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Zebrafish Models of Ribosome-Associated Disorders for Identifying Novel Therapeutics

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Zebrafish Models of Ribosome-Associated Disorders for Identifying Novel Therapeutics

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cell, and Developmental Biology

by

Jason Ear

2015
ABSTRACT OF THE DISSERTATION

Zebrafish Models of Ribosome-Associated Disorders for
Identifying Novel Therapeutics

by

Jason Ear
Doctor of Philosophy in Molecular, Cell, and Developmental Biology
University of California, Los Angeles, 2015
Professor Shuo Lin, Chair

Ribosomes are large complex molecular machineries required for the synthesis of proteins via a process called protein translation. The importance of this molecular machine is conserved and is found within all living cells. Two subunits (designated the small and large subunits) form a functional ribosome. Each subunit is a complex of ribosomal RNA and a variety of proteins. Within the ribosome, amino acids are linked together depending on the order specified by the mRNA molecule.

Due to the importance of ribosomes in maintaining cellular function, one would expect defects in ribosome function, or biogenesis, to have a systemic effect on the organism. However, what is typically seen with disorders involving aberrant ribosome functions, a.k.a. ribosomopathy, is a tissue-specific defect. Specifically, patients normally present themselves with bone marrow
failure, which ultimately leads to severe anemia. Other common features in patients include a shorter stature and a higher predisposition to certain cancers.

Diamond Blackfan anemia (DBA) is the first described ribosome-associated disorder with ribosomal protein S19, RPS19, being the most commonly mutated gene found in patients. Since then, the number of ribosomal proteins found mutated in DBA patients has expanded and includes genes such as RPL11, RPL5, and RPS24. Another ribosomopathy is 5q-syndrome. In this disease, a large chromosomal deletion is found in patients. Within the commonly deleted region (CDR), it is believe that ribosomal protein S14, RPS14, is the candidate gene associated with the macrocytic anemia seen in the disease.

The effects of p53 signaling during ribosome stress have been well documented in both patient bone marrow and in models of ribosomopathies. Aberrant ribosome function has been shown to increase p53 signaling through stabilization of p53 through a RP-MDM2-p53 checkpoint mechanism in the cell. Upon increases in p53 activity, p53-mediated cell cycle arrest and apoptosis occurs. Reversal of this increase in p53 signaling has been shown to relive the phenotypic and anemic defects in cellular and animal models. However, the use of p53 inhibition towards patient care has been cautioned due to risk of cancer progression. p53 is a major tumor suppressor gene in the cell and abolishing its activity has been shown to elevate cancer risk. Patients with malfunctioning ribosome proteins are at a higher risk of tumor development; therefore, targeting p53 may further enhance tumor risk. This dilemma raises the need for novel therapeutics in tackling the treatment of ribosomopathies and to further understand disease pathology.
Animal models are indispensable for understanding the molecular mechanism and pathology of a disease. Zebrafish is an attractive vertebrate model to use in biological studies due to its rapid development, small size, transparent nature, and ease of manipulating gene function. Furthermore, there are many conserved genes between mammals and zebrafish, thereby making the model useful for understanding gene function. The advent of genome-editing technologies applied to the zebrafish model (i.e. TALEN, CRISPR/Cas9) has expanded the use of the zebrafish to model human diseases.

In this work, we model two ribosome-associated disorders in the zebrafish, DBA and 5q-syndrome, via targeted RPL11 and RPS14, respectively. Using these models, we show that a delay in late-stage erythropoiesis occurs and is followed by cell death upon ribosome stress. The delay in late-stage erythropoiesis is independent of p53 signaling and may, in part, be due to overexpression of Lft1. We demonstrated the ability of RAP-011, an activin receptor type IIA ligand trap, to increase erythropoiesis in the zebrafish and restore erythroid levels in our zebrafish models. Furthermore, the effects of RAP-011 are independent of p53 signaling, and thus, offer a new strategy in the treatment of ribosomopathies.
The dissertation of Jason Ear is approved.

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Alvaro Sagasti
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2015
This dissertation is dedicated to my extraordinary parents for their unconditional love, support, and dedication to their children.
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(Chapter 2 is a version of “Ear et. al., RAP-011 Improves Erythropoiesis in Zebrafish Model of Diamond-Blackfan Anemia through Antagonizing Lefty1, Blood (2015)”
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CHAPTER 1

Introduction
1. Erythrocytes, oxygen transport, and anemia

Erythrocytes, a.k.a. red blood cells, are the most common blood cell in the body and function primarily to deliver oxygen to various tissues as they flow through the circulatory system. Erythropoiesis occurs in the bone marrow of adult mammals, and red blood cell enter circulation at the final stages of maturation (Figure 1-1\textsuperscript{1,2}). The mature cells are enucleated and circulate for approximately 100-120 days in the body before being degraded by macrophages\textsuperscript{3}.

Hemoglobin is a metalloprotein found in the cytoplasm of erythroid cells and is responsible for its oxygen carrying ability. Four globular protein subunits (two $\alpha$ subunits and two $\beta$ subunits) are stabilized together via hydrogen bonds to form a functional hemoglobin molecule\textsuperscript{4}. Each protein chain of hemoglobin is tightly associated with a non-protein heme group. As the erythrocytes travel though the alveolar sacs in the lungs, oxygen freely transports across the cell membrane and reversibly binds to the iron atom on the heme group of the protein. Once the oxygen molecule is delivered, the hemoglobin molecule also carries carbon dioxide and nitric oxide waste product generated during cellular respiration from various tissues back to the lungs to be excreted out of the body (Figure 1-1).

Producing sufficient numbers of erythroid cells and hemoglobin levels per cell is crucial for proper oxygen delivery in the body. Approximately 25% of the cells in the human body are red blood cells. When erythroid counts or hemoglobin levels are insufficient, anemia (an inability to deliver adequate oxygen levels to tissues) can occur. Various conditions may lead to anemia, including iron-deficiency, kidney failure, cancer, and bone marrow failure. Given
the crucial role of erythroid cells in the body, understanding the precise mechanism governing normal erythropoiesis and the mechanism underlying erythroid failure during disease conditions will be crucial for the development of novel therapeutics.

2. Hematopoiesis and Erythropoiesis

Hematopoietic stem cells (HSCs) have the ability to give rise to all the different blood lineages, including erythrocytes\textsuperscript{1,5}. These HSCs are derived from a complex differentiation process during development. In mammals, hematopoiesis occurs in two waves (referred to as primitive and definitive) during development (Figure 1-2). The cells produced during primitive (or embryonic) hematopoiesis contribute to the blood cells seen in the early embryo and exist transiently. In mice, this occurs at approximately embryonic day 7.0 (E7.0) on the yolk sac blood islands. The predominant blood cells produced during the primitive wave of hematopoiesis are nucleated primitive erythrocytes, megakaryocytes, and primitive macrophages\textsuperscript{6}.

Definitive hematopoiesis, on the other hand, occurs later in development, beginning at E8.25 in the yolk sac (Figure 1-2)\textsuperscript{7}. The cells that arise from this pool have a limited differentiation potential. Within the embryo proper, HSCs arise in the aorta-gonad-mesonephros (AGM) region at E10.5 and migrate to the fetal liver at E14.5. Within the fetal liver, these early cells differentiate into true HSCs\textsuperscript{8}. These HSCs have long-term repopulating activity within the bone marrow and can differentiate to give rise to all blood cells, including enucleated erythrocytes, lymphocytes, and macrophages. The cells that are produced in the fetal liver are the initial cells that seed the bone marrow of the developing embryo. Just prior to birth, the
main site of blood development switches from the fetal liver to within the bone marrow where the HSCs maintain adequate blood levels throughout the life of the animal (Figure 1-1 and Figure 1-2).

During erythropoiesis, the erythroid cells are derived from HSCs through a complex differentiation process and are regulated through various extrinsic and intrinsic factors (Figure 1-3)\(^1,2\). Within the bone marrow, the multipotent hematopoietic stem cell first differentiates into a multipotent common myeloid progenitor before differentiating to the colony forming the BFU-E and CFU-E progenitors cells, respectively. These CFU-E cells undergo further differentiation into the proerythroblast stage (ProE) followed by transition to the basophilic erythroblast stage (BasoE). This then becomes a polychromatophilic erythroid (PolyE) cell and then an orthochromatic cell (OrthoE). At this stage, the nucleus is expelled and the cells leave the bone marrow, entering circulation as reticulocytes. Once in circulation, the cells undergo further maturation prior to becoming true erythroid cells\(^2\). During the maturation process, heme and globin molecules are synthesized in large quantities to meet the demand needed for proper erythroid function.

Extracellular signals regulating erythropoiesis include growth factors, signaling molecules, and cytokines that are secreted by cells within the niche of the bone marrow microenvironment\(^8,9\). Cells within this microenvironment include adipocytes, vascular cells, macrophages, as well as bone marrow stromal cells (Figure 1-1). In addition to paracrine signaling from cells within the bone marrow, long-range cues can also affect erythropoiesis.
For example, erythropoietin (epo) is secreted by renal cells and stimulate erythropoiesis by promoting the terminal differentiation of CFU-E into mature erythroid cells\textsuperscript{10}.

Intrinsic factors important for erythropoiesis include the activation of key signaling pathways and the regulation of key transcription factors\textsuperscript{8}. Signaling molecules from paracrine and long-range sources are secreted by the supporting cells and can activate various cell signaling pathways such as the SMADs, Jak2/Stat5, MAPK, and PI3K/AKT pathways. Ultimately, activation of these pathways leads to expression of key genes required for erythroid function and development such as globin, heme biosynthesis enzymes, and important transcription factors (i.e. GATA1 and EKLK (erythroid-specific Kruppel-like factor). Dysregulation of these pathways or expression of key transcriptional regulators has been shown to affect the development of erythroid cells. Furthermore, disruption of either GATA1 or EKL leads to defects in both primitive and definitive erythropoiesis\textsuperscript{11}. In many diseases, these extrinsic and intrinsic factors are affected, leading to defects in erythroid development and/or function, thereby causing anemia.

3. Ribosomes, Ribosomopathies, and the p53-pathway

Ribosomes are essential components of every living cell and are required for protein translation. These molecular machineries are composed of a small and large subunit, which recognize a strand of messenger RNA (mRNA) and link together amino acids to generate a growing polypeptide chain. The subunits of ribosomes are composed of ribosomal RNA (rRNA) and ribosomal proteins, intricately assembled to form a functional ribosome\textsuperscript{12}. 
Proper ribosome assembly is an energetic process and requires tight mechanistic control; therefore, defects in this machinery can be detrimental to the cell.

Diseases of aberrant ribosome function or biogenesis, also referred to as ribosomopathies, commonly lead to bone marrow failure disorders. Ultimately, this results in an insufficient amount of erythroid cell production and severe anemia in patients, as seen in two ribosome-associated disorders, Diamond Blackfan anemia (DBA) and 5q-Syndrome. The tissue specific defect associated with these ribosomopathies has remained challenging to understand due to the ubiquitous expression of the affected ribosomes, as one would expect defects in the translational machinery of a cell to have a systemic effect.

Much effort has been put towards understanding the anemic phenotype seen in ribosomopathies. The most widely accepted hypothesis is that upon ribosome stress, an increase in p53-activity leads to cell cycle arrest and apoptosis. While this increase is a ubiquitous response, hematopoietic cells are believed to be more sensitive to p53-activity. Indeed, hematopoietic cells from DBA patients show an increase in levels of p53 protein level. Furthermore, knockdown of ribosomal proteins have been shown to elevate p53-activity in cells through a ribosomal protein (RP)-mdm2-p53 checkpoint mechanism. Reversal of p53-activity was demonstrated to relieve the hematopoietic defects observed in both cellular and animal models of ribosomopathies. While targeting the p53-pathway has yielded promising results in disease models, caution must be taken when targeting this pathway in patients due to the elevated cancer risk associated with those harboring ribosome mutations.
4. Diamond Blackfan Anemia & 5q-Syndrome

Patients with Diamond Blackfan anemia (DBA) present with macrocytic anemia and are commonly seen with short stature and a predisposition to certain cancers\textsuperscript{16}. DBA is the first described ribosome-associated disorder, with mutations in ribosomal protein S19, RPS19, being the first identified. A mutation in this gene is also the most frequently found and is seen in over 25% of patients. Since then, mutations in other ribosomal proteins have been identified, including mutations in ribosomal protein L11 (~5% of patients), L5 (~7%), L35a (~3%), S10 (~3-6%), S7 (~1%), S17 (~1%), S24 (~2%), and S26 (~3-6%)\textsuperscript{24-29}. It has been hypothesized that mutations in genes associated with ribosome biogenesis or function is affected in DBA patients without an identified mutation.

5q-syndrome is a subset of myelodysplastic syndrome (MDS)\textsuperscript{14,15}. Patients with this particular disease have a deletion in the long arm of chromosome 5. Analysis of multiple patients harboring various deletions has led to identification of a commonly deleted region (CDR). The genes located within this critical region were individually characterized in mice models and through an RNAi screen. Through analysis of these knockout mice, it was revealed that a mutation in ribosomal protein S14, RPS14, was sufficient to recapitulate the anemic phenotype seen in patients with 5q-syndrome\textsuperscript{30-32}. Among MDS patients without heterozygous mutation in chromosome 5, some patients also demonstrated decreased levels of RPS14\textsuperscript{32}. Overall, these observations suggest that 5q-syndrome is a ribosome-associated disorder with aberrant expression of RPS14 as the underlying cause of anemia.
5. Advantages of Zebrafish as a Model Organism

Zebrafish offers many advantages as a model organism. Its small size, transparent nature, and rapid development allows for easy manipulation and observation under a microscope. Furthermore, the ability to label specific tissues with a fluorescent maker allows for the unique opportunity to study the development of certain organs and tissues in a live intact animal. For example, transgenic approaches to specifically label blood cells with fluorescent proteins (i.e. EGFP, mCherry) has allowed for the study of cells actively in circulation\(^33\). Other advantages of this model system include ease of performing genetic/chemical screens, gene overexpression via mRNA injections, gene knockdown using morpholino injections, and, with advances in recent genetic technologies (i.e. TALEN, CRISPR/Cas9), the ability to perform gene knockout/knockin\(^34,35\).

Zebrafish is particularly useful in the study of hematopoiesis and blood related disorders\(^36,37\). In addition to having well-defined and specific markers for various blood lineages, the anatomical location and development of these various hematopoietic cells are well characterized. Through mutagenesis screens, many genes have been identified which are important for the development and/or maintenance of hematopoietic cells. These screens have revealed many conserved transcription factors and signaling molecules between mammalian and zebrafish hematopoiesis\(^5,38,39\).

6. Hematopoiesis in the Zebrafish

Similar to mammalian hematopoiesis, zebrafish hematopoiesis also occurs in two waves\(^36,40\). Primitive hematopoiesis occurs in two distinctive sites in the zebrafish embryo (Figure 1-4).
Blood cells are specified along the lateral plate mesoderm, with primitive myeloid cells being generated towards the anterior end of the embryo and primitive erythroid cells along the anterior side of the embryo. Soon after specification, the myeloid cells migrate throughout the embryo while the erythroid cells migrate towards the midline to a region called the intermediate cell mass (ICM). Afterwards, by 24 hours post fertilization (hpf) the cells migrate over the yolk and enter a rudimentary circulation system.

Definitive HSCs are derived from the ventral wall of the dorsal aorta at approximately 30 hpf. After specification, the cells migrate to the caudal hematopoietic tissue (CHT) in the posterior tail. Beginning at 3 days post fertilization (dpf), the development of lymphocytes occurs in the thymus. By 4 dpf, the HSCs migrate to the kidney marrow and maintain blood levels throughout the life of the animal. The kidney marrow is analogous to the bone marrow in mammals.

7. Zebrafish models of ribosomopathies

Zebrafish has been an indispensable model to study ribosome-associated disorders in vivo. Several disease models have been generated in the zebrafish through the use of morpholino knockdown as well as ENU-directed mutagenesis screens. This includes models of RPS19, RPL11, RPS14, RPS24, and RPS29 deficiency. Recently, a model of RPS19 deficiency has been generated through TALEN mediated gene-targeting.

Work using these models has revealed the importance of p53 signaling in mediating the cellular response upon ribosome deficiency. Through morpholino knockdown of RPS19
in zebrafish embryos, it was first demonstrated that the developmental and erythroid defects are mediated, in part, through the p53 pathway\textsuperscript{22}.

8. Scope of the work

In this work, a novel therapeutic (Sotatercept, a.k.a RAP-011) was tested in a zebrafish model of DBA. RAP-011 was shown to elevate erythroid levels in the developing embryo and reverse the anemic phenotype in response to RPS19 or RPL11 deficiency. This response is mediated, in part, through antagonizing lefty1.

In addition, a human disease model was generated through targeting RPS14 using the CRISPR/Cas9 system in zebrafish. This model revealed that a late-stage terminal maturation defect underlies the anemic phenotype and is reversed through administration of RAP-011.
Figure 1-1. Erythroid cells are necessary for oxygen delivery to various tissues. As erythroid cells circulate through the vasculature of the alveolar sac in the lungs, oxygen traverses the cell membrane and binds to the heme group in a hemoglobin molecule. Once oxygen is incorporated, the cells circulate to various tissues to deliver oxygen to cells throughout the body. Waste product such as the carbon dioxide and nitric oxide in circulation is also incorporated onto the hemoglobin molecule and is released into the lungs to be exhaled.

Erythroid cells are produced in the bone marrow of adult mammals. Within the microenvironment of the bone marrow, many cells secrete molecules regulating erythroid development. WBC, white blood cell. RBC, red blood cell.
Figure 1-1. Erythroid cells are necessary for oxygen delivery to various tissues
Figure 1-2. Hematopoiesis occurs in two waves during embryogenesis. Primitive hematopoiesis occurs around E7.0 in the yolk sac before migrating into the mouse embryo. These clusters of cells are referred to as the yolk sac blood islands (YSBI). The erythroid cells produced from these pools are transient and nucleated. At E8.25 definitive hematopoiesis also occurs on the yolk sac; however, these cells are limited in their differentiation potential. Around E10.5, hematopoietic stem cells progenitors are specified in the aorta-gonad-mesonephros (AGM). After specification, the cells migrate to the fetal liver (around E14.5) where they undergo further differentiation into true hematopoietic stem cells. The hematopoietic stem cells in the fetal liver migrate to the bone marrow. Just prior to birth and into adulthood, the main site of hematopoiesis is within the bone marrow.
Figure 1-2. Hematopoiesis occurs in two waves during embryogenesis
**Figure 1-3. Erythroid cells are derived from multipotent hematopoietic stem cells.**

Schematic diagram of the differentiation process of the various blood cells during hematopoiesis. Hematopoietic stem cells have the ability to self-renew and differentiate into all blood lineages (i.e. myeloid cells, lymphoid cells, and erythrocytes). The formation of erythroid cells is a multistep process requiring various growth factors, cytokines, and signaling molecules.
Figure 1-3. Erythroid cells are derived from multipotent hematopoietic stem cells
**Figure 1-4. Blood development in the zebrafish.** Diagram illustrating regions of hematopoiesis in the developing zebrafish embryo. Primitive erythroid cells are first specified from the lateral plate mesoderm in a bilateral stripe pattern, and then migrate towards the midline. Soon after, the cells migrate towards the midline to the intermediate cell mass before entering circulation. The primitive myeloid cells are specified in the anterior side of the embryo. Definitive hematopoietic stem cells bud from the wall of the dorsal aorta and migrate to the caudal-hematopoietic-tissue (CHT). Afterwards, the cells migrate to the kidney marrow where they sustain blood levels throughout the life of the animal. Lymphoid cells are derived from cells within the thymus.
Figure 1-4. Blood development in the zebrafish
Chapter References


CHAPTER 2

RAP-011 Improves Erythropoiesis in Zebrafish Model of Diamond-Blackfan Anemia through Antagonizing Lefty1
Abstract

Diamond-Blackfan Anemia (DBA) is a bone marrow failure disorder characterized by low red blood cell count. Mutations in ribosomal protein genes have been identified in approximately half of all DBA cases. Corticosteroid therapy and bone marrow transplantation are common treatment options for patients; however, significant risks and complications are associated with these treatment options. Therefore, novel therapeutic approaches are needed for treating DBA. Sotatercept, a.k.a ACE-011, (and its murine ortholog RAP-011) acts as an activin receptor type IIA ligand trap, increasing hemoglobin and hematocrit in pharmacologic models, in healthy volunteers, and in patients with β-thalassemia, by expanding late-stage erythroblasts through a mechanism distinct from erythropoietin. Here, we evaluated the effects of RAP-011 in zebrafish models of RPL11 ribosome deficiency. Treatment with RAP-011 dramatically restored hemoglobin levels caused by ribosome stress. In zebrafish embryos, RAP-011 likely stimulates erythropoietic activity by sequestering lefty1 from erythroid cells. These findings identify lefty1 as a signaling component in the development of erythroid cells and rationalize the use of sotatercept in DBA patients.
**Introduction**

Diamond-Blackfan Anemia (DBA) is a bone marrow failure disorder where the predominant defect is in the development of erythroid precursors into mature erythrocytes. While macrocytic anemia is the primary characteristic, physical abnormalities and a predisposition to certain cancers are also observed\(^1,2\). Although the exact mechanism of red blood cell failure is unknown, a defect in ribosome biogenesis or function is believed to be the primary cause of DBA; thus, DBA is classified as part of a group of disorders characterized by malfunctioning ribosomes, also referred to as ribosomopathies. Approximately half of all DBA patients carry a mutation in a gene coding for a ribosomal protein and it is predicted that several unidentified mutations in DBA are involved with ribosome function or biogenesis. A mutation in the ribosomal protein RPS19 was the first identified ribosomal protein aberration discovered in DBA patients\(^3-5\). This gene is the most commonly mutated ribosomal protein found in DBA patients, occurring in approximately 25% of cases\(^6\). Since then, the repertoire of ribosomal proteins found mutated in DBA patients has expanded and includes mutation in RPS24, RPS26, RPS10, RPS35A, RPL5, and RPL11\(^7\).

Defects in ribosomal proteins are expected to have a profound impact on the whole animal and affect various cell types; therefore, the predominantly tissue-specific defect of DBA, and other ribosomopathies, remains an interesting question. Several hypotheses have been proposed to explain the specificity of the disease\(^7,8\). One widely accepted model is that the affected tissues have an increase in p53 activity upon
ribose stress\textsuperscript{9}. Elevation in the p53-pathway leads to cell-cycle arrest and apoptosis, while suppression of the p53-pathway upon ribosome stress has been shown to alleviate both the phenotypic and erythropoietic defects in zebrafish and other models of DBA\textsuperscript{10-12}.

While targeting the p53-signaling pathway has shown promising results in cellular and animal models, caution must be taken into account due to the potential of cancer development in patients\textsuperscript{13}. The p53-pathway is abnormally regulated in many cases of cancer; therefore, targeting this pathway may further increase a patient’s risk for cancer development. Recent studies using cellular and animal models of ribosome deficiency have also demonstrated p53-independent pathways involved in the erythroid defect\textsuperscript{14-16}. Identification of such pathways has created promising new avenues into the treatment of this disease.

Sotatercept (ACE-011) is a ligand trap drug generated from the fusion of the activin receptor type IIA (ActRIIA) extracellular domain to the Fc domain of human IgG\textsuperscript{17-19}. This molecule antagonizes signaling downstream of ActRIIA through sequestering distinct members of the transforming growth factor-\(\beta\) (TGF-\(\beta\)) family, including bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), and Activin molecules, from binding to the endogenous receptor. Injection of RAP-011 (the murine form of ACE-011) into murine models of anemia can restore red blood cell parameters\textsuperscript{17,20}. Furthermore, a study using Sotatercept in healthy volunteers demonstrated that it can increase hemoglobin levels\textsuperscript{18}. Currently, sotatercept is being
evaluated for efficacy in anemia associated with diseases such as β-thalassemia, myelodysplastic syndrome and renal disease. Also, there is an ongoing clinical trial to test the efficacy of sotatercept in the treatment of DBA.

We aim to test the ability of RAP-011 to restore erythroid levels in zebrafish models of ribosome deficiency and to elucidate the role of TGF-β signaling molecules during ribosome stress. Our work demonstrates that RAP-011 can elevate erythroid counts under normal physiological conditions and during ribosome deficiency in zebrafish. The rescue in erythropoiesis occurred at early stages of development, when the erythroid arrest appears to be p53-independent. Finally, we provide evidence to show that RAP-011 can block the function of Lefty1 (Lft1), an activin/nodal signaling antagonist; thus providing a model where RAP-011 can activate or inhibit activin/nodal signaling in a context-dependent manner.
Results:

Rpl11 Deficiency Causes Erythroid-Specific Defect

Although rpl11 genetic mutant is available in zebrafish\textsuperscript{12}, it is less ideal for use in experiments for this study, as only one fourth of the offspring will carry a homozygous mutation and show a phenotype; furthermore, mutations in ribosomal protein genes lead to insufficient protein levels in patients as opposed to a complete absence of the protein. We therefore model rpl11 deficiency in zebrafish embryos by using a translational start codon morpholino to knockdown rpl11 protein levels by approximately 50\% (Figure 2-1A). This level is comparable to levels seen in DBA patients carrying mutations in \textit{RPL11}\textsuperscript{22}. Similar to previous observations, we detected an increase in levels of p53 and p53-target genes such as p21 and mdm2 at our level of rpl11 knockdown (Figure 2-1B)\textsuperscript{12,23}. A mild developmental delay is also observed in embryos by 24 hours post fertilization (hpf). At this stage, a shortened trunk and aplasia in the head can be seen (Figure 2-1C). By 48hpf, knockdown of rpl11 results in mild edema, a defective yolk extension, and a smaller head (Figure 2-1D). \textit{O}-dianisidine staining for mature erythroid cells reveals that morphants had significantly reduced levels of hemoglobin (Figure 2-1E). Because of the close developmental association between vascular and myeloid lineages in the developing embryo, we injected morpholino into \textit{kdrl:dsRED} and \textit{mpo:EGFP} transgenic embryos to observe the effects of ribosome deficiency on endothelial and neutrophil cell development, respectively. Analysis of these embryos reveals no significant changes in the developing vasculature.
or neutrophil cells, suggesting that development of these lineages is normal (Figure 2-1F, G).

Rag1 and c-myb are two markers typically used to label cells of the definitive lineage of hematopoiesis in zebrafish. The expression pattern of rag1 was reduced in morphants compared to control embryos (Supplemental Figure S2-1). Additionally, work by previous groups showed that knockdown of rpl11 led to lower levels of c-myb24. These data suggest that knockdown of rpl11 in zebrafish embryos also affects definitive hematopoiesis. Furthermore, staining by acridine orange showed an elevation of apoptosis upon knockdown of rpl11 (Supplemental Figure S2-2). Overall, insufficient levels of RPL11 in zebrafish embryos led to mild physical abnormalities, apoptosis, hematopoietic defects, and elevated p53 activity; phenotypes which are similar to those observed in DBA patients.

**Maturation and Proliferation of Erythroid Cells is Defective in Rpl11 DBA Model**

Next, we wanted to determine if the defect in erythroid development is due to improper specification and/or differentiation of the erythroid cells. To address specification, we looked at the expression pattern of gata1, one of the earliest markers for erythroid development25. When the expression of gata1 was studied by whole mount in situ hybridization, we did not notice observable differences between control or morphants at the 4 somites stage, suggesting that hematopoietic cells can be specified into the erythroid lineage (Figure 2-2A). We also looked at the expression of gata1 at 30hpf, when circulation is well established. Again, morphants had equivalent levels of gata1
expression compared to control, although there was a delay in the number of cells entering circulation (Figure 2-2B). This was further confirmed by quantification of gata1 transcript levels (Supplemental Figure S2-3). To further characterize the development of erythroid cells, we performed a time course analysis on LCR2:EGFP transgenic embryos (which have EGFP-labeled globin-positive cells)\(^{26}\). When morpholino to rpl11 was injected into LCR2:EGFP embryos, EGFP positive cells were observed in morphants at 20 hpf and an increase in EGFP can be seen in embryos by 24 hpf. Interestingly, by 48 hpf, EGFP was also observed but significantly less compared to control (Figure 2-2C). The remaining EGFP positive cells can be seen circulating throughout the embryo (data not shown), further demonstrating the presence of an intact vasculature. Across all time points analyzed, the average EGFP intensity in morphants was significantly lower than in controls (Figure 2-2D). The lower EGFP intensity and lack of o-dianisidine positive staining suggest a defect in globin production and/or maturation in the erythroid cells. Furthermore, analysis of the size of EGFP positive cells reveal that the cells in morphants are larger compared to control (Figure 2-2E). The circulating erythroid cells are also irregularly shaped (Figure 2-2F). Together, these findings indicate that a late-stage erythropoietic defect may be involved in rpl11 deficiency.

The decrease in EGFP positive cells between 24 and 48 hpf raises the possibility that proliferation may be affected at this stage of development. In zebrafish embryos, proliferating cell nuclear antigen (pcna) was previously reported to label actively dividing cells in the intermediate cell mass (ICM), a site of hematopoietic development
in the developing embryo\textsuperscript{27,28}. Compared to control, morphants had a slight reduction of PCNA in the ICM, suggesting that proliferation of cells in the ICM is affected upon ribosome stress (Figure 2-2G). Collectively, these results suggest that it is not specification of erythrocytes that are affected in rpl11 deficiency, but proliferation and terminal maturation that are dysregulated.

**Delay in Erythroid Maturation is Independent of p53**

The association between ribosome stress and p53 is well-established\textsuperscript{9,10,23}. To determine if the defects in terminal maturation are p53-dependent, we injected morpholino against rpl11 into p53-null zebrafish embryos\textsuperscript{29}. As previously demonstrated, inhibition of p53 partially prevents the physical abnormalities associated with ribosome stress at 24 hpf (Figure 2-3A)\textsuperscript{23}. Transcript levels of p53 and p53-target genes were analyzed by real-time PCR to confirm the decrease in p53 activity (Figure 2-3B). Interestingly, a slight increase in p21 levels was still observed when rpl11 was targeted in the p53 mutant embryos, but the downstream targets mdm2, bax, and puma remain unchanged. At 48 hpf, the developmental abnormalities and lack of globin positive cells was still apparent when rpl11 was targeted in p53-null zebrafish embryos (Figure 2-3C). However, lack of p53 partially restored the erythroid defect at 72 hpf (Figure 2-3D). Collectively, these results suggest that the erythroid defect is p53-independent at early stages of development and become p53-dependent later on.
**Activin Ligand Trap Increases Proliferation of Erythroid Cells in Zebrafish Embryos**

Because RAP-011 was successfully used to treat various models of anemia\textsuperscript{20,30}, we wanted to see if RAP-011 could also be used to restore erythroid levels in our model. Alignment of the activin receptor between zebrafish, mice, and humans showed high similarity (Supplemental Figure S2-4). Therefore, we hypothesized that RAP-011 would also elevate erythroid levels in zebrafish and potentially restore erythroid levels in our rpl11 DBA model.

To test if RAP-011 can function in zebrafish, we injected it into the embryo at the shield stage of development (~4.0 hpf) between individual cells (extracellularly) in order to ubiquitously distribute the drug in the embryos (Supplemental Figure S2-5). Staining against the IgG domain using fluorescent secondary antibodies reveals that, at 24 hpf, IgG and RAP-011 are present throughout the embryo (Supplemental Figure S2-5). Interestingly, at 24hpf, an expansion of the ICM was apparent in RAP-011 injected embryos, but not in IgG injected embryos (Figure 2-4A and B). Furthermore, this expansion occurred dose dependently (Supplemental Figure S2-6). We found that 4ng of RAP-011 injected into the embryos expanded the ICM with minimal developmental defects. Consistent with the notion that RAP-011 functions as a ligand trap, injection of RAP-011 into the developing embryo did not cause an ICM expansion (data not shown).

The expanded cells within the ICM were also EGFP positive as revealed by injecting RAP-011 into LCR2:EGFP transgenic embryos (Figure 2-4A, bottom panel, Figure 2-4C,
and Supplemental Figure S2-5). Probing for gata1 via in situ hybridization further demonstrates that cells within the expanded ICM are indeed of the erythroid lineage (Figure 2-4D). Injection of RAP-011 into mpo:EGFP transgenic fish reveal that cells of the myeloid lineage are also expanded (Supplemental Figure S2-7).

Next, we wanted to determine if the expansion of cells within the ICM is due to an increase in cell proliferation. Higher levels of pcna were observed in RAP-011 injected embryos compared to IgG or uninjected control (Figure 2-4E). These experiments suggest that RAP-011 can function to increase erythroid levels in zebrafish embryos by increasing cell proliferation, similar to observations made in cell culture systems\textsuperscript{17,31}.

**Erythroid Levels Can Be Recovered Using RAP-011**

To test if RAP-011 can be used to recover the erythroid cells in zebrafish deficient for rpl11, we injected RAP-011 into rpl11 morphant embryos. Treatment with RAP-011 partially restored erythroid cells, as shown by the presence of positively stained cells with o-dianisidine. Furthermore, no phenotypic rescue was observed, as the defective yolk extension and head development are still present. No rescue of erythroid cells was observed upon treatment with IgG (Figure 2-5A). Staining for pcna suggest that RAP-011 restores erythroid count by promoting the proliferation of cells upon ribosome stress (Figure 2-5B). Gene expression analysis of p53, p21, and mdm2 indicate no changes in the expression levels of these genes, suggesting that RAP-011 works independently of the p53-pathway (Supplemental Figure S2-8).
We further tested this drug on the previously described rpl11 and rps19 genetic models of ribosome deficiency in zebrafish\textsuperscript{12,32}. Injection of RAP-011 into both rpl11 and rps19 mutant embryos restored hemoglobin levels (Figure 2-5C and D). Together, these data suggest that RAP-011 treatment promoted erythroid maturation and proliferation in our models.

Next, we wanted to test if RAP-011 could be used in combination with other treatments. Dexamethasone has been shown to restore erythropoiesis in cellular and zebrafish models of DBA\textsuperscript{12,33}. When rpl11 deficient embryos were treated with both dexamethasone and RAP-011, an additive effect on erythroid levels was observed compared to either treatment alone (Supplemental Figure S2-9).

**Lefty1 is Expressed within tissues surrounding the ICM and Up-Regulated upon Rpl11 Deficiency**

Previous work showed that RAP-011 influences the hematopoietic niche within the bone marrow to elicit the increase in erythropoiesis\textsuperscript{31}. RAP-011 sequesters extracellular ligands from binding to receptors on the cell surface. RAP-011 might also function in a similar manner in the developing embryo (Figure 2-6A). Because the expanded ICM can first be observed between 18-20 hpf, we looked at the expression of various members of the TGF-\(\beta\) family in tissues surrounding the ICM at this stage. BMP4, LFT1, GDF6a, and GDF11 were detected in the tissues surrounding the ICM (Figure 6B and Supplemental Figure S2-10). To determine which factor may be involved in the delayed maturation of erythroid cells upon ribosome deficiency we
measured transcript levels of these factors in rpl11 morphants. Interestingly, Lft1 was upregulated in morphants compared to control (Figure 2-6C). Elevated Lft1 expression was also observed in rpl11 and rps19 mutant zebrafish (Supplemental Figure S2-11). This raises the possibility that Lft1 can inhibit globin production in erythroid cells.

**RAP-011 Antagonizes the Effects of Lefty1 Overexpression**

To test if overexpression of Lft1 can delay the maturation of erythroid cells, we overexpressed Lft1 in zebrafish embryos. We used a construct to facilitate the temporal expression of Lft1, thereby allowing us to overcome the gastrulation defects associated with Lft1 overexpression by mRNA injection, and to overexpress Lft1 when globin expression occurs (Supplemental Figure S2-12). This plasmid was injected into transgenic zebrafish embryos with cre-recombinase being driven under the control of the heat shock promoter. When Lft1 is overexpressed by heat shocking the embryos at approximately 14-18hpf, reduced globin levels in erythroid cells was observed, demonstrating the inhibitory effects of Lft1 on globin production (Figure 2-6D). We then injected RAP-011 into the Lft1 temporally overexpressing embryos, and saw a recovery of o-dianisidine positive staining (Figure 2-6D).

Previous work showed that overexpression of the extracellular domain of the activin receptor can antagonize the effects of Lft1.\textsuperscript{34} We therefore tested if RAP-011, which has the extracellular domain of the activin receptor type IIA, can also antagonize the effects of Lft1 overexpression \textit{in vivo}. We injected \textit{in vitro} transcribed capped mRNA coding for Lft1 into the developing embryos and saw that overexpression of Lft1 by mRNA
injection caused developmental defects and a reduction in mesoderm-derived tissues (Supplemental Figure S2-13)\textsuperscript{35}. As expected from a reduction in mesoderm levels, there is also a reduction in the development of erythroid cells. When RAP-011 or IgG was injected into zebrafish embryos overexpressing Lft1, RAP-011 treated embryos showed recovery of erythroid cells (Supplemental Figure S2-13). Overall, these finding indicate that RAP-011 can function in zebrafish embryos and antagonize the effects of Lft1.

**Discussion**

We demonstrated that haploinsufficiency of ribosomal proteins caused an erythroid-specific defect in zebrafish embryos. Rpl11 deficiency causes an erythroid-specific defect though both a p53-dependent and p53-independent pathway, although this defect appears to be p53-independent at the earlier stages of development. Both p53-dependent and p53-independent mechanism in the pathogenesis of ribosome deficiency has been previously described\textsuperscript{12,14,16,23}.

Through analysis of gata1 expression, the specification of erythroid cells appears to be unaffected. Injection of morpholino into LCR2:EGFP embryos and analysis of pcna expression reveal that terminal maturation and proliferation are defective. Using the activin ligand trap RAP-011, we demonstrated that treatment of zebrafish embryos with RAP-011 could increase erythropoiesis and partially restore hemoglobin levels in embryos deficient for ribosomal proteins, rationalizing the use of Sotatercept in DBA patients. The incomplete rescue in erythroid levels may be due to several factors: 1) not all RAP-011 expanded cells can mature into erythroid cells and 2) protein translation is not fully rescued. Interestingly, L-Leucine was shown to overcome defects in zebrafish
models of ribosome deficiency and restore erythroid levels\textsuperscript{32,36}. This raises the possibility of using Sotatercept in combination with L-Leucine treatment in DBA patients.

Lft1, an antagonist to activin/nodal signaling, was upregulated in our RPL11 deficient zebrafish embryos, as well as in the RPL11 and RPS19 mutant embryos, albeit to a lower degree. We believe that differences in gene expression level between the knockout and knockdown may be due to the presence of maternal transcripts in the genetic model. Differences between the two RPL11 zebrafish models have been previously described\textsuperscript{24}. We demonstrated that this elevation of Lft1 could block the maturation of erythroid cells, potentially through antagonizing activin signaling. Past work in cell culture demonstrated the importance of activin signaling for the induction of globin during erythroid development\textsuperscript{37-42}. Using RAP-011, we can antagonize the effects of Lft1 overexpression. Others have demonstrated that Lft1 is a member of the TGF-\beta family of signaling molecules, and provided evidence that suggest Lft1 inhibits activin receptor signaling by binding to the activin receptors without activating it\textsuperscript{35}. RAP-011 may therefore enhance activin receptor signaling through sequestering Lft1.

Biochemical assays to identify if Lft1 can interact with RAP-011 \textit{in vitro} proved technically challenging. However, our rescue experiments, as well as work from other groups, support an \textit{in vivo} interaction between Lft1 and the activin receptor\textsuperscript{34,35}. We cannot rule out that the effects of Lft1 on erythroid development in our model are specific to the primitive stages erythropoiesis. The exact role of Lft1 in hematopoiesis, especially definitive hematopoiesis, remains an interesting question.
Other members of the TGF-β family cannot be ruled out to have a role in contributing to the erythroid defect. In some instances, members of the TGF-β family are not regulated at the transcriptional level, but rather at the post-translational level, such as through the cleavage of a repression domain by matrix metalloproteinases (MMPs)\(^{43,44}\). Upon increased MMP activity, latent TGF-β molecules are converted to active TGF-β. An increase in MMP9 was previously observed in response to rpl11 deficiency\(^{12,24}\). In our model, an increase in MMP9 is also observed (data not shown). The precise role of this elevated MMP9 has yet to be determined, but we speculate that this elevation in MMP9 may activate latent members of the TGF-β family in the ICM. This increase in TGF-β activity may explain the elevation of p21 independent of p53\(^{45}\). Interestingly, MMP9 is expressed within a subset of myeloid cells within the ICM\(^{46}\); therefore, it may be possible these myeloid cells secret MMP9, thereby activating the latent TGF-β family molecules, which in turn, effect the development of erythroid cells. Such a mechanism would be analogous to the regulation of erythroid cells by macrophages in the bone marrow\(^{47}\).

In the current study, we have provided a model where members of the TGF-β family of signaling molecules are misregulated during ribosome stress (Figure 2-7). These molecules affect the development of erythroid cells by blocking proliferation, delaying globin production and/or inducing cell-cycle arrest and apoptosis. RAP-011 binds to and sequesters several of these molecules, thus, restoring erythroid development. Understanding how an activin ligand trap such as RAP-011 can promote erythropoiesis
remains difficult to explain, given the contribution of activin molecules to erythroid development\textsuperscript{17,48}. Our work provides evidence to suggest RAP-011 can function to enhance activin signaling by antagonizing activin inhibitors such as Lft1. Therefore, RAP-011 can promote an increase in erythroid counts by maintaining homeostasis.

In humans, Lft1 (also called EBAF or TGF-\(\beta\)4) is highly expressed in women with abnormal endometrial bleeding and has been correlated with infertility\textsuperscript{49-53}. Also, expression of Lft1 has been observed in colonic, duodenal, and ovarian adenocarcinomas\textsuperscript{54}. The antagonistic relationship between Lft1 and RAP-011 raises the interesting possibility of using Sotatercept to treat these diseases where the expression of Lft1 is misregulated.

Finally, our studies demonstrate that protein drugs can be effectively evaluated in zebrafish disease models, offering a unique tool to study their mechanisms of action.
Methods

Zebrafish Stains and Husbandry

Zebrafish protocol and maintenance was performed using methods approved by the University of California, Los Angeles Institutional Animal Care and Use Committee (UCLA-IACUC).

Zebrafish Injections

Rpl11 was targeted in embryos by injecting a translational start codon morpholino (5’-CTTCTTCTCGCTCTGGTCCGCCATG-3’) into the cell at the one-cell stage. Unless noted, morpholino was used at ~1.0ng per embryo. mRNA (20pg) injection was also done at the one-cell stage into the cell of the developing embryo. RAP-011 and mouse IgG was injected at 4ng per embryo at 4.0hpf.

Quantitative PCR

Total RNA was extracted in Trizol Reagent (Life Technologies) according to manufacturer’s protocol. cDNA was synthesized using Superscript III (Life Technologies) and oligoDt (Life Technologies) following manufacturer’s protocol. Quantitative real-time PCR was performed using FastStart SYBR Green Master (Roche). Primer sequences can be found in supporting documents (Table S2-1).

Whole-Mount In Situ Hybridization
Embryos were fixed overnight at 4°C using 4%-paraformaldehyde (PFA) in PBS. After fixation, PFA was washed using four (5 min each) washes of PBS followed by two washes (5 min each) with 100% Methanol. Embryos were stored at -20°C. Whole-mount in situ hybridization was performed using labeled probed as previously described\textsuperscript{21}.

\textbf{o-Dianisidine staining}

Embryos were fixed at room temperature for 2 hour using 4%-paraformaldehyde (PFA) in PBS followed by three washes (5 min each) with PBS. Embryos were incubated in \textit{o}-dianisidine staining solution (40%Ethanol, 0.65% H\textsubscript{2}O\textsubscript{2}, 10mM Na-Acetate, and 0.6mg/ml \textit{o}-dianisidine (Sigma)) in the dark for 30 mins. After incubation, embryos were washed four times with PBS and then placed into bleach solution (1% KOH, 3%H\textsubscript{2}O\textsubscript{2}) for 20 mins to remove pigmentation. Embryos were then washed with PBS and immediately imaged.

\textbf{Flow Cytometry}

Embryos were removed from chorion and yolk protein was removed mechanically in 4°C calcium-free ringers solution. After deyolking, embryos were dispersed into single cell using a 0.5% trypsin solution. Trypsinization was stopped using DMEM supplemented with 10% FBS. Cells were then pelleted, resuspended in PBS with 1% BSA, and filtered with a 40 micron mesh.

\textbf{Immunostaining}
Embryos were fixed and dehydrated into methanol as described above. For 4.0hpf embryos, methanol was removed using four washes (5 min each) with PBS to fully rehydrate embryos. Embryos were rinsed once with blocking buffer (PBS with 0.1% Tween, 1% Triton Tx-100, 1% DMSO, 2mg/ml BSA) then blocked for 2 hrs at 4°C. RAP-011, RAP-536, and mouse IgG were detected using a fluorescently-conjugated secondary antibody raised against mouse IgG (Sigma). Secondary antibody was diluted in blocking buffer and incubated with embryos overnight at 4°C. Embryos were washed four times (1 hr each) with blocking buffer at 4°C then two times with PBS (30 min each) at 4°C. Embryos were imaged immediately.

For staining in 24hpf embryos, embryos were rinsed twice with 100% acetone at 4°C to remove methanol, then incubated in acetone at -20°C for 20mins prior to rehydration. After acetone treatment, embryos were rehydrated and blocked as described above. Detection of GFP was done using a rabbit anti-GFP antibody (life technologies) diluted in blocking buffer. Embryos were incubated in diluted antibodies overnight at 4°C. Excess anti-GFP antibody was removed by washing with blocking buffer four times (1 hr each) at 4°C. Anti-rabbit IgG and anti-mouse IgG fluorescently-conjugated secondary antibody were diluted into blocking buffer and incubated with embryos overnight at 4°C. Excess antibody was removed as described above and embryos were immediately imaged.

**Zebrafish embryos heat shock**
Embryos were placed into 6-well tissue culture plates containing 3ml of fish water. Plates were then floated in a 38.5°C water bath for 30mins. After heat shock, plates were kept at 28.0°C until desired time point for analysis.

**Wright Giemsa Staining**

Circulating erythroid cells were collected by puncturing the heart of zebrafish embryos and allowing cells to pool in a solution of PBS supplemented with 1% BSA. Cells were dried onto glass slides using a 42°C heat block. Cells were methanol fixed for 10mins on slides before submerging into Wright Giemsa stain (Sigma). Staining was done for 20 mins at room temperature. After staining, slides were rinsed with distilled water and immediately imaged.

**Construct Cloning for mRNA Injection and Probes**

Plasmids for generating mRNA and antisense probe were made using Gateway technology (Life Technologies). Genes were amplified using primers flanked with attB1 forward or attB2 reverse sequences. After amplification, PCR product used in a BP clonase II reaction with pDONR221 (Life Technologies). For generating DIG labeled antisense probes, plasmids were linearized and synthesized using T7 RNA polymerase (Promega) and DIG RNA Labeling Mix (Roche). Cloning primers used can be found in supporting documents (Table S2-2)

For mRNA synthesis, middle entry clones with the gene of interest were used in a multi-site gateway reaction using LR clonase II plus (Life Technologies). The plasmid was
recombined into a destination vector containing a SP6 promoter and a 6Xmyc tag for c-terminal fusion. Plasmid was linearized using NotI and capped mRNA was synthesized using mMessage mMACHINE SP6 Transcription Kit (Ambion). RNA was purified using LiCl precipitation and suspended in RNase-free water.

**Western Blot**

Embryos were deyolked as above and cells were lysed by boiling in Laemmli buffer. Anti-rpl11 (Abcam), Anti-Tubulin (Sigma), and Anti-EGFP (Life Technologies) antibodies were used.
Figure 2-1. Knockdown of Rpl11 in Zebrafish Embryos Model the Erythroid and Developmental Defect in DBA. Rpl11 in embryos was targeted using a translational start codon morpholino. (A) Western Blot against Rpl11 in 24 hpf embryos injected with various doses of morpholino. (B) Transcript quantification of p53, mdm2, and p21 at 24 hpf. (C, D) Morphological features of morphants at 24 hpf, C, and 48 hpf, D. Arrowheads point to the defective head development and shorted tail extension at 24 hpf. At 48 hpf, a smaller head, edema, and defective yolk extension was observed (arrowhead) in morphants. (E) α-dianisidine staining of zebrafish embryos at 48 hpf. (F) Flk:dsred transgenic zebrafish at 48 hpf. (G) Mpo:EGFP transgenic zebrafish at 48hpf.
Figure 2-1. Knockdown of Rpl11 in Zebrafish Embryos Model the Erythroid and Developmental Defect in DBA.
Figure 2-2. Terminal Differentiation and Proliferation, but not Specification, are Defective in RPL11 Deficiency. Erythroid cells at various stages of development were analyzed. (A, B) In situ hybridization for gata1 expression. A, 4 somites stage. B, 30 hpf. (C) Time course of morphants in LCR2:EGFP transgenic zebrafish. (D, E) Flow cytometry analysis of EGFP positive cells. D, EGFP intensity. E, Cell Size, measured by forward scatter. *, p<.05 (F) Wright Giemsa staining of isolated erythroid cells collected from zebrafish embryos. (G) In situ hybridization for pcna in uninjected or morpholino injected embryos. Observed phenotype: G, uninjected=24/24 morphants=23/25.
Figure 2-2. Terminal Differentiation and Proliferation, but not Specification, are Defective in RPL11 Deficiency.
Figure 2-3. Inhibition of p53 Partially Restored Erythroid Levels at Later Stages of Development. Rpl11 morpholino was injected in p53-null zebrafish embryos. (A) Morphology of p53-null zebrafish embryos at 24 hpf. Arrowhead indicates the recovery of aplasia in the head. (B) Transcript quantification for p53 and genes downstream of p53: mdm2, p21, bax, and puma at 24 hpf. (C) Top, morphology of p53-null zebrafish embryos at 48 hpf. Arrowhead points to the presence of a smaller head, edema, and defective yolk extension. Bottom, o-dianisidine staining of embryos reveals lack of hemoglobin positive cells. (D) O-dianisidine staining of wt, top, or p53-null, bottom, embryos at 72 hpf. Arrowhead points to the recovery of erythroid cells in p53-null embryos. Observed phenotype: D, wt+rpl11 MO=40/40 p53(-/-)+rpl11 MO=30/40.
Figure 2-3. Inhibition of p53 Partially Restored Erythroid Levels at Later Stages of Development.
Figure 2-4. RAP-011 Expands Erythroid Cells in Zebrafish Embryos. (A, B) Expansion of ICM observed in RAP-011 treated embryos but not in IgG control. Arrow points to the ICM region. (C) Western blot of EGFP from LCR2:EGFP embryos treated with IgG or RAP-011. (D) *In situ* hybridization for gata1 expression in IgG or RAP-011 treated embryos at 24 hpf. (E) *In situ* hybridization for pcna expression in IgG or RAP-011 treated embryos at 24 hpf. Arrows in D and E point to the expanded ICM region. Observed Phenotype: D, wt+IgG=30/30, wt+RAP-011=26/30, E, wt+IgG=29/30, wt+RAP-011=27/29.
Figure 2-4. RAP-011 Expands Erythroid Cells in Zebrafish Embryos.
Figure 2-5. RAP-011 Can Restore Erythroid Levels in Ribosome Deficient Embryos. (A) Rpl11 morpholino injected embryos were treated with IgG or RAP-011. Staining of 48 hpf embryos with o-dianisidine shows a partial recovery (seen in ~87.0% of embryos, p<.05) of globin positive cells, arrows. (B) In situ hybridization for pcna expression in morpholino injected embryos, 24 hpf, treated with IgG or RAP-011. Rescue by pcna staining seen in ~87.2% of embryos, p<.05. (C, D) o-dianisidine staining of RPL11 or RPS19 mutant embryos treated with RAP-011. Observed phenotype: A, Rpl11 MO+IgG=32/32 Rpl11 MO+RAP-011=54/62, B, Rpl11 MO+IgG=58/58 Rpl11 MO+RAP-011=41/47, C, Rpl11(-/-)=15/15 Rpl11(-/-)+RAP-011=10/10, D, Rps19(-/-)=8/8 Rps19(-/-)+RAP-011=12/12.
Figure 2-5. RAP-011 Can Restore Erythroid Levels in Ribosome Deficient Embryos.
Figure 2-6. RAP-011 Antagonizes the Function of Lft1. (A) Illustrated cross-section of ICM region. Secreted factors from somites, neurotube, notochord, mesenchyme, and endothelial cells can affect erythroid development. (B) Left, In situ hybridization for GDF6a, GDF11, Lft1, and BMP4 at 18 hpf. Right, illustration of the expression pattern of various TGF-β family members. (C) Transcript quantification of TGF-β family members in rpl11 morphants compared to control embryos at 48 hpf. (D) hsp:cre-dsRed transgenic embryos injected with plasmid for the temporal expression of Lft1. Embryos were also treated with IgG or RAP-011. o-dianisidine staining of embryos at 48 hpf. Arrows points to elevated erythroid cells seen in caudal vein and head region (D’). Observed phenotype: D, Uninjected (no heatshock)=30/30, Uninjected (heatshock)=26/26, Injected (no heatshock)=26/28, Injected (heatshock)=25/30, Injected (heatshock)+IgG=23/25, Injected (heatshock)+RAP-011=23/30.
Figure 2-6. RAP-011 Antagonizes the Function of Lft1.
Figure 2-7. Model of Erythroid Failure during Ribosome Stress in Zebrafish Embryos and Rescue with RAP-011. Multiple defects lead to the erythroid failure in zebrafish embryos upon ribosome deficiency. i) Ribosome stress lead to lower rates of protein synthesis. ii) Increase in p53 activity in erythroid cells lead to cell-cycle arrest and cell-death following the delay in maturation. iii) Lft1 blocks the erythroid promoting activity of activin/nodal signaling. iv) Elevated MMP9 activity converts latent TGF-β to its active form. Activated TGF-β / TGF-β superfamily members induce cell-cycle arrest in erythroid cells. v) RAP-011 sequesters and block active TGF-β superfamily members and Lft1 from binding to the endogenous receptor.
Figure 2-7. Model of Erythroid Failure during Ribosome Stress in Zebrafish Embryos and Rescue with RAP-011.
Supplemental Figure S2-1. Expression of rag1 in morphants vs. uninjected control at 4 dpf.
**Supplemental Figure S2-2.** Acridine orange staining in morphants vs. uninjected control at 48 hpf.
Supplemental Figure S2-3. Relative fold induction of gata1 transcripts upon rpl11 deficiency at 30 hpf.
**Supplemental Figure S2-4.** Extracellular domain alignment of the activin receptor type IIa between human, mouse, and zebrafish. Zebrafish has two copies of the receptor, ACVR2Aa and ACVR2Ab. Alignment was performed using Clustal Omega.

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**Supplemental Figure S2-5.** (A) Rpl11 morpholino was injected into the cell at one-cell stage. RAP-011, or IgG, was injected into the embryo at approximately 4.0 hpf. (B) Immunostaining using anti-mouse IgG to detect RAP-011 or IgG injected into the embryo at 4.0 hpf. (C) RAP-011 or IgG was injected into LCR2:EGFP zebrafish embryos. At 24 hpf, immunostaining for the IgG domain or GFP was performed. White bar indicate expanded EGFP expression.
Supplemental Figure S2-6. RAP-011 expands the ICM in a dose dependent manner. At levels above 4.0ng, toxicity from the drug becomes apparent (i.e. edema, craniofacial defects). No significant levels of abnormalities were observed in 20ng of IgG injected embryos.
Supplemental Figure S2-7. mpo:EGFP transgenic embryos were injected with RAP-011 or IgG as a control. An expansion in the number of EGFP positive cells was seen.
Supplemental Figure S2-8. Fold change expression level of p53, p21, and mdm2 in morphants treated with RAP-011 compared to morphants treated with IgG.
**Supplemental Figure S2-9.** Treatment of zebrafish with dexamethasone and RAP-011 have a additive effect (arrows). LCR2:EGFP transgenic embryos were treated with RAP-011, 50μM dexamethasone, or both.
**Supplemental Figure S2-10.** Expression pattern of various members of the TGF-β family of signaling molecules was analyzed by whole-mount *in situ* hybridization. Age of embryos are approximately 18-20 hpf.
Supplemental Figure S2-11. Relative fold induction of Bmp4, Lft1, gdf6a, and gdf11 transcripts upon rpl11 or rps19 ribosomal protein deficiency. Fold induction is calculated as mutants over sibling control.
**Supplemental Figure S2-12.** (A) Diagram of plasmid used to temporally overexpress Lft1 in zebrafish embryos. (B) Plasmid was injected into hsp:cre-dsRED transgenic embryos and heat shocked at 18 hpf. By 24 hpf (6 hours post heat shock) induction of cre-dsRED can be observed. At 48 hpf, EGFP was expressed at lower levels in heat shocked embryos compared to non-heat shocked control (C) Function of construct was further validated by co-injecting cre mRNA with plasmid. Co-injection with cre caused growth defects in embryos.
**Supplemental Figure S2-13.** (A) Injection of Lft1 mRNA caused growth defects and lack of globin positive cells in a concentration dependent manner. (B) anti-myc blot of embryos injected with mRNA for myc-tagged Lft1 or mCherry. (C) o-dianisidine stain of Lft1-myc injected embryos treated with IgG or RAP-011.
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**Table S2-2.** Primers used for cloning. All forward primers were flanked with *attB1* Fwd sequence: `GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACC`. Reverse primers were flanked with *attB2* sequence: `GGGGACCACTTTGTACAAAAAAGCAGGCTCCACC`.

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Chapter References


30. Mulivor AW, Barbosa D, Kumar R, Sherman ML, Seehra J, Pearsall RS. RAP-011, a Soluble Activin Receptor Type IIa Murine IgG-Fc Fusion Protein, Prevents Chemotherapy Induced Anemia. ASH Annual Meeting Abstracts. 2009;114(22):161-.


CHAPTER 3

A Zebrafish Model of 5q-Syndrome using CRISPR/Cas9 to Target RPS14
Abstract

5q-syndrome is a distinct form of myelodysplastic syndrome (MDS) where a deletion on chromosome 5 is the underlying cause. Various hematological features are associated with the disease, including severe macrocytic anemia. Genetic mapping and studies using various models provide evidence that the ribosomal protein RPS14 is a candidate gene for the erythroid failure. Targeted disruption of RPS14, as well as of many other ribosomal proteins, causes an increase in p53 activity and p53-mediated apoptosis of the erythroid cell. However, due to the high risk for cancer development in patients with aberrant ribosome function, targeting the p53 pathway is not a viable treatment option. To better understand the pathology of RPS14 deficiency in 5q-deletion, we generated a zebrafish model harboring a mutation in the RPS14 gene. This model mirrors the anemic phenotype seen in 5q-syndrome and, moreover, the anemia is due to a late-stage erythropoietic defect. Finally, we demonstrated the ability of RAP-011, an activin receptor type IIA ligand trap, to reverse the anemia in our zebrafish model.
**Introduction**

Chromosome 5q-deletion syndrome, a.k.a. 5q-syndrome, is a hematological disorder characterized by a deletion in part of the long arm of chromosome 5. It is characterized as a distinct form of myelodysplastic syndrome (MDS). Approximately 10 to 20% of all MDS cases are caused by chromosomal deletions. Patients with 5q-syndrome have macrocytic anemia, and commonly present themselves with other hematological phenotypes (i.e. thrombocytosis, megakaryocyte hyperplasia). Unlike MDS patients without 5q deletion, there is a low rate of progression to acute myeloid leukemia (AML). Lenalidomide, a thalidomide analog, has been shown to increase erythropoiesis in patients with 5q-syndrome; however, in some cases, there is a deleterious effect, which leads to thrombocytopenia and neutropenia. These side effects raise a need for further mechanistic studies on 5q-syndrome and the identification of novel therapeutics.

A commonly deleted region (CDR) of 5q-syndrome has been narrowed to a 1.5 Mb locus on the chromosome. Several genes in this CDR have been suggested to play a role in the pathogenesis of the disease, with haploinsufficiency of ribosomal protein S14 (RPS14) contributing to the anemic phenotype. Its expression is severely down regulated in 5q-deletion patients. Knockdown of RPS14 using RNAi in CD34+ hematopoietic progenitor cells severely affected erythroid differentiation. Furthermore, a small chromosomal deletion in a mouse model containing a deletion of RPS14 was shown to have macrocytic anemia. The correlation between RPS14 and
5q-syndrome has led the disorder to be classified as a ribosomopathy (a disorder due to aberrant ribosome function or biogenesis).

Haploinsufficiency of ribosomal proteins has been implicated in several bone marrow failure disorders such as Diamond Blackfan anemia (DBA), Dyskeratosis congenital (DKC), Schwachmann-Diamond Syndrome (SDS), and Cartilage-hair hypoplasia (CHH). Malfunctioning ribosomal proteins have been shown to cause an increase in p53-activity, leading to cell cycle arrest and apoptosis through the ribosomal protein (RP)-mdm2-p53 checkpoint mechanism\(^9,13\). Indeed, in the mouse model of 5q-syndrome, an elevated level of p53 and cell death is observed. Breeding the model into p53-null background mice reverses many aspect of the disease\(^12\). While this may suggest targeting p53 to be a potential therapeutic strategy, caution is warranted in this approach due to the risk in tumor progression in targeting this pathway. Recently, L-leucine treatment was demonstrated to restore erythroid levels in a zebrafish model of RPS14 deficiency through a p53-independent mechanism\(^14\).

Here, through the use of CRISPR/Cas9 gene targeting, we report the generation of a zebrafish model of RPS14 deletion that mirrors the anemic phenotype of patients with 5q-syndrome. Using this model, we demonstrated that a p53-independent mechanism leads to the delay in erythroid maturation. Furthermore, this delay is reversed upon treatment with RAP-011, an activin receptor type 2A ligand trap.

Results
**CRISPR/Cas9 Targeting of RPS14**

Deletion of the CDR on chromosome 5 is expected to lead to halpoinsufficient levels of RPS14. The zebrafish RPS14 gene is located on chromosome 21 and shares over 99% amino acid identity to the human RPS14 (Supplemental Figure 3-1). To target the RPS14 gene in zebrafish using CRISPR/Cas9, we generated a guide-RNA (gRNA) to a region on exon2 of the gene. To facilitate validation of nuclease activity and screening, we targeted near an AvrII restriction digest site on exon 2 (Figure 3-1A). Injection of the synthesized gRNA along with Cas9 mRNA into the zebrafish embryos successfully targeted the AvrII restriction enzyme cut site, as indicated by the presence of an upper band after restriction digest of the PCR product (Figure 3-1B). Interestingly, a portion of injected embryos showed an erythroid defect similar to that observed in RPS14 morphants (Figure 3-1C)\(^{14}\). This suggests that our constructs were successful in targeting the RPS14 gene.

Injected embryos were raised to adulthood and screened for transmission into germline using the above restriction digest procedure on F1 embryos (Figure 3-1D). Embryos from germline-transmitted fish were raised to adult, and genotyped by fin clip. Positive fish were identified containing insertions and deletions, thus confirming the successful targeting of RPS14 in zebrafish (Figure 3-1E).

**Deficiency of RPS14 Leads to Morphological Defects**

Three mutant lines were outcrossed to generate working stocks of fish for further analysis (Figure 3-1E and F). Two lines had the introduction of an early stop codon
(RPS14Δ4bp and RPS14ΔINS) and one line (RPS14Δ21bp) carried an inframe deletion (Figure 3-1F).

When the RPS14ΔINS were incrossed, at 24 hours post fertilization (hpf), aplasia in the developing head of the mutant embryos can be observed (Figure 3-2A). Later on, embryos displayed decreased pigmentation levels and a defective yolk extension at 30 hpf (Figure 3-2B). By 48 hpf, there is a slight recovery of pigmentation; however, the embryos had a smaller head, edema, and retained a defective yolk extension (Figure 3-2C). Overtime, the severity of the phenotypes increases and the mutation become lethal around 6 to 7 days post fertilization (dpf) (Figure 3-2D through F). Reduced levels of RPS14 transcript was confirmed though mRNA in situ hybridization (Figure 3-2G).

These morphologically abnormal embryos were confirmed to be mutants for RPS14 through PCR validation (Figure 3-H). The RPS14Δ4bp also displayed similar morphological abnormalities to the RPS14ΔINS line (Supplemental Figure 3-2).

No observable defects were present in the RPS14Δ21bp (Supplemental Figure 3-2). In fact, homozygous carriers of this mutation were able to grow to adulthood and produce homozygous progeny (Supplemental Figures 3-3). Overall, these data suggest that a null mutation in RPS14 leads to the morphological defects seen in zebrafish embryos.

**Erythroid Defect is observed upon RPS14 Deficiency**
Next, we sought to study the effects of RPS14 deficiency on the development of erythroid cells. RPS14ΔINS embryos at 48 hpf and 3 dpf showed decreased levels of o-dianisidine staining positive cells, suggesting a defect in the production of mature erythroid cells in the developing embryos (Figure 3-3A and B). Similar erythroid defects were also observed in the RPS14 Δ4bp line, but not in the RPS14Δ21bp line (Supplemental Figure 3-2). Because no obvious defects between the RPS14ΔINS and RPS14Δ4bp can be detected, the RPS14ΔINS was selected for the remainder of experiments, due to ease of genotyping.

Next, to determine if the defect in erythroid development is due to improper specification of the erythroid lineage, we performed whole mount in situ hybridization (WISH) for gata1 expression on zebrafish embryos. There was no observable difference in expression levels between mutants and sibling controls, suggesting that upon RPS14 deficiency, specification of hematopoietic cells into the erythroid lineage is unaffected (Figure 3-3C).

Normal specification of the erythroid lineage, but a failure to produce mature erythroid cells maybe be due to defects in the production of sufficient globin levels or early apoptosis of the erythroid cells. To help address this issue, we bred the RPS14 mutation into the LCR:EGFP transgenic fish line which specifically label all globin positive cells with GFP. Interestingly, in mutants, we observed a similar degree in the amount of GFP-positive cells between mutants and sibling control at both 24 and 48 hpf, albeit GFP-intensity was lower in mutants (Figure 3-3D and E). At 3dpf, there appears to be less
GFP positive cells (Figure 3-3F). These data suggest that a defect in the terminal maturation followed by cell death of erythroid cells occurs upon RPS14 deficiency.

**p53-activity is upregulated in embryos deficient for RPS14**

Haploinsufficiency of ribosome proteins has been shown to lead to elevated levels of p53 and activation of the p53 pathway. As expected, a mutation in RPS14 led to increased levels of p53 and the p53-target genes p21, mdm2, puma, and bax (Figure 3-4A). Interestingly, when we analyzed the expression of p53, p21, and mdm2 by WISH, we observed ubiquitous overexpression of these genes (Figure 3-4B). Therefore, in response to a deficiency in RPS14, a systemic increase in p53-activity is observed.

To determine if the delay in erythroid maturation is due to an activation of p53 signaling, morpholino (MO) was used to knockdown p53 in RPS14 mutants. O-dianisidine staining of mutants treated with p53 MO revealed no observable changes in hemoglobin levels (Figure 3-4C).

**RAP-011 treatment of RPS14 mutants reverses the anemic phenotype**

RAP-011 was previously shown to restore erythroid levels in a zebrafish model of DBA through a p53-independent mechanism\(^\text{15}\). We, therefore, tested if RAP-011 has the potential to recover the anemic phenotype in our RPS14 mutant model. When RPS14 mutant fish were treated with RAP-011, an increase in o-dianisidine positive staining was observed compared to untreated control (Figure 3-4C).
Discussion

In this work, we successfully targeted RPS14 in the zebrafish using CRISPR/Cas9 gene-targeting technology. Upon RPS14 deficiency, gross morphological defects can be observed. Furthermore, the impaired growth is accompanied with severe anemia.

The anemia observed upon RPS14 deficiency is due to a late-stage maturation delay in the erythroid cell, specifically, an inability to produce sufficient hemoglobin level. Morpholino knockdown of p53 did not restore hemoglobin levels in our model, suggesting a p53-independent mechanism for the delay in terminal maturation. It was reported in a recent model of RPS14 deficiency in mice that a translational defect in addition to p53 activation underscores the erythroid differentiation defect. Furthermore, inhibition of p53 only partially restores translational levels\textsuperscript{16}.

We further show that the delay in erythroid maturation is reversed upon treatment with RAP-011. Treatment with RAP-011 appears to be specific to the erythroid cells, as the morphological defects were not rescued.

Recent works comparing morpholino vs. genetic mutants has raise concern regarding the off-target effects of morpholino\textsuperscript{17}. The phenotype in our model mirrors the anemia observed upon knockdown of RPS14 in zebrafish using morpholino\textsuperscript{14}. Thus, our work validates previous work in zebrafish using morpholino against RPS14 and adds a new tool in the field for the study of ribosome function and ribosome associated disorders.
Materials and Methods

Cas9 mRNA and guide RNA synthesis

Zebrafish optimized cas9 mRNA was in vitro transcribed from pGH-T7-zCas9 as previously described\(^\text{18}\). 200 pg of Cas9 mRNA was injected per embryo at the one-cell stage.

RPS14 gRNA was synthesized using a protocol similar to previously described methods\(^\text{19}\). RPS14 gene specific sequence was flanked with a T7 promoter site at the 5’ region and a scaffold sequence on the 3’ region (Oligo: ATTTAGGTGACACTATAGAAGAGCAGGTCATCAGCTGTTTTAGAGCTAGAAATAGC). This oligo was used in a PCR reaction to generate template for guide RNA synthesis. Guide RNA was in vitro transcribed using MAXIscript T7 Kit (Life Technologies). After synthesis, DNA template was removed using TURBO DNase (Life Technologies) and purified using phenol chloroform. 50pg of gRNA was injected per embryos at the one-cell stage.

PCR validation, Sequencing Mutation, and Genotyping of embryos

To extract genomic DNA, embryos (or tail fin clips) were lysed in 50mM NaOH by boiling at 95°C for 1hr. After boiling, lysate were neutralized with 10% volume 1M Tris (pH=8.0). Crude lysates were then used in a PCR reaction.

To validate function successful targeting of RPS14 and identify founder fish, the region flanking the targeted region was first PCR amplified using RPS14 Fwd: CTGTTGACTGTGTGTTTCAGCA and RPS14 Rev: TCAACATTTCAATGCAACAAAC
primers. Afterwards, PCR product was used in a restriction digest reaction using AvrII (NEB).

To determine the sequence of mutants, PCR products from positive carriers were used in a TOPO-TA reaction (Life Technologies) according to manufacturers' direction.

After generating a stable mutant line, fish lines were genotype using primers RPS14 gel screen Fwd: ATGGCTCCTCGCAAGGTAAGG and RPS14 gel screen Rev: TGTGCACACAAAGACACTGTT. The PCR product was then run on a TBE-PAGE gel.

**o-Dianisidine staining**

Embryos were first fixed at room temperature for 2 hour using 4%-paraformaldehyde (PFA) in PBS. Next, three washes (5 min each) with PBS was performed to remove PFA. Embryos were then incubated in an o-dianisidine staining solution (40%Ethanol, 0.65% H₂O₂, 10mM Na-Acetate, and 0.6mg/ml o-dianisidine (Sigma)) in the dark for 30 mins followed by four washes times with PBS. To remove pigmentation, embryos were placed into a bleach solution (1% KOH, 3%H₂O₂) for 20 mins. Finally, embryos were washed with PBS and immediately imaged.

**mRNA Probes and In Situ Hybridization**

Plasmids for antisense probe were made using Gateway technology (Life Technologies). Genes were amplified using primers flanked with attB1 forward or attB2 reverse sequences and then used in a BP clonase II reaction with pDONR221 (Life
Technologies). To generate DIG labeled antisense probes, plasmids were linearized and synthesized using T7 RNA polymerase (Promega) with DIG RNA Labeling Mix (Roche).

For hybridization, embryos were first fixed overnight at 4°C using 4%-paraformaldehyde (PFA) in PBS. After fixation, PFA was washed four times (5 min each) with PBS, followed by two washes (5 min each) with 100% Methanol. Embryos were stored at -20°C. Whole-mount in situ hybridization was performed using labeled probed as previously described20.

**Real-time quantitative PCR**

To generate cDNA for real-time quantitative PCR analysis, total RNA was first isolated from whole embryos using TRIzol Reagent (Life Technologies) following manufacture’s protocol. Next, cDNA was reverse transcribed using Oligo(dT) and SuperScript III reverse transcriptase (Life Technologies).

For quantitative PCR analysis, cDNA was used in a PCR reaction using FastStart SYBR Green Master (Roche).
**Figure 3-1. Targeting RPS14 using CRISPR/Cas9.** A) Diagram of targeted region on RPS14 gene. B) Validation of gene targeting in injected embryos. C) A percentage of embryos injected with Cas9 mRNA and gRNA showed reduced o-dianisidine staining. D) Illustration of screening procedure (*top*). Clutch of embryos positive for RPS14 mutation were raised to adults and genotyped by fin clip (*bottom*). E) Three mutant alleles were maintained for further analysis. F) Predicted protein sequence of RPS14 mutant (*indicates early termination of protein sequence*).
Figure 3-1. Targeting RPS14 using CRISPR/Cas9

A. Schematic representation of RPS14 and its exons, showing the Cas9 nuclease region and the guide RNA sequence targeting the AvrII cut site.

B. Photographs of zebrafish embryos. Uninjected embryos (left) and gRNA/Cas9 injected embryos (right) at 48 hours post-fertilization (hpf).

C. Flowchart diagram illustrating the generations from F0 embryos to F1 adults, showing the targeting and editing process.

D. Sequence comparisons of RPS14 WT, RPS14A4bp, RPS14A21bp, and RPS14INS variants with the corresponding Cas9 cleavage sites.
Figure 3-2. RPS14 deficiency leads to morphological defects. A-F) Morphological phenotype of RPS14ΔINS mutants compared to sibling control. G) In situ hybridization of RPS14 transcript levels in mutants compared to sibling control. H) Mutants were validated via genotyping. Genotype is of 48 hpf embryos.
Figure 3-2. RPS14 deficiency leads to morphological defects
Figure 3-3. RPS14 mutants have defects in erythroid maturation. A, B) o-dianisidine staining of RPS14 mutants at 48 hpf, A, or 3 dpf, B. C) *In situ* hybridization of gata1 expression in RPS14 mutants compared to sibling control. D-F) RPS14 mutant line was bred into a LCR2:GFP transgenic line. Double positive carriers were incrossed and RPS14 null mutants were analyzed for GFP expression. D, 24 hpf. E, 48 hpf. F, 3 dpf.
Figure 3-3. RPS14 mutants have defects in erythroid maturation
Figure 3-4. Delay in erythroid maturation is independent of p53-activity and reversed upon RAP-011 treatment. A) Real-time quantitative PCR for p53, p21, mdm2, and bax in RPS14 mutants compared to sibling control. B) *In situ* hybridization for p53, p21, and mdm2 in mutant embryos. C) *o*-dianisidine of RPS14 mutants treated with p53 morpholino (p53 MO) or RAP-011 at 48 hpf.
Figure 3-4. Delay in erythroid maturation is independent of p53-activity and reversed upon RAP-011 treatment
Supplemental Figure 3-1. Alignment of Human and Zebrafish RPS14 protein.

Alignment of human and zebrafish protein sequence using clustal omega. Over 99% sequence identity between the two species.
Supplemental Figure 3-1. Alignment of Human and Zebrafish RPS14 protein

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Supplemental Figure 3-2. Mutant alleles of RPS14. A and B) Morphological phenotype of RPS14Δ4bp mutants at 30 hpf, A, and 48 hpf, B. C) o-dianisidine staining of RPS14Δ4bp mutants at 48 hpf. D) Genotype of individual mutants confirmed by PCR. E and F) Morphological phenotype of RPS14Δ421bp mutants at 30 hpf, E, and 48 hpf, F. G) o-dianisidine staining of RPS14Δ21bp mutants at 48 hpf. H) Genotype of individual embryos confirmed by PCR.
Supplemental Figure 3-2. Mutant alleles of RPS14
Supplemental Figure 3-3. RPS14Δ21bp are viable as adults. A) RPS14Δ21bp male and female fish at approximately 3 months of age. Wild type (wt) fish are also provided for comparison. B) Homozygous RPS14Δ21bp fish were incrossed and PCR genotyping was performed on embryos at 48 hpf. Left of Marker, from wt incross. Right of Marker, from mutant incross.
Supplemental Figure 3-3. RPS14Δ421bp are viable as adults
Chapter References


CHAPTER 4

Concluding Remarks
In this dissertation, I have studied the effects of aberrant ribosome function on erythroid development. In Chapter 1, I present a review on two diseases associated with malfunctioning ribosomes, Diamond Blackfan anemia and 5q-Syndrome. Additionally, I discuss the use of zebrafish as an animal model to study human diseases. In Chapter 2, I describe a zebrafish model of DBA using morpholino to knockdown rpl11 to haploinsufficient levels. Zebrafish deficient in rpl11 displayed delayed erythroid maturation and reduced erythroid counts. Injection of morpholino into p53-null zebrafish revealed that the delay in erythroid maturation is independent on p53 signaling. RAP-011 is an activin receptor type IIA ligand trap drug and can increase erythroid parameters in zebrafish. This is similar to observations made in human studies using Sotatercept, the human version of RAP-011. Furthermore, I demonstrated the ability of RAP-011 to reverse the anemic phenotype in our zebrafish model of DBA. Surprisingly, deficiency of rpl11 led to an increase in lft1 expression. The effects of lft1 overexpression can be reverse with RAP-011. In Chapter 3, I describe a zebrafish model of 5q-syndrome through RPS14 knockout. Gene targeting using CRISPR/Cas9 was more attractive in this model due to the lack of available antibodies for RPS14 detection. Without available antibodies, titrating RPS14 morpholino to achieve haploinsufficient levels remains challenging. Using this model, I demonstrated that RPS14 deficiency causes a delay in erythroid maturation similar to observations made in our DBA model. This delay in erythroid maturation could be reversed using RAP-011. However, much still remains to be learned about the mechanism of erythroid failure and the function of RAP-011. In Chapter 4, I will discuss possible future studies that can address these issues. Furthermore, I will discuss the implications of this work.
**Lft1 function in erythroid development**

Activin signaling has been demonstrated to be crucial in the induction of erythroid differentiation. Therefore, understanding how a ligand trap such as RAP-011 can promote erythropoiesis has been difficult to explain. Lft1 is a member of the TGF-β family of signaling molecules and has been shown to inhibit activin signaling. It has been suggested that Lft1 inhibits activin receptor via binding to the activin receptor and preventing activin molecules from binding. It was also shown that overexpressing the extracellular domain of the activin receptor type IIA could reverse the effects of Lft1 overexpression. RAP-011 may also be reversing the effects of Lft1 overexpression via a similar mechanism to ectopic expression of activin receptor type IIA extracellular domain. If this is true, then RAP-011 may promote erythropoiesis by enhancing activin receptor signaling. To test if RAP-011 can indeed promote activin signaling, it would be necessary to study, at the cellular level, the activity of downstream molecules in activin receptor signaling. This will include measuring the phosphorylation state of SMAD molecules, observing SMAD cellular localization, and analyzing target-gene activation.

It will be necessary to determine if the interaction between Lft1 and RAP-011 is direct or if the antagonist relationship is due to an indirect interaction. *In vivo* work with Lft1 and activin receptor overexpression suggests that the interaction is direct; thus, the interaction between RAP-011 and Lft1 is also hypothesized to be direct. Biochemical interaction assays such as co-immunoprecipitation or surface plasmon resonance (SRP) imunoassays will aid in elucidating this question.
MMP9 and Other TGF-β family molecules during erythroid development

Furthermore, it would be interesting to see if other members of the TGF-β family are involved in the pathogenesis of erythroid failure in our model. Although our model did not show upregulation in gene expression of many members of the TGF-β family of molecules, they cannot be excluded from playing a role in the erythroid failure. In fact, many members in the TGF-β family are not regulated at the gene expression level; they are regulated post-translationally through cleavage of the inhibitory domain. Removal of the inhibitory domain occurs through proteolytic cleavage by matrix metalloproteinases (MMPs).

Previous work on MMP-9 and TGF-β demonstrated that increase activity of MMP9 could activate TGF-β molecules. Increase in TGF-β can lead to induction of cell cycle arrest through increase expression of the cell cycle inhibitor p21.

In our model, there was a significant increase in the expression level of MMP9. Therefore, it may be possible that TGF-β activity is elevated through enhance MMP9 activity and this leads to cell cycle arrest in the erythroid cells. To test this hypothesis, it will be necessary to inhibit the function of MMP9 in our model and observe the effects on erythroid development. This can be easily achieved through chemical inhibition or gene knockdown of MMP9 using morpholino.

Furthermore, it will be interesting to see if this increase in TGF-β signaling is synergistic with p53 activity. It has been observed that TGF-β signaling and p53 interact
synergistically to enhance p21 gene expression. It may, therefore, be possible that these two signaling pathways act in combination, which leads to the cycle arrest and apoptosis seen in our model. A suboptimal dose of a p53 inhibitor and a TGF-β signaling inhibitor can be applied in combination in our model to test this hypothesis.

**Implications of this work**

In this body of work, we have identified a novel therapeutic strategy that can be used in the treatment of anemia associated with aberrant ribosomal proteins such as those seen during DBA and 5q-syndrome. A key feature for the appeal of using RAP-011 in therapeutic strategy is that it functions independently of p53 signaling; therefore, tumor risk should not be affect using this drug. Furthermore, this work has shown Lft1 to be a molecule that may be involved in the erythroid failure in DBA. Due to the antagonist relationship between Lft1 and RAP-011, RAP-011 may also be applied to disorders with elevated levels of Lft1. Such disorders include certain cancers and endometrial bleeding. Furthermore, our model of 5q-syndrome offers a new tool to further study the affects of aberrant ribosome function in an *in vivo* model. In summary, our work has demonstrated the importance of zebrafish as a model for human disorders and to understand the function of therapeutic drugs.