromilas, A. E., Hershey, J. W. B., and Sonenberg, N. (1995). Abrogation of translation initiation factor eIF-2 phosphorylation causes malignant transformation of NIH 3T3 cells. The EMBO Journal *14*, 3828-3834.

Draptchinskaia, N., Gustavsson, P., Andersson, B., Pettersson, M., Willig, T.-N., Dianzani, I., Ball, S., Tchernia, G., Klar, J., Matsson, H., *et al.* (1999). The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia. Nature Genetics *21*, 169-175.

Fukuchi-Shimogori, T., Ishii, I., Kashiwagi, K., Mashiba, H., Ekimoto, H., and Igarashi, K. (1997). Malignant transformation by overproduction of translation initiation factor eIF4G. Cancer Research *57*, 5041-5044.

Lambertsson, A. (1998). The *Minute* Genes in *Drosophila* and Their Molecular Functions. Advances in Genetics *38*, 69-134.

Lazaris-Karatzas, A., Montine, K. S., and Sonenberg, N. (1990). Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. Nature *345*, 544-547.

Lin, S., Long, W., Chen, J., and Hopkins, N. (1992). Production of germ-line chimeras in zebrafish by cell transplants from genetically pigmented to albino embryos. Proceedings of the National Academy of Sciences *89*, 4519-4523.

Moore, J. L., Aros, M., Steudel, K. G., and Cheng, K. C. (2002). Fixation and decalcification of adult zebrafish for histological, immunocytochemical, and genotypic analysis. BioTechniques *32*, 300-304.

Oliver, E. R., Saunders, T. L., Tarlé, S. A., and Glaser, T. (2004). Ribosomal protein L24 defect in Belly spot and tail (*Bst*), a mouse *Minute*. Development *131*, 3907-3920.

Rajasekhar, V. K., Viale, A., Socci, N. D., Wiedmann, M., Hu, X., and Holland, E. C. (2003). Oncogenic Ras and Akt Signaling Contribute to Glioblastoma Formation by Differential Recruitment of Existing mRNAs to Polysomes. Molecular Cell *12*, 889-901.

Török, I., Herrmann-Horle, D., Kiss, I., Tick, G., Speer, G., Schmitt, R., and Mechler, B. M. (1999). Down-Regulation of RpS21, a Putative Translation Initiation Factor Interacting with P40, Produces Viable Minute Imagos and Larval Lethality with Overgrown Hematopoietic Organs and Imaginal Discs. Molecular and Cellular Biology *19*, 2308-2321.

Wasser, J. S., Yolken, R., Miller, D. R., and Diamond, L. (1978). Congenital Hypoplastic Anemia (Diamond-Blackfan Syndrome) Terminating in Acute Myelogenous Leukemia. Blood *51*, 991-995.

Watson, K. L., Konrad, K. D., Woods, D. F., and Bryant, P. J. (1992). *Drosophila* homolog of the human S6 ribosomal protein is required for tumor suppression in the hematopoietic system. Proceedings of the National Academy of Sciences *89*, 11302-11306.

Chapter 4

Overview & Discussion

1. Overview of Results

The preceding work has demonstrated the power of the zebrafish system in identifying novel cancer genes. A collection of over 500 zebrafish insertional mutant lines displaying homozygous embryonic lethal phenotypes was screened for cancer predisposition by observing the adult heterozygous carriers for evidence of external lumps. We reasoned that since most mammalian tumor suppressors identified to date are homozygous lethal (Jacks, 1996), this screen would yield novel tumor suppressors. Indeed, a new class of tumor suppressors, the ribosomal proteins (rps) was identified. Furthermore, the human relevance of the screen was validated by the fact that an *NF2* mutant line was also identified as tumor-prone. Mutation of this known tumor suppressor gene leads to tumors of the nervous system in human patients with neurofibromatosis type II, and mutation of its paralog in zebrafish leads to spindle-cell neoplasms resembling nerve sheath tumors. Tumors arising in human carriers of an *NF2* mutation are often a consequence of loss of the remaining wild-type allele (Ruttledge et al., 1994). This remains to be demonstrated in our zebrafish model. In contrast, we have definitively shown that loss of heterozygosity (LOH) does *not* occur in tumors arising in *rp* heterozygous lines. The *rp* mutations are loss-of-function mutations, as the homozygous mutants display a dramatic reduction in the mRNA encoding the rp. The consequence of this message reduction is a defect in ribosome integrity, as evidenced by aberrant polysome profiles and a reduction in abundance of the associated rRNA. Since loss-of-function mutations lead to cancer in the heterozygous adults, the *rp* genes are *bona fide* tumor suppressor genes. Loss of both copies of an *rp* gene, however, is expected to be cell lethal. Consistent with this, our finding that LOH does not occur in the tumors suggests that the *rp* genes are haploinsufficient.

The finding that many, but not all, *rp* heterozygous lines are tumor-prone led us to ask whether the high-tumor lines could be distinguished phenotypically from the non-tumor-prone lines, thereby providing a hint as to the mechanism of tumorigenesis. We found that a severe growth impairment was common to the most highly tumor-prone lines, though this defect was not sufficient for tumorigenesis in some lines. The growth defect was also detected at the cellular level for at least one high-tumor line, *hi10* (*rpL36a*). A likely explanation for this growth defect is a general impairment of protein translation, the cellular process common to all of the rps. While a translation defect was not detected at the level of the polysome profiles, we cannot rule out that a global defect in translation has more subtle effects, as discussed below. Even if a translation defect were demonstrated, we do not know whether this, in turn, directly causes tumors later in life. The correlation between growth impairment and tumor susceptibility, however, strongly suggests that there may be a novel mechanistic link between translation and cancer that has yet to be elucidated.

2. Ribosomal Proteins as Haploinsufficient Tumor Suppressors

The most significant and intriguing novel finding of this work has been the identification of many ribosomal proteins as haploinsufficient tumor suppressors in the zebrafish. The classic mechanism of loss of tumor suppression is based upon the "two-hit model" proposed by Alfred Knudsen in his analysis of the age of onset of hereditary and nonhereditary forms of human retinoblastoma (Knudsen, 1971). He postulated that both copies of a tumor suppressor gene must be mutated in order to initiate tumor formation. This was later demonstrated to be essentially true in the case of retinoblastoma upon the cloning and mutational analysis of *RB*, the gene mutated in the disease (Friend et al., 1986). Many other familial cancer syndromes also

adhere to this model: one mutated copy of a tumor suppressor gene is inherited, thus sensitizing the somatic cells of the carrier to tumor formation upon the second hit (Russo et al., 2000). LOH can occur as a result of chromosomal loss, mitotic recombination, deletion, or point mutation (Wijnhoven et al., 2001).

In recent years, there has been an abundance of evidence to suggest that loss of the second allele of many tumor suppressor genes is not necessary to promote tumorigenesis (Santarosa and Ashworth, 2004). For example, $p27^{Kip1}$, a cyclin-dependent kinase inhibitor that blocks cell proliferation, has been demonstrated to be haploinsufficient insufficient for tumor suppression (Fero et al., 1998). Other haploinsufficient tumor suppressors have been found to have a variety of functions, which may or may not be directly tied to control of cell proliferation. For example, several genes involved in DNA repair, such as *Blm* and *Fen1*, have been found to be haploinsufficient tumor suppressors, in that loss of one allele enhances intestinal tumor formation in *Apc* heterozygous mice (Goss et al., 2002; Kucherlapati et al., 2002). It is easy to imagine how an impairment of DNA repair might lead to a mutator phenotype that accelerates the progression of tumors initiated by the loss of other tumor suppressors. Several known tumor suppressors, such as p53 and ATM, have also been shown to be haploinsufficient (Spring et al., 2002; Venkatachalam et al., 1998). Although loss of heterozygosity of these genes, especially *p53*, is commonly associated with cancer, the reduced dosage in the presence of only one allele can also promote tumorigenesis. In these cases, an impaired induction of apoptosis in response to DNA damage may allow cells with mutations to survive and sustain further changes that promote their proliferation.

The emerging theme seems to be that dosage is critical for the tumor suppressive functions of certain genes. Since the ribosomal proteins are extremely abundant in the cell,

comprising 5 to 10% of cellular protein (Kenmochi et al., 1998), a reduction in the dosage of one rp is likely to have enormous repercussions that we are only beginning to understand. How this reduction of *rp* gene dosage leads to a loss of tumor suppression is unknown, but the answer may involve a control mechanism that integrates the signals for cell growth and cell proliferation. At this point, it is only possible to speculate on such a mechanism by analyzing our data in light of the published literature.

3. Possible Molecular Mechanisms of Tumorigenesis in Ribosomal Protein Mutants

There are two opposing hypotheses to explain the tumors arising from haploinsufficiency of certain rps, though it is possible that they are not mutually exclusive. First, because so many of our *rp* mutant lines are tumor-prone, one obvious possibility is that the tumorigenic effect is related to the role of these proteins in translation. For example, a general reduction in translational capacity might lead to the inefficient production of some key negative regulator of proliferation, or an alteration in the balance of positive and negative regulators that would lead to improper control of cell proliferation. At the other extreme is an alternative model that takes into account only the extraribosomal functions of the rps. As discussed in Chapter 1, these functions may include the regulation of cell growth and apoptosis (Wool, 1996); thus, reducing the gene dosage may disrupt these functions sufficiently to cause cancer. Since not all of our *rp* mutant lines are tumor-prone, it is possible that mutation of only the rps with such critical extraribosomal roles would lead to cancer.

An example of an extraribosomal function that is particularly relevant to cancer may be that of L5, L11, and L23, all of which have been shown to bind MDM2 (or its human homolog HDM2) and inhibit its function (Zhang and Zhang, 2005). Since MDM2 normally targets the

major tumor suppressor p53 for ubiquitination and destruction (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997), the involvement of some rps in potentially regulating this process may be a key to understanding the mechanism of tumorigenesis in the *rp* mutant zebrafish lines. The interaction of L5 with MDM2 has actually been known for well over a decade (Marechal et al., 1994), though hints as to the functional consequences of this interaction have only been revealed recently. Lohrum et al. were the first to report an interaction between L11 and HDM2 (Lohrum et al., 2003). They showed that, similar to the tumor suppressor ARF, L11 could inhibit HDM2 function, and overexpression of L11 in ARF-null U2OS cells could induce a p53-dependent cell cycle arrest. Furthermore, treatment of these cells with low levels of actinomycin D, an inhibitor of RNA polymerase I (and therefore ribosome biogenesis), enhanced the L11-HDM2 interaction and p53 stabilization (Lohrum et al., 2003). Thus, there may exist a p53-dependent checkpoint that results in cell cycle arrest in response to abnormalities in ribosome biogenesis.

Zhang et al. later reported similar results and mapped the interaction of L11 to the central domain of HDM2, a region of the protein that had not been previously assigned any functional significance (Zhang et al., 2003). The fact that ARF and L11 bind different regions of HDM2 may explain the fact that ARF is able to inhibit the nuclear export of HDM2 (Zhang and Xiong, 1999), whereas L11 is not. The same group later reported that L23 also binds the central region of HDM2, but to a site that is distinct from the L11 binding site (Jin et al., 2004). L23 was shown to have a role similar to that of L11 in regulating the stability of p53 via its binding of HDM2 (Jin et al., 2004). Interestingly, while inhibition of ribosome biogenesis with actinomycin D stabilizes the L23-HDM2 interaction, inhibition of general translation with pactamycin or cycloheximide does not, again suggesting that this pathway may be activated in

response to a defect in ribosome synthesis (Dai et al., 2004). Furthermore, the L23-HDM2 interaction is not affected by gamma-irradiation, suggesting that this response is independent of the p53 response induced by DNA damage (Dai et al., 2004). Coming full circle, L5 was also shown to bind the central domain of HDM2 and have functions similar to L11 and L23 (Dai and Lu, 2004). Furthermore, L5, L11, and L23 can all bind simultaneously to HDM2. (Dai and Lu, 2004; Dai et al., 2004).

Among the rp interactors of HDM2 identified thus far, the Hopkins insertional mutant collection contains only L11. As shown in Chapter 3, the *L11* heterozygous fish do not appear to be tumor-prone. At first glance, this appears to contradict the hypothesis that reduction of L11 levels should lead to reduced p53 stability, and therefore cancer. But, there are several ways that this apparent contradiction can be reconciled. First, there may be compensation for the loss of the *L11* allele at the transcriptional level in the *L11* heterozygotes. As demonstrated in the case of the *S19* heterozygous mouse, an increase in the mRNA encoding S19 leads to an S19 protein level that is similar to wild-type controls (Matsson et al., 2004; Matsson et al., 2006). The mechanism of this transcriptional regulation is as yet unknown. Thus, determining the levels of L11 message and protein in the *L11* heterozygous fish is an important experiment that has yet to be done. Furthermore, if this type of transcriptional regulation occurs with other *rp* genes, it may provide an explanation as to why such *rp* heterozygous zebrafish lines are not tumor-prone. Another possible explanation that needs to be investigated is that there may exist another compensatory mechanism in the *L11* heterozygous fish that boosts the levels of the other critical rps, L5 and L23. Since overexpression of each protein individually can stabilize p53, it is possible that upregulation of these rps is sufficient to compensate for the reduction in L11.

Another connection between a ribosomal protein and the p53 pathway was recently described by Takagi et al., who found that p53 translation is directly regulated by L26 (Takagi et al., 2005). Upon DNA damage, L26 was found to bind the 5' untranslated region of the p53 message and enhance its translation. Overexpression of L26 enhances p53 expression and siRNA knockdown of L26 blocks p53 induction after DNA damage (Takagi et al., 2005). The same study also found that nucleolin had the opposite effects. The authors propose that L26 may displace nucleolin from binding the stem loop structure in the p53 5' UTR, or may simply allow a bypass of this stem loop structure that normally impedes translation. The signal transduction pathway that leads to the preferential association of L26 has yet to be elucidated.

While the role of L5, L11, L23, and L26 in the p53 pathway is intriguing, one may wonder why mutation of any *other rps* would lead to cancer in zebrafish. The answer may lie in the fact that synthesis of ribosomal proteins is coordinately regulated at the level of translation, since the messages all contain TOP sequences, as discussed in Chapter 1 (Meyuhas, 2000). It is possible that certain rps with critical extraribosomal functions are normally produced in excess with respect to the other rps. A reduced level of one rp might then lead to a reduced level of all the rps, forcing all available rps to assemble into ribosomes to maintain adequate translational capacity, and fewer free rps to perform the extraribosomal functions. To put it another way, there may exist a threshold level of each rp that must be met for the proper functioning of the translational machinery and/or the proper execution of extraribosomal functions. Reduction of the level of a particular rp by about half in the *rp* heterozygous fish (assuming no transcriptional compensation) may be enough in some, but not all, cases to be below this threshold.

One piece of evidence that supports the involvement of p53 in the tumorigenesis of the *rp* mutant lines is that zebrafish *p53* mutants develop predominantly the same tumor type as the *rp*

heterozygotes, zMPNSTs (Berghmans et al., 2005). As demonstrated in Chapters 2 and 3, this tumor type is exceedingly rare in both the control population of adult zebrafish as well as in fish identified with external lumps. It is difficult to believe, then, that the predisposition of both *rp* and *p53* mutant fish to zMPNSTs is a mere coincidence. Furthermore, preliminary data from our laboratory suggest that the zMPNSTs arising in the *rp* heterozygotes have no point mutations in the *p53* gene (Alyson Wilbanks, unpublished results), suggesting that *p53* need not be mutated in *rp* mutant fish to induce tumor formation. In addition, p53 protein cannot be detected in the tumors, even though p53 message is present (Alyson Wilbanks and Kevin Lai, unpublished results), suggesting that p53 translation may be impaired. The connection between rps and p53 is currently being explored further by generating compound mutants in zebrafish and analyzing the time to tumor onset. If indeed the rps and p53 are acting in the same pathway, one might not expect to see an acceleration of tumorigenesis in the compound mutants. In addition, occupancy of the p53 message in polysomes should be analyzed more carefully in the *rp* heterozygotes and tumors, in order to investigate the status of p53 translation.

The possibility that p53 translation may be impaired could be indicative of a general phenomenon that occurs as a result of impaired translational capacity. This brings us back to one of the original hypotheses stated above, that a general reduction in translation could lead to the inefficient production of some key regulator of cell proliferation or cell cycle progression. Precedent for this model comes from an analysis of p21 translation in tumor cells. Specifically, Beuvink et al. showed that the rapamycin derivative, RAD001, sensitized tumor cells to apoptosis following DNA damage by inhibiting the translation of p21 (Beuvink et al., 2005). Since RAD001 inhibits mTOR, a reduction in global translation can result, as described in Chapter 1. The actual effect of RAD001 on global translation demonstrated by Beuvink et al.

was very subtle, but the p21 message was noticeably shifted into smaller polysomes upon RAD001 treatment. Coupled with the short half-life and low abundance of p21, this small shift was enough to reduce p21 protein levels significantly, thus inhibiting its induction upon treatment with the DNA-damaging agent cisplatin (Beuvink et al., 2005). There is at least one other published instance of translational control superseding control at the transcriptional level: Rajasekhar et al. found that, upon oncogenic signaling by *Ras* and *Akt*, the profile of messages associated with polysomes changes dramatically (Rajasekhar et al., 2003). That is, specific messages are recruited to the ribosome for translation. This effect happens much faster than any changes in the total cellular levels of the mRNAs, suggesting that altered translation of particular messages might play a key role in driving cellular transformation (Rajasekhar et al., 2003).

Thus, the mechanism of tumorigenesis in the *rp* heterozygous fish may involve both the role of the rps in translation *and* their extraribosomal functions. The finding that all high-tumor *rp* lines have a growth impairment suggests that a translation deficiency may be a primary defect of these fish that enables tumor formation later in life. However, since growth impairment is not sufficient for tumorigenesis in some *rp* mutant lines, other changes are likely to be involved. It is clear that the tumors are *not* impaired for translation, as abundant polysomes can be detected in the tumor tissue (Kevin Lai, unpublished results). Therefore, the general growth defect of the whole organism must have been "fixed" in a subset of cells that give rise to these tumors. In a background of impaired global translation, one can imagine that the cellular levels of a key regulator, such as p53, could be reduced. Since p53 itself is haploinsufficient, as discussed above, this could lead to an inability to induce apoptosis of cells that sustain mutations during the lifetime of the fish.

It is important to note that, though many rps have been assigned extraribosomal functions, it is unclear whether these functions are active in physiologically normal situations or only active in response to some stress. For example, the function may be triggered by an acute defect in translation or ribosome biogenesis. In the case of L5, L11, and L23, for example, it appears to be the latter. An acute impairment of ribosomal assembly, perhaps due to a scarcity of nutrients, may result in a transient overabundance of free rps, which could then perform functions outside of their normal role in the ribosome. In the tumor-prone *rp* heterozygous fish, it is possible that these acute stresses are the initiating events in tumorigenesis. The *rp* heterozygous fish may have a translation defect that results in the slower growth of the organism, but the fish appear otherwise normal, morphologically and histologically, before they develop tumors. Upon an acute stress, they may not be able to activate the extraribosomal functions that arrest the cell cycle, thus starting down the road towards tumorigenesis. The signaling pathways that lead to the activation of these functions are currently poorly understood. Considering the known key role that mTOR plays in integrating nutrient availability signals with cell growth, this should be an area of focus in defining these pathways.

4. The Future of the Zebrafish as a Gene Discovery Tool in Cancer Research

The discovery of a tumor suppressive role for ribosomal proteins in the zebrafish highlights the utility of this model organism in elucidating the molecular basis of cancer. While the current preferred cancer model, the mouse, has greatly increased our understanding of the molecular actions of known human tumor suppressors and oncoproteins, it has not been as successful at identifying novel cancer genes. Forward genetic screens are beginning to be performed in the mouse (Clark et al., 2004; Shima et al., 2003; Zohn et al., 2005), but such

screens are inherently more expensive and require more laboratory space than zebrafish screens. Since we and others have shown that the functions of known cancer genes are conserved in the zebrafish, the fish will continue to be a useful tool in identifying novel pathways in the development of human cancer.

The finding that many zebrafish insertional mutant lines with mutations in ribosomal protein genes are cancer prone was the result of a screen that identified adult fish with large external lumps. Although this finding confirmed the validity of our screening approach to identify novel cancer genes, it is possible that we missed a number of lines that are predisposed to tumor types that do not present as external lumps. The finding that the *NF2* mutant line was tumor-prone was the result of a focused effort to look for the tumor phenotype at the histological level, as we had expected this line to be tumor-prone based on the known tumor suppressive function of NF2 in humans. Although two *NF2* heterozygous fish had presented with lumps in our initial cancer screen, this was not an unusual number of tumor-bearing fish to find within one family, as it is not uncommon for two fish in the same family to each have a seminoma, for example. The NF2 tumors, however, turned out to be a much rarer tumor type resembling a nerve sheath tumor.

Thus, we are extending our effort to screen the Hopkins collection of zebrafish insertional mutants for other cancer genes, mutation of which predispose the fish to tumor types that are only detected histologically. To this end, we have already fixed over 10,000 adult fish at 22 to 26 months of age, similar to the age range in which the *rp* heterozygous lines were analyzed. This collection is comprised of approximately 15-30 fish from each of 465 lines, representing 335 gene loci. Approximately 5800 of these fish have been analyzed histologically thus far. We have already found at least one additional line that appears to be tumor-prone. Specifically,

heterogyzotes for a mutation in the *hagoromo* gene are predisposed to the development of neuroblastomas, a rare tumor type within our colony. The tumor phenotype has been confirmed to be present in fish bearing other alleles of this mutation in the Hopkins collection. *hagoromo* was one of the first mutants identified in the Hopkins screen and is one of the few lines in the collection with a dominant phenotype: heterozygous adults have aberrant stripe patterns. The cloned gene was found to encode an F-Box/WD40-repeat containing protein, though still very little is known about its function. Its homologs in other vertebrates appear to be required for pattern formation of various distinct structures (Kawakami et al., 2000). A direct link to cancer has not been previously described. It is as yet unknown whether the homozygotes, which are viable, are predisposed to tumors as well. This exciting finding obviously demonstrates the value of continuing to screen the Hopkins collection for other tumor-prone lines.

The utility of the zebrafish as a gene discovery tool can be further extended to screen for genes that modulate the cancer phenotype of mutants in known cancer genes. As demonstrated by Patton and colleagues, mutation of p53 enhances the tumorigenicity of oncogenic BRAF, inducing malignant melanoma, whereas expression of mutant BRAF alone induces only nevi formation (Patton et al., 2005). Similarly, in the mouse, mutation of *p53* cooperates with *NF1* mutation in the development of MPNSTs (Cichowski et al., 1999; Vogel et al., 1999). As cancer is known to be a disease that results from successive mutations conferring a growth advantage upon a subset of cells (Hanahan and Weinberg, 2000), it will be important to understand the order of these molecular events and the various combinations of acquired mutations that can lead to cancer. As new cancer models are generated in the zebrafish, they can be used as the starting background for chemical or insertional mutagenesis to identify suppressors or enhancers of the

cancer phenotype. For example, rps may impinge upon the p53 pathway in ways that are yet to be discovered, and performing screens in *p53*- or *rp*-mutant backgrounds may be enlightening.

References

Berghmans, S., Murphey, R. D., Wienholds, E., Neuberg, D., Kutok, J. L., Fletcher, C. D. M., Morris, J. P., Liu, T. X., Schulte-Merker, S., Kanki, J. P., *et al.* (2005). *tp53* mutant zebrafish develop malignant peripheral nerve sheath tumors. Proceedings of the National Academy of Sciences *102*, 407-412.

Beuvink, I., Boulay, A., Fumagalli, S., Zilbermann, F., Ruetz, S., O'Reilly, T., Natt, F., Hall, J., Lane, H. A., and Thomas, G. (2005). The mTOR Inhibitor RAD001 Sensitizes Tumor Cells to DNA-Damaged Induced Apoptosis through Inhibition of p21 Translation. Cell *120*, 747-759.

Cichowski, K., Shih, T. S., Schmitt, E., Santiago, S., Reilly, K., McLaughlin, M. E., Bronson, R. T., and Jacks, T. (1999). Mouse Models of Tumor Development in Neurofibromatosis Type 1. Science *286*, 2172-2176.

Clark, A. T., Firozi, K., and Justice, M. J. (2004). Mutations in a Novel Locus on Mouse Chromosome 11 Resulting in Male Infertility Associated with Defects in Microtubule Assembly and Sperm Tail Function. Biology of Reproduction *70*, 1317-1324.

Dai, M.-S., and Lu, H. (2004). Inhibition of MDM2-mediated p53 Ubiquitination and Degradation by Ribosomal Protein L5. The Journal of Biological Chemistry *279*, 44475-44482.

Dai, M.-S., Zeng, S. X., Jin, Y., Sun, X.-X., David, L., and Lu, H. (2004). Ribosomal Protein L23 Activates p53 by Inhibiting MDM2 Function in Response to Ribosomal Perturbation but Not to Translation Inhibition. Molecular and Cellular Biology *24*, 7654-7668.

Fero, M. L., Randel, E., Gurley, K. E., Roberts, J. M., and Kemp, C. J. (1998). The murine gene $p27^{Kip1}$ is haplo-insufficient for tumour suppression. Nature *396*, 177-180.

Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M., and Dryja, T. P. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature *323*, 643-646.

Goss, K. H., Risinger, M. A., Kordich, J. J., Sanz, M. M., Straughen, J. E., Slovek, L. E., Capobianco, A. J., German, J., Boivin, G. P., and Groden, J. (2002). Enhanced Tumor Formation in Mice Heterozygous for *Blm* Mutation. Science *297*, 2051-2053.

Hanahan, D., and Weinberg, R. A. (2000). The Hallmarks of Cancer. Cell *100*, 57-70.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature *387*, 296-299.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Letters *420*, 25-27.

Jacks, T. (1996). Tumor suppressor gene mutations in mice. Annual Review of Genetics *30*, 603-636.

Jin, A., Itahana, K., O'Keefe, K., and Zhang, Y. (2004). Inhibition of HDM2 and Activation of p53 by Ribosomal Protein L23. Molecular and Cellular Biology *24*, 7669-7680.

Kawakami, K., Amsterdam, A., Shimoda, N., Becker, T., Mugg, J., Shima, A., and Hopkins, N. (2000). Proviral insertions in the zebrafish *hagoromo* gene, encoding an F-box/WD40-repeat protein, cause stripe pattern anomalies. Current Biology *10*, 463-466.

Kenmochi, N., Kawaguchi, T., Rozen, S., Davis, E., Goodman, N., Hudson, T. J., Tanaka, T., and Page, D. C. (1998). A Map of 75 Human Ribosomal Protein Genes. Genome Research *8*, 509-523.

Knudsen, A. G. (1971). Mutation and Cancer: Statistical Study of Retinoblastoma. Proceedings of the National Academy of Sciences *68*, 820-823.

Kubbutat, M. H. G., Jones, S. N., and Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Kucherlapati, M., Yang, K., Kuraguchi, M., Zhao, J., Lia, M., Heyer, J., Kane, M. F., Fan, K., Russell, R., Brown, A. M. C., *et al.* (2002). Haploinsufficiency of Flap endonuclease (*Fen1*) leads to rapid tumor progression. Proceedings of the National Academy of Sciences *99*, 9924-9929.

Lohrum, M. A. E., Ludwig, R. L., Kubbutat, M. H. G., Hanlon, M., and Vousden, K. H. (2003). Regulation of HDM2 activity by the ribosomal protein L11. Cancer Cell *3*, 577-587.

Marechal, V., Elenbaas, B., Piette, J., Nicolas, J.-C., and Levine, A. J. (1994). The Ribosomal L5 Protein Is Associated with mdm-2 and mdm-2-p53 Complexes. Molecular and Cellular Biology *14*, 7414-7420.

Matsson, H., Davey, E. J., Draptchinskaia, N., Hamaguchi, I., Ooka, A., Levéen, P., Forsberg, E., Karlsson, S., and Dahl, N. (2004). Targeted Disruption of the Ribosomal Protein S19 Gene Is Lethal Prior to Implantation. Molecular and Cellular Biology *24*, 4032-4037.

Matsson, H., Davey, E. J., Fröjmark, A. S., Miyake, K., Utsugisawa, T., Flygare, J., Zahou, E., Byman, I., Landin, B., Ronquist, G., *et al.* (2006). Erythropoiesis in the *Rps19* disrupted mouse: Analysis of erythropoietin response and biochemical markers for Diamond-Blackfan anemia. Blood Cells, Molecules, and Diseases *36*, 259-264.

Meyuhas, O. (2000). Synthesis of the translational apparatus is regulated at the translational level. European Journal of Biochemistry *267*, 6321-6330.

Patton, E. E., Widlund, H. R., Kutok, J. L., Kopani, K. R., Amatruda, J. F., Murphey, R. D., Berghmans, S., Mayhall, E. A., Traver, D., Fletcher, C. D. M., *et al.* (2005). BRAF Mutations Are Sufficient to Promote Nevi Formation and Cooperate with p53 in the Genesis of Melanoma. Current Biology *15*, 249-254.

Rajasekhar, V. K., Viale, A., Socci, N. D., Wiedmann, M., Hu, X., and Holland, E. C. (2003). Oncogenic Ras and Akt Signaling Contribute to Glioblastoma Formation by Differential Recruitment of Existing mRNAs to Polysomes. Molecular Cell *12*, 889-901.

Russo, A., Zanna, I., Tubiolo, C., Migliavacca, M., Bazan, V., Latteri, M. A., Tomasino, R. M., and Gebbia, N. (2000). Hereditary common cancers: molecular and clinical genetics. Anticancer Research *20*, 4841-4851.

Ruttledge, M. H., Sarrazin, J., Rangaratnam, S., Phelan, C. M., Twist, E., Merel, P., Delattre, O., Thomas, G., Nordenskjöld, M., Collins, V. P., *et al.* (1994). Evidence for the complete inactivation of the NF2 gene in the majority of sporadic meningiomas. Nature Genetics *6*, 180-184.

Santarosa, M., and Ashworth, A. (2004). Haploinsufficiency for tumour suppressor genes: when you don't need to go all the way. Biochimica et Biophysica Acta *1654*, 105-122.

Shima, N., Hartford, S. A., Duffy, T., Wilson, L. A., Schimenti, K. J., and Schimenti, J. C. (2003). Phenotype-Based Identification of Mouse Chromosome Instability Mutants. Genetics *163*, 1031-1040.

Spring, K., Ahangari, F., Scott, S. P., Waring, P., Purdie, D. M., Chen, P. C., Hourigan, K., Ramsay, J., McKinnon, P. J., Swift, M., and Lavin, M. F. (2002). Mice heterozygous for mutation in *Atm*, the gene involved in ataxia-telangiectasia, have heightened susceptibility to cancer. Nature Genetics *32*, 185-190.

Takagi, M., Absalon, M. J., McLure, K. G., and Kastan, M. B. (2005). Regulation of p53 Translation and Induction after DNA Damage by Ribosomal Protein L26 and Nucleolin. Cell *123*, 49-63.

Venkatachalam, S., Shi, Y.-P., Jones, S. N., Vogel, H., Bradley, A., Pinkel, D., and Donehower, L. A. (1998). Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. The EMBO Journal *17*, 4657-4667.

Vogel, K. S., Klesse, L. J., Velasco-Miguel, S., Meyers, K., Rushing, E. J., and Parada, L. F. (1999). Mouse Tumor Model for Neurofibromatosis Type 1. Science *286*, 2176-2179.

Wijnhoven, S. W. P., Kool, H. J. M., Teijlingen, C. M. M. v., Zeeland, A. A. v., and Vrieling, H. (2001). Loss of heterozygosity in somatic cells of the mouse: An important step in cancer initiation? Mutation Research *473*, 23-36.

Wool, I. G. (1996). Extraribosomal functions of ribosomal proteins. Trends in Biochemical Sciences *21*, 164-165.

Zhang, Y., Wolf, G. W., Bhat, K., Jin, A., Allio, T., Burkhart, W. A., and Xiong, Y. (2003). Ribosomal Protein L11 Negatively Regulates Oncoprotein MDM2 and Mediates a p53-Dependent Ribosomal-Stress Checkpoint Pathway. Molecular and Cellular Biology *23*, 8902-8912.

Zhang, Y., and Xiong, Y. (1999). Mutations in Human ARF Exon 2 Disrupt Its Nucleolar Localization and Impair Its Ability to Block Nuclear Export of MDM2 and p53. Molecular Cell *3*, 579-591.

Zhang, Z., and Zhang, R. (2005). p53-Independent Activities of MDM2 and Their Relevance to Cancer Therapy. Current Cancer Drug Targets *5*, 9-20.

Zohn, I. E., Anderson, K. V., and Niswander, L. (2005). Using genomewide mutagenesis screens to identify the genes required for neural tube closure in the mouse. Birth Defects Research Part A: Clinical and Molecular Teratology *73*, 583-590.

Appendix A

Polysome Arrays

Kevin Lai
Nancy Hopkins and Jacqueline A. Lees

Preface

This appendix provides additional data not shown in Chapter 3. It also outlines an experiment in progress that follows logically from the results presented in Chapter 3. The proposed experiment is designed to determine definitively whether the tumor-prone heterozygous *rp* mutant fish have a defect in translation that underlies their growth impairment.

Results & Discussion

It is well-accepted that a decrease in global protein synthesis can lead to growth impairment, as in the case of the *Drosophila Minute* mutants that have lost one copy of a ribosomal protein (Lambertsson, 1998). We aimed to show that this is true in the case of *rp* heterozygous zebrafish, and reasoned that polysome profiling would yield a definitive answer. In the homozygous *rp* mutant embryos, the translation defect was very obvious from the polysome profiles (Figure 3.1). When we analyzed mixtures of embryos highly enriched for *rp* heterozygotes, however, the shape of the profile was very similar to that of a wild-type-enriched sample (Figure A.1). The observed difference in the overall height of the peaks was likely due to the fact that the heterozygote-enriched sample had less starting material since the embryos were smaller (Figure A.1). Importantly, there was no difference in the number of higher-order polysomes detected, which is a measure of translational capacity. As discussed in Chapter 3, a global translation defect can be manifested in a very subtle manner in a polysome profile, and it is possible that the translation phenotype in the fish cannot be detected by this method.

One possibility may be that the translation defect in the *rp* heterozygotes only affects the selection of messages that are translated. There is precedent for this mechanism in the literature, as described previously (Beuvink et al., 2005; Rajasekhar et al., 2003). To test this hypothesis,

we are performing microarray analysis of mRNAs isolated from polysome fractionated samples. The experimental plan is outlined in Figure A.2. We are again using wild-type-enriched and *hi258* heterozygote-enriched embryo mixtures since we are able to isolate polysomes from them easily. We also tried using individual 34-day-old fish, the advantage of which was having a pure heterozygous or wild-type sample since we were able to take a tail biopsy for genotyping before performing the polysome fractionation. Unfortunately, since the overall rate of translation is much lower at this point than in 4-day-old embryos, the profiles were dominated by the monosome peak, and there were too few higher-order polysome peaks to be able to isolate a useful amount of RNA (data not shown). Thus, we proceeded with the wild-type-enriched and the *hi258* heterozygote-enriched embryo mixtures, pooling the monosome fractions and polysome fractions separately. The associated mRNAs were then labeled and hybridized to a zebrafish genome array containing nearly 15,000 transcripts.

While these experiments are in progress, it is useful to provide a brief analysis of the expected outcomes. It will be of interest to determine the identity of messages that are preferentially associated with either the monosome or the polysome fractions in the heterozygotes, and how this distribution is different from the wild-types. It is commonly accepted that, unless stalling of the ribosome on the mRNA occurs, messages associated with polysomes are translated at a higher rate than those associated with the monosome fraction. If there is a general defect in translation in the *rp* heterozygotes, we might expect to find a shift of messages that are normally polysome-bound in the wild-type to be in the monosome fraction of the heterozygotes. That is, the heterozygotes may be using most of the available translational capacity to synthesize proteins that are required in abundance. Perhaps, then, the messages that are normally in the monosome fraction of the wild-types would be absent completely from the

ribosomes of the heterozygotes. The messages in the heterozygote polysomes would then encode only the most extremely abundant proteins in the cell. Obviously, if some key protein such as p53 were not made in sufficient quantities, this translation defect would predispose the fish to tumorigenesis.

Alternatively, a more selective shift in polysome occupation of messages might be detected in the heterozygotes. Translation of certain messages is known to be regulated by sequence elements in the 5' untranslated region. For example, messages encoding all ribosomal proteins and many other factors involved in translation have a 5'-terminal oligopyrimidine motif that allows them to be preferentially translated under conditions favoring growth (Meyuhas, 2000). In addition, the secondary structure of the 5' UTR of many messages is a strong determinant of the efficiency of their translation. For example, messages encoding growth-promoting proteins such as cyclin D1, c-myc, and VEGF have 5' UTRs with complex secondary structures, making them less competitive for translation than other messages with simpler 5' UTR structures (Graff and Zimmer, 2003; Koromilas et al., 1992). A defect in translation in the *rp* heterozygotes might make the cells less sensitive to the signals that cue the translation of particular subsets of messages, resulting in an abnormal balance of proteins that may, for example, regulate cell proliferation.

The polysome arrays may reveal a subset of proteins that are aberrantly translated in the *rp* heterozygotes, which may provide a clue as to which pathways are most affected in these fish. Meanwhile, a candidate approach can be taken to determine whether certain messages are impaired for translation in the heterozygotes by performing RT-PCR for the selected message on the same RNA samples that were isolated for the microarrays. These experiments may therefore

provide evidence of a translation impairment in the *rp* heterozygotes that only affects certain messages selectively.

Materials & Methods

Sorting of rp Heterozygous Embryos and Polysome Microarrays

Adult fish heterozygous for the *hi258* insertion were outcrossed to non-transgenic T-AB fish. Embryos were sorted at 4 dpf by the presence or absence of a swim bladder. These latter groups were subsequently sorted into two further groups based on the size of the embryos. Polysome fractionation was performed on the wild-type-enriched and heterozygote-enriched mixtures as described in Chapter 3. Monosome and polysome fractions were pooled separately, and RNA was extracted from the fractions with Trizol LS (Invitrogen, Cat. No. 10296-010) following the manufacturer's modified protocol for samples contaminated with polysaccharides. RNA was checked for integrity and concentration with an Agilent BioAnalyzer, and labeled and hybridized to Affymetrix GeneChip Zebrafish Genome Arrays containing oligonucleotide probe sets for over 14,900 zebrafish transcripts.

Acknowledgements

We thank Christian Petersen and Philip Sharp for helpful advice and equipment for polysome fractionation analysis. We thank Manlin Luo of the MIT BioMicro Center for labeling and hybridizing RNA samples to microarrays. We thank Charlie Whittaker for analysis of microarray data and helpful advice.

Figure A.1: Polysome profiles do not reveal an overt translation defect in *hi258* (*rpL35*) heterozygous embryos.

(A) A polysome profile was generated from an embryo mixture enriched in wild-type embryos with possible contamination of ~10% *hi258* (*rpL35*) heterozygotes.

(B) A polysome profile of an embryo mixture enriched in *hi258* (*rpL35*) heterozygotes with possible contamination of ~10% wild-type embryos appears very similar to the wild-type-enriched embryo mixture.

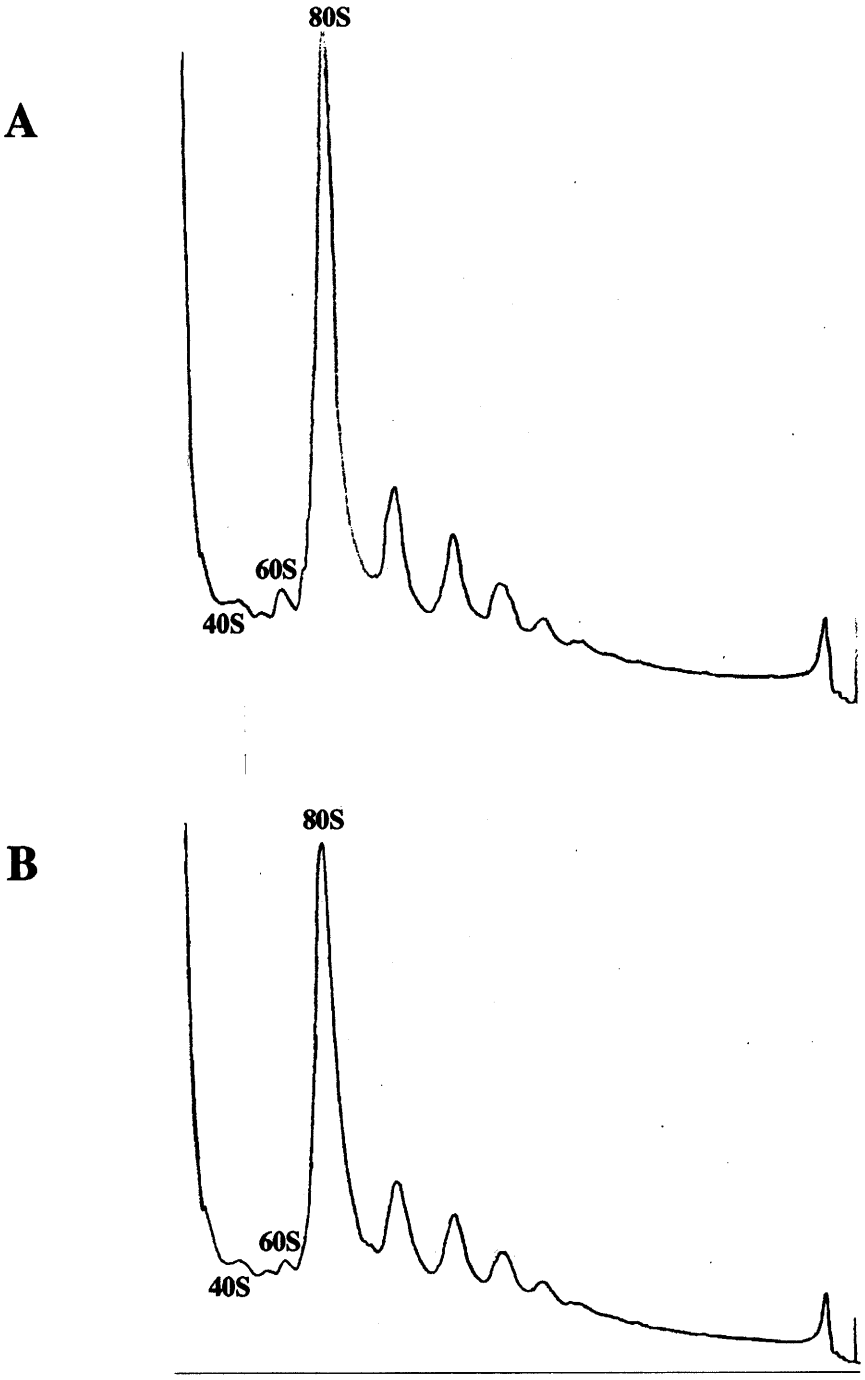
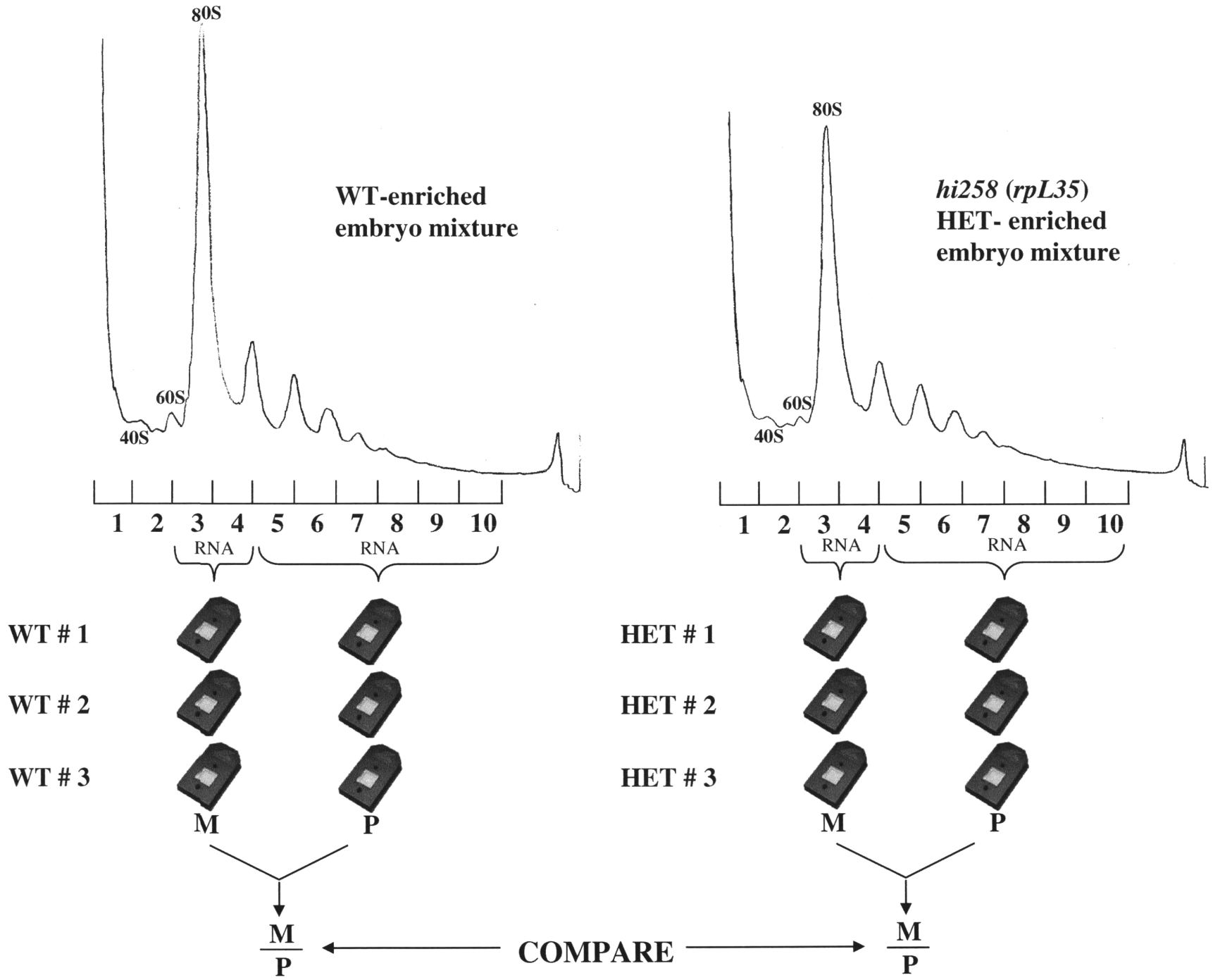

A
80S
60S
40S
B
80S
60S
40S

Figure A.2: An experimental scheme to compare the identity of messages translated in *rp* heterozygous fish versus their wild-type siblings.

Polysome fractionation is performed on mixtures enriched in wild-type or *hi258* (*rpL35*) heterozygous embryos. The monosome fractions (Fractions 3 and 4) are collected and pooled. Likewise, the polysome fractions (Fractions 5 – 10) are collected and pooled separately. RNA is isolated from each pool, labeled, and hybridized on a zebrafish genome microarray. The absolute level of each message will be averaged from triplicate experiments. The ratio of message level in the monosome fractions ("M") to the level in the polysome fractions ("P") will be calculated, and this ratio will be compared between the wild-type and heterozygote samples for each gene. In this manner, we may determine whether certain messages are, for example, enriched in polysomes in the wild-types, but enriched in monosomes of the heterozygotes.


80S
60S
40S
WT-enriched
embryo mixture
1 2 3 4 5 6 7 8 9 10
RNA
RNA
80S
60S
40S
hi258 (rpL35)
HET- enriched
embryo mixture
1 2 3 4 5 6 7 8 9 10
RNA
RNA
WT # 1
WT # 2
WT # 3
HET # 1
HET # 2
HET # 3
M
P
M
P
M
P
COMPARE
M
P

References

Beuvink, I., Boulay, A., Fumagalli, S., Zilbermann, F., Ruetz, S., O'Reilly, T., Natt, F., Hall, J., Lane, H. A., and Thomas, G. (2005). The mTOR Inhibitor RAD001 Sensitizes Tumor Cells to DNA-Damaged Induced Apoptosis through Inhibition of p21 Translation. Cell *120*, 747-759.

Graff, J. R., and Zimmer, S. G. (2003). Translational control and metastatic progression: Enhanced activity of the mRNA cap-binding protein eIF-4E selectively enhances translation of metastasis-related mRNAs. Clinical and Experimental Metastasis *20*, 265-273.

Koromilas, A. E., Lazaris-Karatzas, A., and Sonenberg, N. (1992). mRNAs containing extensive secondary structure in their 5' non-coding region translate efficiently in cells overexpressing initiation factor elF-4E. The EMBO Journal *11*, 4153-4158.

Lambertsson, A. (1998). The *Minute* Genes in *Drosophila* and Their Molecular Functions. Advances in Genetics *38*, 69-134.

Meyuhas, O. (2000). Synthesis of the translational apparatus is regulated at the translational level. European Journal of Biochemistry *267*, 6321-6330.

Rajasekhar, V. K., Viale, A., Socci, N. D., Wiedmann, M., Hu, X., and Holland, E. C. (2003). Oncogenic Ras and Akt Signaling Contribute to Glioblastoma Formation by Differential Recruitment of Existing mRNAs to Polysomes. Molecular Cell *12*, 889-901.

Appendix B

A Screen for Cell Cycle Genes in the Zebrafish

Kevin Lai
Nancy Hopkins and Jacqueline A. Lees

Introduction

The Hopkins collection of insertional mutant zebrafish lines (Amsterdam et al., 2004) represents a unique resource that can be re-screened for genes involved in specific developmental pathways or cellular processes. This collection is distinct from those generated by chemical mutagenesis (Driever et al., 1996; Haffter et al., 1996) because approximately two-thirds of the insertional mutants have non-specific phenotypes, including widespread necrosis, smaller head and eyes, and malformed bodies. Mutants with such phenotypes were discarded from the chemical screens due to the assumption that many of the mutated genes would be "housekeeping" genes, and the effort required to positionally clone such genes was deemed too time-consuming, considering the expected result. The relative ease of cloning genes disrupted by viral insertions has allowed the Hopkins laboratory to maintain all such "non-specific" mutants. The assumption that these would generally be housekeeping genes turned out to be correct, with genes encoding DNA polymerases, translational machinery components, etc., among them. Amongst this set, however, were genes that were of particular interest to us: known cell cycle regulators, such as cyclin B1 and aurora B kinase (Amsterdam et al., 2004). Importantly, another feature of the Hopkins collection is that 20% of the genes were novel or poorly characterized at the time that they were cloned. Thus, there was a possibility that amongst these novel genes were novel regulators of the cell cycle.

In order to screen for such cell cycle genes, assays commonly used in mouse and human cell culture studies were adapted for use in zebrafish embryos. We settled upon using an antibody against phosphorylated histone H3, which labels cells in G2/M phase of the cell cycle. More specifically, histone H3 is phosphorylated on serine 10 during late G2 and becomes dephosphorylated at anaphase of mitosis (Crosio et al., 2002). The sensitivity of this screen was

verified by staining known cell cycle mutants in the Hopkins collection, and a pilot screen was performed on several mutants, including some with insertions in novel genes. A postdoctoral fellow, Christopher Sansam, has since taken the lead on this screen and also added a treatment with gamma radiation to screen in parallel for mutants with a cell cycle checkpoint defect. As this latter work will be the subject of an upcoming publication, only the efforts in setting up the initial screen will be described here.

Results

Assay Development: BrdU Incorporation

The first goal of this cell cycle screen was to optimize specific and robust assays that could be used to screen a large number of mutant lines efficiently. Embryos were successfully stained with an antibody against proliferating cell nuclear antigen (PCNA) to label cells in S phase (data not shown). Similarly, bromodeoxyuridine (BrdU) incorporation was also successful in displaying the cells of the embryo that had gone through S phase (Figure B.1). The results of the BrdU incorporation assay in wild-type embryos are consistent with what is expected: At 24 hpf, the level of staining is very high, especially in the head and central nervous system (Figures B.1A-C). As the embryo ages, a smaller proportion of the cells are proliferating (Figures B.1D-I). By 96 hpf (Figures B.1J-L), BrdU incorporation is relatively low, except in the region of the arches and internal organs, which are undergoing rapid development at this time (Kimmel et al., 1995). These results indicate that the BrdU incorporation assay produces an accurate profile of the proliferation status of all cells within the whole wild-type embryo.

When the BrdU incorporation assay was performed on mutants, however, the staining was generally poor (data not shown). The tested mutants were generally those that had non-

specific defects such as general necrosis, since this subset of mutants would be of particular interest in an eventual cell cycle screen. Among the offspring of heterozygous intercrosses, the phenotypically wild-type embryos incorporated BrdU quite well, similar to the levels shown in Figure B.1; however, BrdU-positive staining was barely detectable, if not completely absent, among the homozygous mutant embryos. This lack of BrdU incorporation was observed in most such non-specific mutants, regardless of whether the mutated gene encoded a known cell cycle regulator or a protein with a housekeeping function. Therefore, this assay was deemed too non-selective to be used in a cell cycle screen.

An Antibody Stain for Phosphorylated Histone H3 Is Suitable for Use in Screening

Another assay, whole-mount immunostaining for phosphorylated histone H3 (pH3), proved to be more selective. Since the anti-pH3 antibody specifically labels cells in G2/M phase of the cell cycle, a smaller proportion of the cells in the embryo is stained, compared to BrdU incorporation (Figures B.2A and B). The embryos were stained between 24 and 36 hpf since the number of pH3-positive cells in wild-type embryos is optimal during this time, allowing easy detection of both an increase or a decrease in staining. Similar to the BrdU incorporation assay, the number of pH3 positive cells decreases progressively at 48, 72, and 96 hpf (data not shown). The pH3 staining assay was then tested on several known cell cycle mutants in the Hopkins collection, in order to demonstrate that the assay yields the expected results. For example, embryos that are homozygous mutant for cyclin B1 have a markedly reduced level of pH3 staining (Figures B.2C and D). This is to be expected, since this mitotic cyclin is required for entry into mitosis (Ohi and Gould, 1999). Similarly, mutants in aurora B kinase show a dramatic reduction in pH3 staining (Figures B.2E and F), again as expected since histone H3 is known to

be a target of this kinase (Crosio et al., 2002). In contrast, embryos mutant for polo-like kinase display an increase of pH3-positive cells (Figures B.2G and H). Mutations of this kinase in other organisms lead to mitotic arrest (Descombes and Nigg, 1998; Llamazares et al., 1991), which is consistent with the observed increase in pH3 staining.

A Pilot Cell Cycle Screen Using an Anti-Phosphorylated Histone H3 Antibody

Since the pH3 staining assay proved robust and reasonably sensitive, a pilot screen was performed with selected mutants from the Hopkins collection. This initial set consisted of 49 clutches of about 30-60 embryos, each generated from a mating of heterozygous adults. The clutches were coded and screened blindly for differences in pH3 staining in a quarter of the embryos in each clutch. Among the selected set of mutants were some known cell cycle genes as positive controls, other known genes with non-specific/necrosis phenotypes, and some novel or poorly characterized genes. In some cases, there were multiple clutches from the same family or from another allele of the same mutant. The screen was done in collaboration with James Amatruda and Kathleen Pfaff from the laboratory of Leonard Zon, who were performing a similar pH3 screen at the time. We screened each clutch independently, assigning a call of an increase, a decrease, or no difference in pH3 staining in the mutant embryos. As shown in Table B.1, the calls were generally consistent among the three of us, and also consistent among clutches of the same mutant. The well-characterized cell cycle mutants tested earlier (Figure B.2) also yielded the same results, further demonstrating the reproducibility of this assay. In addition, survivin 1 mutants showed a decrease in pH3 staining as expected, since the protein functions in the passenger protein complex with aurora B (Vagnarelli and Earnshaw, 2004). Among the remainder of the mutants screened, most showed no difference in pH3 staining

(Table B.1), indicating that the assay is quite selective; therefore, a screen of the entire Hopkins collection of mutants would likely yield a manageable number of true cell cycle mutants that could be characterized further. The few mutants that did display a difference in pH3 staining (NDC80/HEC, RNA polymerase II subunit B, and ribosomal protein S15a) generally showed a slight decrease.

Discussion

This study has demonstrated that the zebrafish is amenable to screens for cell cycle genes. Zebrafish embryos incorporate BrdU and can be labeled with an antibody against phosphorylated histone H3 (pH3), to detect cells that have gone through S phase or are in G2/M phase, respectively. Since most zebrafish mutants with general necrotic phenotypes were unable to incorporate BrdU, this assay does not selectively detect cell cycle mutants. On the other hand, pH3 staining was much more specific since we were able to identify known cell cycle mutants in a blind pilot screen by observing increases or decreases in pH3 staining relative to wild-type embryos. Since the pilot screen yielded specific and highly reproducible results, the pH3 assay is a viable method to screen the entire collection of Hopkins insertional mutants for novel cell cycle genes.

Among the mutants identified in the pilot screen with defects in phosphorylation of H3 and no previously known role in the cell cycle, all had a slight reduction in staining. One possibility is that this decrease in pH3-positive cells is a consequence of the non-specific cell death occurring in these embryos. This is a caveat of this assay that must be taken into account if upon screening the entire collection of Hopkins mutants, we find that many have reduced levels of pH3 staining. Further characterization of mutants with increases in pH3 staining would

therefore deserve higher priority since these are much more likely to be specific cell cycle defects.

On the other hand, decreases in pH3 staining might actually be specific defects as well, because not *all* of the necrotic mutants in the pilot screen displayed such a reduction. For example, mutants in a DNA polymerase subunit (*hi1703*) already have necrosis in the head and eyes at the time the embryos were fixed for the pilot screen, yet they showed no difference in pH3 staining compared to wild-type (Table B.1). At the time that this pilot screen was performed, the Ndc80/HEC protein was thought to have a role in chromosome segregation (Zheng et al., 1999), but its mechanism of action was unknown. In recent years, it has been found to be a critical member of a complex that is involved in kinetochore assembly, chromosome congression, and the spindle checkpoint (McCleland et al., 2003). Furthermore, blocking the function of Ndc80/HEC results in early mitotic exit (McCleland et al., 2003), which is consistent with the reduced pH3 phenotype in zebrafish mutants. Thus, this example demonstrates that the zebrafish pH3 phenotype may shed light on the function of poorly characterized genes that may be identified in a large-scale pH3 screen.

In addition, the finding that mutants in two "housekeeping" genes (an RNA polymerase II subunit and a ribosomal protein) displayed a reduction in pH3 staining should not be summarily dismissed based on the gene mutated. Since we now know that many ribosomal proteins are haploinsufficient tumor suppressors, it is feasible that they may play a role in the cell cycle, if perhaps indirectly. Many of the homozygous ribosomal protein mutants, in fact, generally have a reduction in pH3 staining (Kevin Lai, unpublished results), though the significance of this finding is not yet known. Therefore, there is utility to screening the entire collection of Hopkins

mutants, not only the ones mutated in novel genes, since we might uncover a previously unknown cell cycle role for some known genes.

In any case, any mutants identified in an eventual large-scale screen that have either decreases or increases in pH3 staining should be re-tested using an independent cell cycle assay. A straightforward assay that works reproducibly is fluorescence-activated cell sorting (FACS) after labeling cells from a dissociated embryo with propidium iodide as a marker for DNA content (Kevin Lai, unpublished results). In fact, this is another assay that could be used to screen the entire collection of Hopkins mutants, though it is slightly more time-consuming than the pH3 assay. An advantage of FACS, however, is that it reveals the full cell cycle profile of the cells in the embryo, i.e., the relative proportion of cells in G1, S, or G2/M phase.

Christopher Sansam has now continued the pH3 screen and has used FACS analysis to confirm some of the cell cycle defects identified by pH3 staining. He has also adapted the pH3 staining assay to screen for genes involved in the DNA damage checkpoint. Clutches of embryos from heterozygous intercrosses are treated with gamma radiation before fixation and pH3 staining. Since wild-type embryos undergo cell cycle arrest and display a reduction in pH3 staining after irradiation (Christopher Sansam, unpublished results), the clutches are screened for the presence of homozygous mutants that maintain high levels of pH3 staining, suggesting that they have a defect in the DNA damage checkpoint. Eventually, the mutants identified in both the general mitosis screen and the checkpoint screen should be further characterized in other systems, such as cultured cells, in which the cell cycle is better understood and methods for experimental manipulation are well-developed. The zebrafish has proven to be a useful discovery tool for genes involved in vertebrate development, and now also has the potential to be a powerful system in which to identify novel cell cycle genes.

Materials & Methods

BrdU Incorporation and Immunostaining

Adult T-AB wild-type fish were mated, and embryos were collected, staged within 4 hpf, and treated with 1-phenyl-2-thiourea, as described previously (Westerfield, 2000). Embryos were dechorionated by hand. At selected time points, they were treated for 15min with 10mM BrdU in 15% DMSO/fish water on ice, or for 3 to 8hrs with 10mM BrdU in fish water at 28.5°C. Embryos were washed several times with fish water and fixed with 4% paraformaldehyde for 4hrs at room temperature. Embryos were washed 4 × 5min in PBS + 0.1% Tween 20 (PBST) and 5min in water. Embryos were permeabilized with acetone for 5 to 15min, then washed 5min each in water, PBST, and 2M hydrochloric acid. Embryos were incubated in 2M hydrochloric acid for 1hr at room temperature, then washed 5min in PBST and 5min in PBSDTT (PBS + 1% DMSO + 0.1% Tween 20 + 0.5% Triton X-100). Embryos were blocked in PBSDTT + 10% normal goat serum for 2hrs at room temperature. Embryos were incubated overnight at 4°C with anti-BrdU antibody (Becton Dickinson Immunocytometry Systems, Cat. No. 347580) in PBSDTT with 3% normal goat serum.

Embryos were washed 5min, then 6 × 15min in PBSDTT + 3% normal goat serum + 0.1M sodium chloride. Embryos were incubated for 4hrs at room temperature in a 1:20 dilution of a biotinylated goat anti-mouse secondary antibody (Sigma, Cat. No. B7264) in PBSDTT + 3% normal goat serum. Embryos were washed 5min, then 6 × 15min in PBSDTT + 0.1% normal goat serum + 0.1M sodium chloride, followed by 2 × 15min in PBSDTT. The VectaStain ABC kit (Vector Laboratories, Cat. No. PK4002) and DAB substrate kit (Vector Laboratories, Cat. No. SK4100) were used according to the manufacturer's protocol to visualize the BrdU-positive cells. Embryos were cleared in 50% glycerol for 2hrs before photographing.

Anti-Phosphorylated Histone H3 (pH3) Staining

Homozygous mutant embryos were generated along with phenotypically wild-type sibling controls by intercrossing heterozygous carriers. Embryos were staged within 4 hpf and treated with 1-phenyl-2-thiourea, as described previously (Westerfield, 2000). They were dechorionated by hand before fixation at selected time points. Embryos were fixed in 4% paraformaldehyde for 4hrs at room temperature or at 4°C overnight. Embryos were washed 4 × 5min in PBS with 0.1% Tween 20 (PBST), then 5min in water or stored in PBST at 4°C for up to 2 weeks.

Fixed embryos were permeabilized in acetone for 15min, then washed 5min with water and 5min with PBS containing 1% DMSO, 0.1% Tween 20, and 0.5% Triton X 100 (PBSDTT). Embryos were blocked in PBSDTT + 10% normal goat serum for 2hrs. Embryos were incubated with a 1:200 dilution of a rabbit polyclonal antibody against phosphorylated histone H3 (Santa Cruz Biotechnology, Inc., Cat. No. sc-8656-R) in PBSDTT + 3% normal goat serum at 4°C overnight on a rotating platform shaker. Embryos were washed 2 × 1min and 6 × 15min in PBSDTT with 3% normal goat serum and 0.1M sodium chloride. Embryos were incubated for 4hrs at room temperature with a goat anti-rabbit secondary antibody (Sigma, Cat. No. B7389) diluted 1:20 in PBSDTT with 3% normal goat serum. Embryos were washed 2 × 1min and 6 × 15min in PBSDTT with 0.1% normal goat serum and 0.1M sodium chloride, then 2 × 30min in PBSDTT. The VectaStain ABC kit (Vector Laboratories, Cat. No. PK4002) and DAB substrate kit (Vector Laboratories, Cat. No. SK4100) were used according to the manufacturer's protocol to visualize the pH3-positive cells. Embryos were cleared in 50% glycerol for 2hrs before photographing.

Pilot pH3 Screen

Heterozygotes from selected lines were mated to generate clutches of 30 to 60 embryos containing ~25% homozygous mutants. Clutches of embryos were coded to conceal the identity of the mutated gene. Embryos were fixed and stained with an antibody against pH3, as described above. Clutches of stained embryos were visualized with a dissecting microscope and screened for the presence of embryos among each clutch that had increased or decreased pH3 staining relative to the remaining embryos in the clutch. Three independent observers made assessments which were considered in making a consensus pH3 call.

Acknowledgements

We thank James F. Amatruda and Kathleen L. Pfaff from the laboratory of Leonard I. Zon for participating in the pilot pH3 screen, and for providing technical advice on antibody staining. We thank Adam Amsterdam for mating fish and coding the clutches of embryos used in the screen. Wenbiao Chen and Ulrike Ziebold provided some of the initial protocols, which were developed into their current form.

Figure B.1: BrdU incorporation in T-AB wild-type embryos at selected time points.

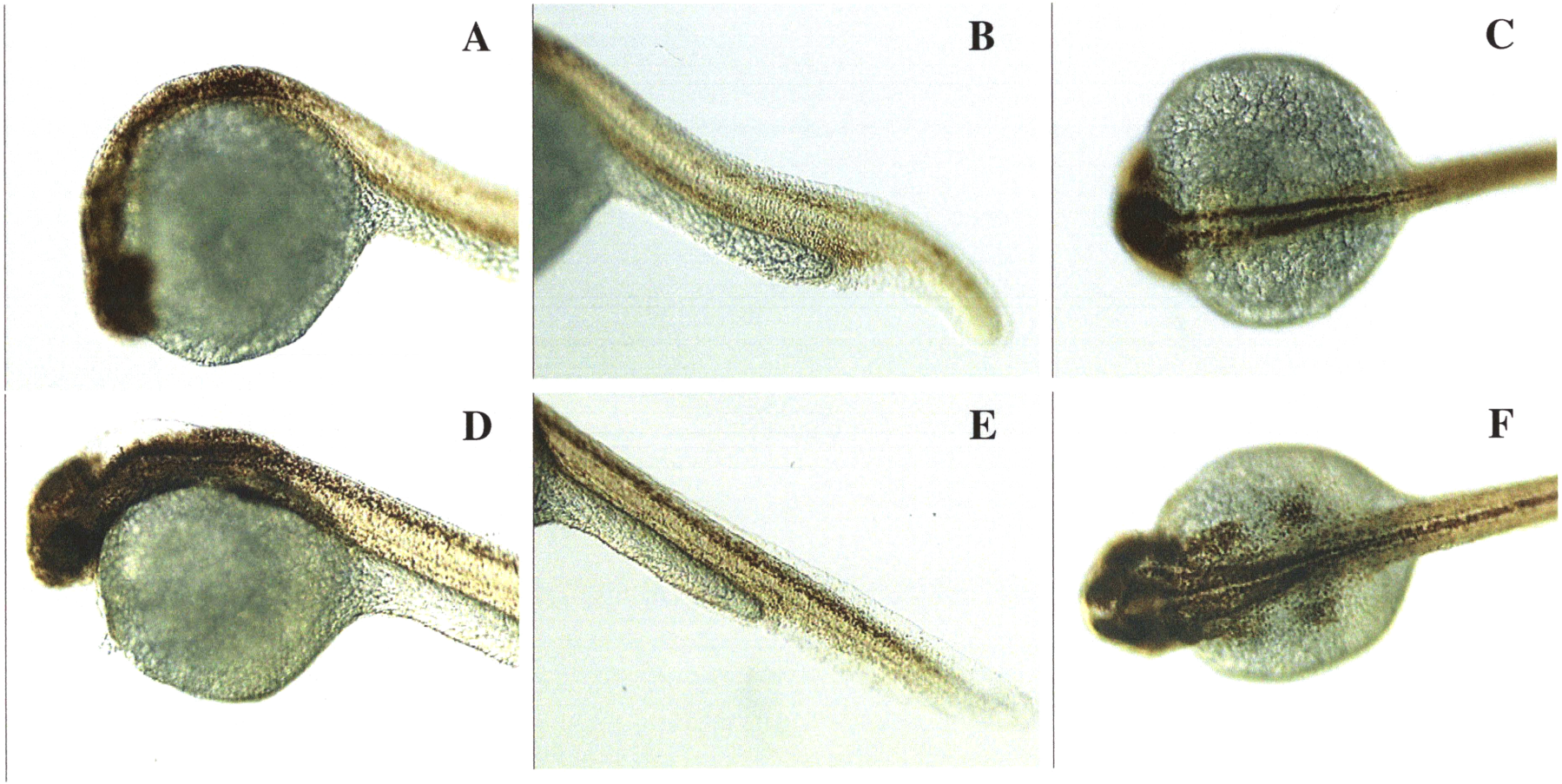
T-AB wild-type embryos were treated with BrdU at selected time points, then fixed and immunostained for BrdU-positive cells. Note that BrdU-positive staining decreases globally over the course of development and becomes localized to specific developing structures.

(A-C) 24 hpf, lateral (A and B) and dorsal (C) views.

(D-F) 48 hpf, lateral (D and E) and dorsal (F) views.

(G-I) 72 hpf, lateral (G and H) and dorsal (I) views.

(J-L) 96 hpf, lateral (J and K) and dorsal (L) views.


A
B
C
D
E
F

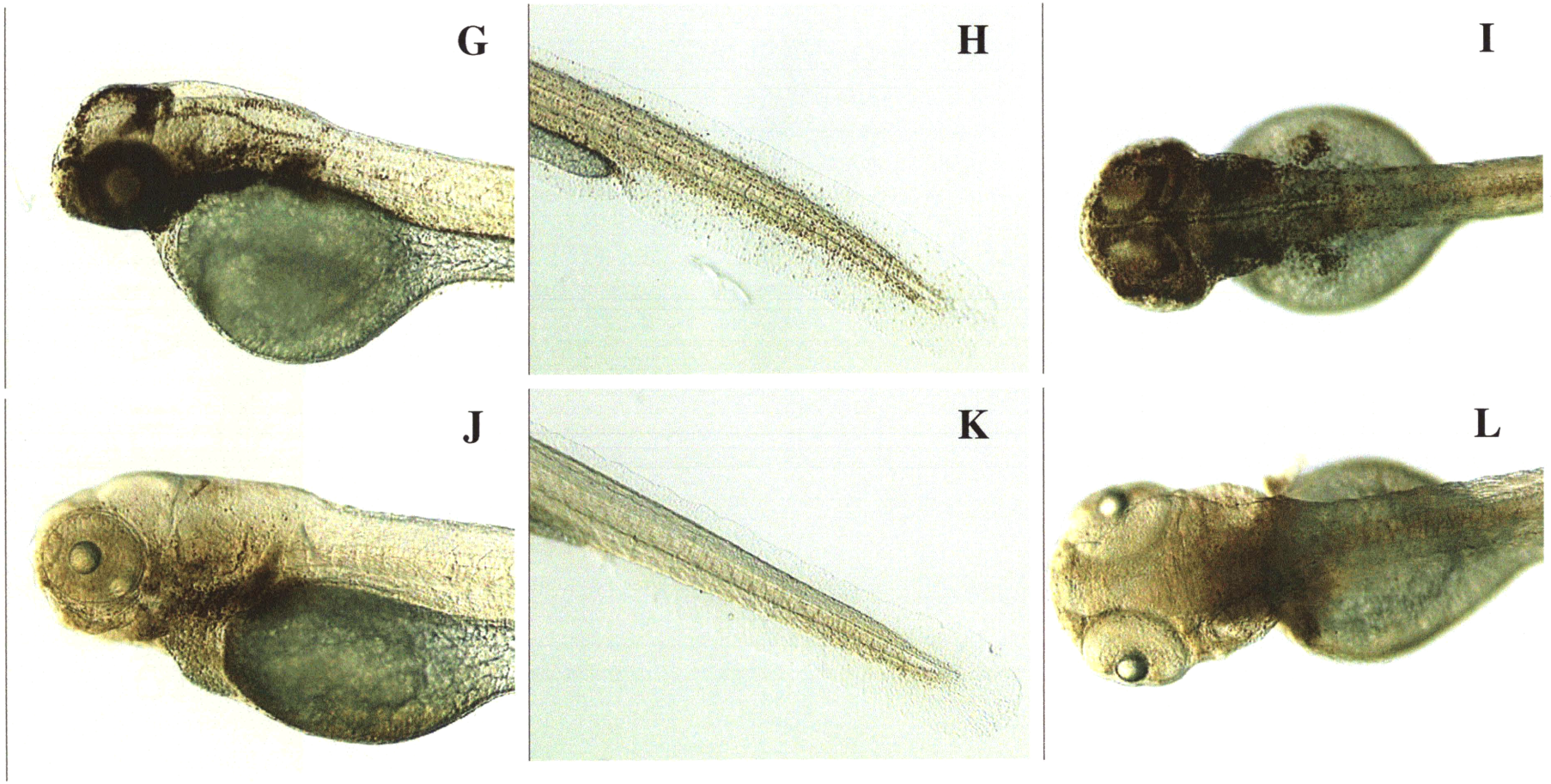

G
H
I
J
K
L

Figure B.2: Anti-phosphorylated histone H3 (pH3) staining in wild-type embryos and selected cell cycle mutants.

Lateral views of embryos stained with an antibody against phosphorylated histone H3 are shown.

(A and B) pH3-positive cells are distributed throughout a wild-type embryo at 32 hpf.

(C and D) pH3 staining is markedly decreased at 32 hpf compared to wild-type in an embryo homozygous mutant for cyclin B1 (*hi2734*).

(E and F) pH3 staining is even more severely reduced at 32 hpf in an embryo homozygous mutant for aurora B (*hi1045*), a kinase that phosphorylates histone H3.

(G and H) pH3 staining is increased compared to wild-type at 28 hpf in an embryo homozygous for polo-like kinase (*hi1856*).

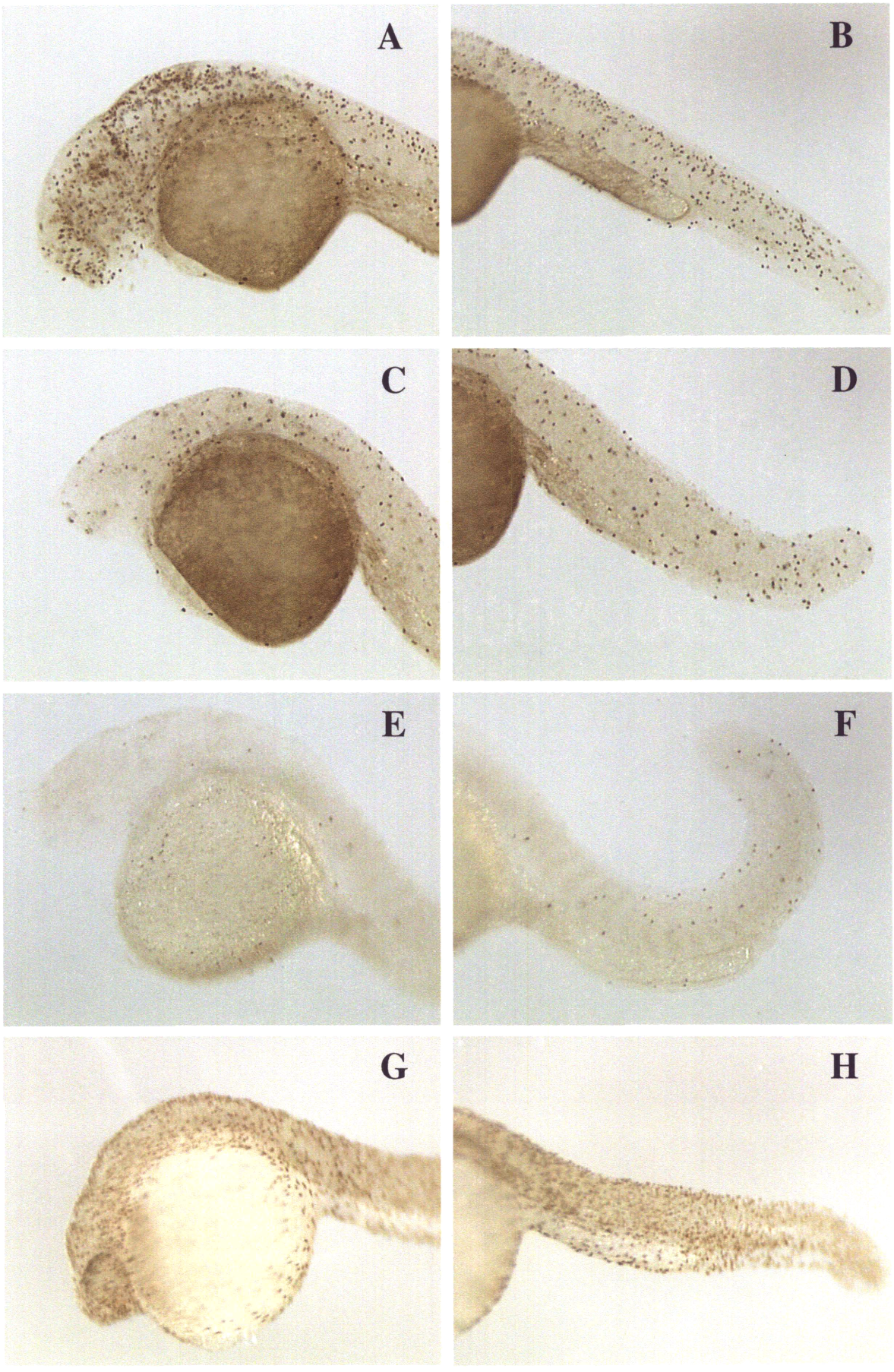

A
B
C
D
E
F
G
H

Table B.1: Pilot cell cycle screen of mutants from the Hopkins zebrafish insertional mutant collection using an antibody against phosphorylated histone H3 (pH3).

Heterozygous carriers of mutations in the indicated genes were intercrossed to yield clutches of embryos that were expected to consist of 25% homozygous mutants. The clutches were fixed between 24 and 36 hpf and stained with a pH3 antibody. The clutches were coded to allow for screening without knowledge of the mutated gene. Kevin Lai, James Amatruda, and Kathleen Pfaff independently scored the clutches for the presence of homozygous mutant embryos that displayed an increase or decrease in pH3 staining. A final consensus pH3 call was made after a discussion amongst the three participants, before the mutants were decoded. The mutants in known cell cycle genes served as positive controls, and yielded the expected results. Several other mutants showed slight decreases in pH3 staining.

Gene Mutated	Line-Clutch	Kevin	James	Kathleen	Consensus pH3 call
aurora B kinase	hi3986-1	decrease	decrease	decrease	**decrease**
aurora B kinase	hi3986-2	decrease	(not seen)	decrease	**decrease**
cyclin A2	hi2696-1	no difference	increase	slight increase	**slight increase**
cyclin B1	hi2734-1	decrease	decrease	decrease	**decrease**
cyclin B1	hi2734-2	decrease	decrease	decrease	**decrease**
deadeye (NIC96-like)	hi4-1	no difference	no difference	no difference	**no difference**
deadeye (NIC96-like)	hi4-2	no difference	no difference	no difference	**no difference**
deadeye (NIC96-like)	hi821B-1	no difference	no difference	no difference	**no difference**
deadeye (NIC96-like)	hi821B-2	no difference	(not seen)	no difference	**no difference**
DNA polymerase epsilon, subunit B	hi1703-1	no difference	no difference	no difference	**no difference**
DNA polymerase epsilon, subunit B	hi1703-2	no difference	no difference	no difference	**no difference**
DNA polymerase epsilon, subunit B	hi1703-3	no difference	no difference	no difference	**no difference**
DNA polymerase epsilon, subunit B	hi1703-4	no difference	no difference	no difference	**no difference**
MCM3	hi3068-1	no difference	no difference	no difference	**no difference**
MCM7	hi1411-1	no difference	no difference	no difference	**no difference**
MCM7	hi1411-2	no difference	no difference	no difference	**no difference**
MCM7	hi1411-3	no difference	no difference	no difference	**no difference**
MCM7	hi2704-1	no difference	no difference	no difference	**no difference**
MCM7	hi2704-2	no difference	no difference	no difference	**no difference**
MBD2-interacting zinc finger protein	hi286-1	no difference	no difference	no difference	**no difference**
MBD2-interacting zinc finger protein	hi286-2	no difference	no difference	no difference	**no difference**
MBD2-interacting zinc finger protein	hi286-3	no difference	no difference	no difference	**no difference**
myb binding protein (P160) 1a-like	hi1552-1	no difference	no difference	no difference	**no difference**
novel (ars2-like)	hi2249-1	no difference	no difference	no difference	**no difference**
novel (ars2-like)	hi2249-2	no difference	slight decrease	no difference	**no difference**
novel (MGC1346-like)	hi1373-1	no difference	no difference	no difference	**no difference**
nuclear protein NP95	hi272-1	no difference	no difference	no difference	**no difference**
nuclear protein NP95	hi272-2	no difference	no difference	no difference	**no difference**
nuclear protein NP95	hi3020-1	no difference	no difference	no difference	**no difference**
polo-like kinase	hi1856-1	increase	increase	increase	**increase**
polo-like kinase	hi1856-2	increase	increase	increase	**increase**
polo-like kinase	hi1856-3	increase	increase	increase	**increase**
inhibitor of apoptosis protein	hi2026-1	no difference	no difference	no difference	**no difference**
inhibitor of apoptosis protein	hi2026-2	no difference	no difference	no difference	**no difference**
NDC80 / HEC (highly expressed in cancer)	hi2080-1	slight decrease	slight decrease	slight decrease	**slight decrease**
RNA polymerase II subunit B	hi2635-1	slight decrease	slight decrease	slight decrease	**slight decrease**
RNA polymerase II subunit B	hi2635-2	slight decrease	slight decrease	slight decrease	**slight decrease**
ribosomal protein LP1	hi1444-1	no difference	no difference	no difference	**no difference**
ribosomal protein LP1	hi1444-2	no difference	no difference	no difference	**no difference**
ribosomal protein S15A	hi2649-1	slight decrease	slight decrease	decrease	**slight decrease**
ribosomal protein S15A	hi2649-2	no difference	no difference	slight decrease	**no difference**
ribosomal protein S15A	hi2649-3	slight decrease	slight decrease	decrease	**slight decrease**
survivin 1	hi1326-1	decrease	decrease	decrease	**decrease**
survivin 1	hi1326-2	decrease	decrease	decrease	**decrease**
survivin 1	hi2111-1	decrease	slight decrease	decrease	**decrease**
survivin 1	hi2111-2	slight decrease	(not seen)	decrease	**slight decrease**
wee1	hi2479-1	uninterpretable*	uninterpretable*	uninterpretable*	**uninterpretable***
wee1	hi2479-2	uninterpretable*	uninterpretable*	uninterpretable*	**uninterpretable***
wee1	hi2479-3	uninterpretable*	uninterpretable*	uninterpretable*	**uninterpretable***

*wee1 embryos displayed early morphological defects and were too necrotic to be reliably scored for pH3 staining at 24 to 36 hpf.

References

Amsterdam, A., Nissen, R. M., Sun, Z., Swindell, E. C., Farrington, S., and Hopkins, N. (2004). Identification of 315 genes essential for early zebrafish development. Proceedings of the National Academy of Sciences *101*, 12792-12797.

Crosio, C., Fimia, G. M., Loury, R., Kimura, M., Okano, Y., Zhou, H., Sen, S., Allis, C. D., and Sassone-Corsi, P. (2002). Mitotic Phosphorylation of Histone H3: Spatio-Temporal Regulation by Mammalian Aurora Kinases. Molecular and Cellular Biology *22*, 874-885.

Descombes, P., and Nigg, E. A. (1998). The polo-like kinase Plx1 is required for M phase exit and destruction of mitotic regulators in *Xenopus* egg extracts. The EMBO Journal *17*, 1328-1335.

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C. F., Malicki, J., Stemple, D. L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Rangini, Z., *et al.* (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. Development *123*, 37-46.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Eeden, F. J. M. v., Jiang, Y.-J., Heisenberg, C.-P., *et al.* (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. Development *123*, 1-36.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. Developmental Dynamics *203*, 253-310.

Llamazares, S., Moreira, A., Tavares, A., Girdham, C., Spruce, B. A., Gonzalez, C., Karess, R. E., Glover, D. M., and Sunkel, C. E. (1991). *polo* encodes a protein kinase homolog required for mitosis in *Drosophila*. Genes and Development *5*, 2153-2165.

McCleland, M. L., Gardner, R. D., Kallio, M. J., Daum, J. R., Gorbsky, G. J., Burke, D. J., and Stukenberg, P. T. (2003). The highly conserved Ndc80 complex is required for kinetochore assembly, chromosome congression, and spindle checkpoint activity. Genes and Development *17*, 101-114.

Ohi, R., and Gould, K. L. (1999). Regulating the onset of mitosis. Current Opinion in Cell Biology *11*, 267-273.

Vagnarelli, P., and Earnshaw, W. C. (2004). Chromosomal passengers: the four-dimensional regulation of mitotic events. Chromosoma *113*, 211-222.

Westerfield, M. (2000). The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), 4th edn (Eugene, OR: Univ. of Oregon Press).

Zheng, L., Chen, Y., and Lee, W.-H. (1999). Hec1p, an Evolutionarily Conserved Coiled-Coil Protein, Modulates Chromosome Segregation through Interaction with SMC Proteins. Molecular and Cellular Biology *19*, 5417-5428.