INVESTIGATION OF NOVEL DRUGS TO RESCUE RIBOSOMAL PROTEIN DEFICIENCIES

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Abstract

Mutations in ribosomal protein genes cause a complex category of diseases, known as ribosomopathies. Diamond-Blackfan anemia is the best studied example of these syndromes and characterized as a rare, but severe congenital hypoplastic anemia, which often goes along with short stature, craniofacial and other physical abnormalities, and even an increased risk to develop cancer. Other ribosomopathies might differ from Diamond-Blackfan anemia in their clinical phenotypes and associated genes, however, the common root of all of them lies in a defect in ribosome biogenesis and/or function.[1]

The main regulator of the pathophysiology of these diseases is p53. The role of p53 in ribosomal protein deficiencies have been identified by means of rescuing the defect by crossing an *rps29*$^{-/-}$ and *p53*$^{-/-}$ zebrafish. The zebrafish is a perfect model organism for development and disease that can be applied in a big variety of scientific research topics. The zebrafish model is also of big interest in the field of genetics as well as hematopoiesis, so that it covers exactly those requirements that are critical in inheritable ribosomal protein deficiencies, such as Diamond-Blackfan anemia.[2]

Phenothiazine derivatives, primarily used as antipsychotics, have been found to be able to rescue hemoglobin in ribosomal protein deficient model systems, but the drawback of these compounds is that they cross the blood-brain barrier and thereby cause severe negative side effects.[3] To investigate novel derivatives that have the same positive effects on the hemoglobin levels of ribosomal protein deficient patients, but do not cross the blood-brain barrier, an in vivo drug screen of eight different phenothiazine derivatives was performed in the *rps29*$^{-/-}$ zebrafish model system for Diamond-Blackfan anemia. Selected compounds were then tested in a behavior assay in 6-day old wild type zebrafish and finally confirmed in a human sh19CD34^{+} cell model. The drug 088-2 was effective, but did not produce the best results in increasing the hemoglobin levels of *rps29* mutant zebrafish embryos, however, this phenothiazine derivative did not alter the fish' behavior, suggesting that the compound does not go into the brain. Further tests in human cell models are still necessary.

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List of Abbreviations

RP	Ribosomal protein
rRNA	Ribosomal ribonucleic acid
mRNA	Messenger ribonucleic acid
DBA	Diamond-Blackfan anemia
HSC	Hematopoietic stem cell
S	Svedberg unit
MW	Molecular weight
Da	Dalton (unit)
tRNA	Transfer ribonucleic acid
RPS	Ribosomal protein of the small subunit
RPL	Ribosomal protein of the large subunit
rDNA	Ribosomal de(s)oxyribonucleic acid
NCL1	Nucleolin 1
NPM1	Nucleophosmin
eIF	Eukaryotic initiation factor
GTP	Guanosine triphosphate
POL	Polymerase
MDM2	Mouse double minute 2
HDM2	Human double minute 2
TS	Tumor suppressor
TF	Transcription factor
MDS	$5q^-$ Myelodysplastic syndrome
SDS	Schwachmann-Diamond syndrome
TCS	Treacher Collins syndrome
DKC	Dysteratosis congenita
CHH	Cartilage-hair hypoplasia
DBAR	Diamond-Blackfan Anemia Registry
CBC	Complete blood count
MCV	Mean corpuscular volume
MCH	Mean corpuscular hemoglobin

MCHC	Mean corpuscular haemoglobin concentration
eADA	Erythrocyte adenosine deaminase
HgbF	Fetal hemoglobin
HgbA	Adult hemoglobin
HLA	Human leukocyte antigen
dH	Degree of hardness
WGD	Whole-genome duplication
TSD	Teleost-specific genome duplication
hpf	Hours post fertilization
PTZ	Phenothiazine
TFP	Trifluoperazine
FLU	Fluphenazine
E3	Fish water
BBB	Blood-brain barrier
HotSHOT	Hot sodium hydroxide and Tris
USP	United States Pharmacopeia
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
EDTA	Ethylenediaminetetraacetic acid
Tris	Tris(hydroxymethyl)-aminomethane
TAE	Tris acetate EDTA
EtBr	Ethidium bromide
NaOAc	Sodium acetate
H_2O_2	Hydrogen peroxide
RT	Room temperature
PBS-T	Phosphate buffered saline-Tris
PFA	Paraformaldehyde
DMSO	Dimethyl sulfoxide
o/n	overnight
NaCl	Sodium chloride
KCl	Potassium chloride
$NaHCO_3$	Sodium hydrogen carbonate

dH_2O	Distilled water
$CaCl_2.2H_2O$	Calcium chloride dihydrate
Tris-HCl	Tris hydrogen chloride
RIPA	Radio-Immunoprecipitation Assay
BSA	Bovine serum albumin
DPBS	Dulbecco's phosphate buffered saline
TBS-T	Tris buffered saline-Tween 20
HEK 293T	Human Embryonic Kidney Cells 293T
FACS	Fluorescence Activated Cells Sorting
GFP	Green Fluorescence Protein
FHCRC	Fred Hutchinson Cancer Research Center
FBS	Fetal bovine serum
MOI	Multiplicity of infection

1 Introduction

1.1 Ribosomal Protein Deficiencies

Ribosomal protein (RP) deficiencies, collectively known as ribosomopathies, are characterized by mutations or defects in ribosome biogenesis and/or function and can present in various complex diseases with very different clinical phenotypes. Symptoms most often include craniofacial or other physical abnormalities and bone marrow failure, leading to defects in erythropoiesis.[4]

As the word itself already suggests, define ribosomopathies in general those medical conditions in which primarily ribosomes are affected. Although ribosomopathies can show different and very specific clinical phenotypes, depending on the gene(s) involved, do all of these have in common that certain genetic mutations result in impaired ribosome function. Whether this is due to an imbalance in or insufficiency of the individual ribosomal compounds, in ribosome assembly, to alterations in ribosomal structure or to other abnormalities, are all of these syndromes caused by a failure at any of the multiple steps involved in the production of ribosomes.[5] But, apart from this common feature, differ clinical manifestations of ribosomal dysfunctions a lot.[6]

Ribosomes are large and complex molecules that are composed of many different proteins and sets of specific ribosomal ribonucleic acids (rRNAs). They are crucial for every cell as they serve as molecular machinery for protein production. Correspondingly, any dysregulation of the biogenesis of ribosomes can have severe impacts on the whole organism and be the leading cause of many hereditary diseases, such as ribosomopathies.[7]

1.1.1 Ribosome Biogenesis

The biogenesis of ribosomes is a sophisticated process that includes several steps, starting with the precursor rRNA being packaged in a large ribonucleoprotein particle that contains numerous specific RPs from the cytoplasm. Within the nucleolus, the ribonucleoprotein particle is processed into immature small and large ribosomal subunits that are then transported separately through nuclear pores into the cytoplasm, where both subunits associate to finally form a functional ribosome. Ribosomal biogenesis is evolutionary highly conserved within eukaryotic organisms.[7] So, in the case of humans as well as in all other mammals one 40S small subunit and one 60S large subunit conjoin on an messenger ribonucleic acid (mRNA) strand to form a complete 80S ribosome molecule.[8]

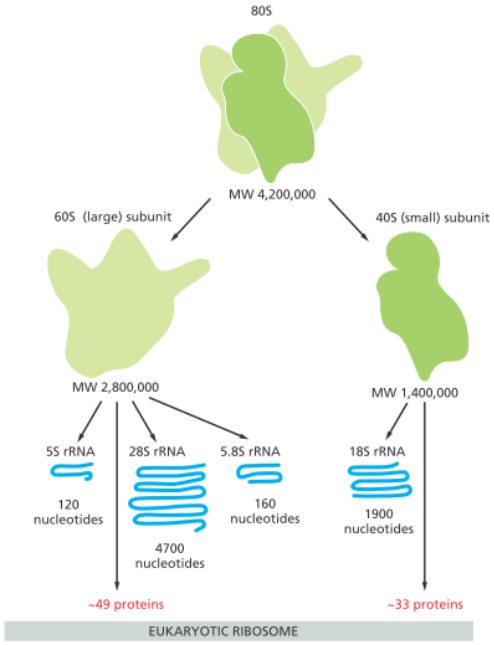

Figure 1: A simplified schematic of eukaryotic ribosome composition.
The functional 80S eukaryotic ribosome has an estimated molecular weight (MW) of 4,200,000 daltons (Da) and contains a 60S large subunit of 2,800,000 Da as well as a 40S small subunit of 1,400,000 Da. While the large subunit is made of 5S rRNA with 120 nucleotides, 28S rRNA with 4,700 nucleotides and 5.8S rRNA with 160 nucleotides and has about 49 proteins (L1, L2, L3,…, L49; "L" for large subunit) in total, is the small subunit composed of 18S rRNA with 1,900 nucleotides and about 33 proteins (S1, S2, S3,…S33; "S" for small subunit).[8]

Ribosome biogenesis is a strongly coordinated process that comprises multiple steps: In the nucleolus, rDNA is transcribed into 47S pre-rRNA, which is then processed into 18S, 5.8S and 28S rRNA with the support of the intracellular proteins nucleolin 1 (NCL1) and nucleophosmin 1 (NPM1). The synthesis of 5S rRNA takes place in the nucleoplasm. Ribosomal proteins of small and large subunits (RPS and RPL) are synthesized in the cytoplasm and transported into the nucleus, where they associate with the previously processed rRNAs. While RPS and 18S rRNA form a

pre-40S small ribosomal subunit, conjoin RPL, 28S rRNA, 5.8S rRNA as well as 5S rRNA to form a pre-60S large ribosomal subunit. The immature subunits pass through nuclear pores and are exported into the cytoplasm, where they form the translation machinery together with mRNAs, transfer ribonucleic acids (tRNAs) and eukaryotic translational initiation and elongation factors (eIF2, eIF3, eIF4E). eIF2, eIF3, tRNA and guanosine triphosphate (GTP) are incorporated into a pre-40S ribosomal subunit to form a pre-43S complex. After that, eIF4E associates with this pre-43S complex, resulting in a 48S complex with mRNA. Finally, a pre-60S large subunit and a 48S complex form a complete 80S ribosome. (see **Figure 2**)[9]

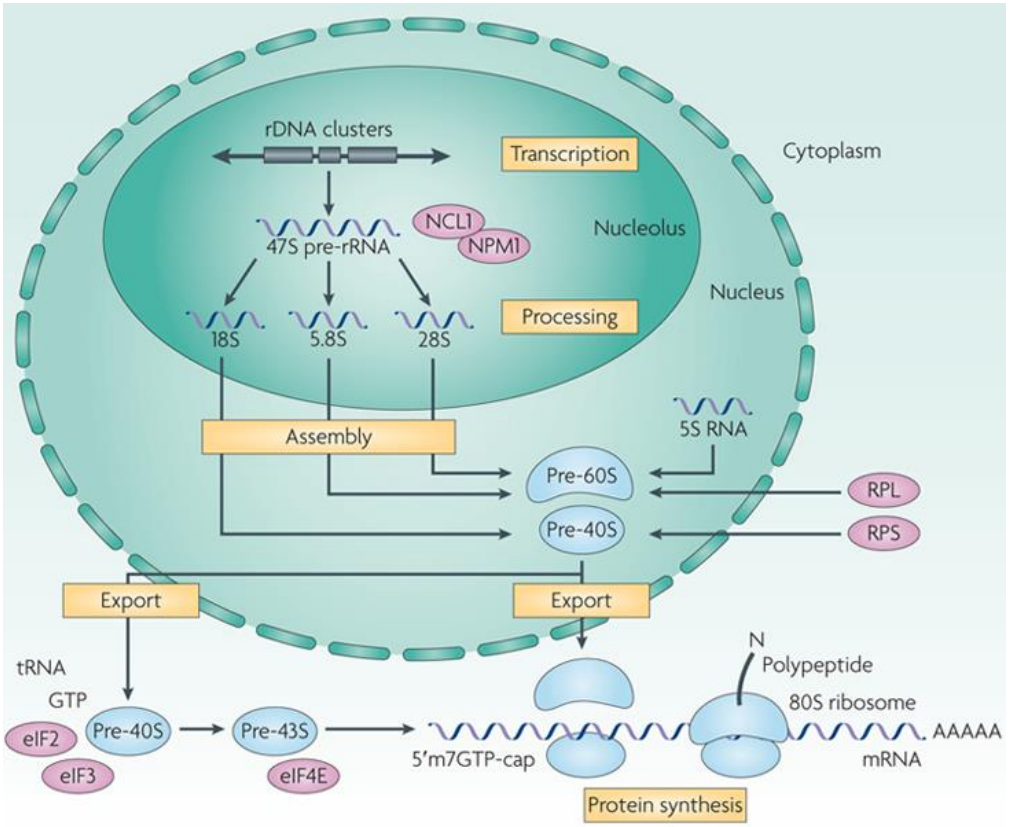

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Figure 2: A schematic of ribosome biogenesis.
The synthesis of ribosomes contains not only one, but multiple cellular events, of which most happen within the nucleolus, apart from 5S rRNA transcription and RP production. Before the individual ribosomal subunits can be exported into the cytoplasm to become active in protein synthesis, certain sets of rRNA and RP have to be produced, which then associate with a pre-40S subunit, transcriptional initiation and elongation factors, pre-60S subunit, as well as an mRNA molecule, resulting in functional ribosome complexes.[9]

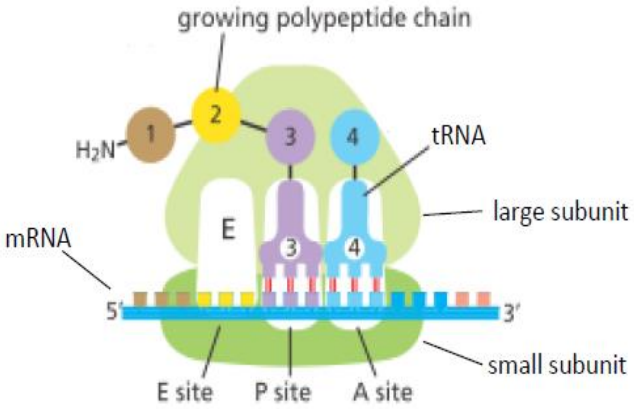
Furthermore, ribosome biogenesis belongs to the cellular processes with the highest energy demand. Because ribosomes build a fundamental molecular framework for protein production and for cell growth or division, and hence are of such big importance for every cell, ribosome biogenesis must be tightly regulated. Accordingly, it is reasonable that a great amount of cellular energy is invested into the synthesis of ribosomes. In eukaryotes, ribosome biogenesis is coordinated by the three RNA polymerases (POL I, POL II and POL III) as well as by more than two hundred of assembly factors, which ensure that the ribosomes are assembled correctly so that they can function in a proper way.[10]

1.1.2 Ribosome Function

Both ribosomal subunits have individual functions that are all essential for the translational process of mRNA into final proteins. The small subunit is responsible for procuring a site on which tRNAs can bind correctly to nucleotide triplets, the codons, of the mRNA. The large subunit, in contrast, supports the linking of the amino acids into a polypeptide chain by catalyzing the formation of peptide bonds.[8] This also explains, why an imbalance in the individual ribosomal compounds is very likely to have a drastic impact on ribosome function, and as a consequence poses a major cause of RP deficiencies or dysfunctions.

The two individual ribosomal subunits remain separated until the initiation of protein synthesis. Functional ribosomes have four binding sites that are crucial for the regulation of the translational process. One site is specifically for mRNA molecules and three, the so called A-, E- and P-sites, for tRNA molecules. When the ribosomal subunits join on an mRNA molecule, usually close to the 5'-end, the mRNA molecule is pulled through the ribosome complex. Triplets of nucleotides of the mRNA form a codon that contains a certain amino acid sequence. When the ribosome encounters a start codon, translation is initiated and protein synthesis can start. Within the core of the ribosome, the nucleotide sequences become translated into the corresponding amino acid sequences with the help of tRNAs that have the function to add the correct amino acids to each other so that a growing polypeptide chain forms.

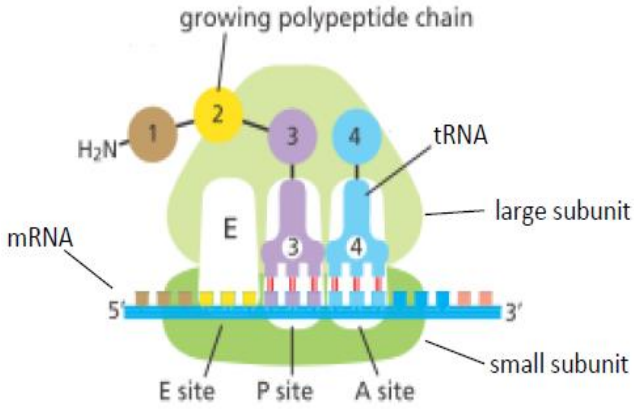
This process continues until a stop codon is encountered, which causes the of the protein. The two subunits separate again and are available for a new trans-



lational process. (see

Adapted from B. Alberts, A. Johnson, J. Lewis, D. Morgan, M. Raff, K. Roberts and P. Walter (2015)

Figure 3)[8]



Adapted from B. Alberts, A. Johnson, J. Lewis, D. Morgan, M. Raff, K. Roberts and P. Walter (2015)

Figure 3: A simplified model of a functional ribosome during translation.
A suitable tRNA binds to the A-site, where it translates the nucleotide sequence of the mRNA into the corresponding amino acid sequence and then this tRNA is moved to the P-site, where its polypeptide is linked to a growing peptide chain via peptide bonds. Finally the tRNA is moved to the E-site before it is released from the ribosome complex.

Due to the complexity of ribosome molecules and their biogenesis and function, there is a certain risk that some proportion of ribosomes is not properly assembled or becomes damaged by external influences during its lifecycle. To avoid that these defective ribosomes can also use cellular energy, which might lead to the production

of non-functional or even worse, of detrimental proteins, a surveillance system recognizes and marks the flawed ribosomes for being degraded by exosomes.[7]

1.1.3 Ribosomal Protein Dysfunction and the Role of p53

Even though several hundreds of factors (transcriptional regulators, enzymes, etc.) support the multiple cellular events during ribosome synthesis and ensure correct rRNA processing, folding as well as assembly with RPs, mistakes can still happen.[11]

However, the number of patients suffering from any type of ribosomopathies is relatively low, despite of the pivotal role of ribosomes in the life of all cells. Most probably, this is due to the fact that severe abnormalities and changes in the process of ribosome biogenesis are lethal, presumably already during a very early stage of embryonic development, so that such cases are never diagnosed.[10]

Many studies have proven that there is a link between ribosome biogenesis and p53 activity.[4] p53 is a tumor suppressor (TS), which is responsible for cell cycle arrest and apoptosis and hence a very important safety regulator for the prohibition of excessive or abnormal cell growth and proliferation.

But on the other hand, an overexpression in the pro-apoptotic p53, results in increased death of not just potentially harmful cells, which also must be prevented. For this reason, there is a negative feedback loop, controlled by mouse double minute 2 (MDM2), or in humans by human double minute 2 (HDM2) protein, which binds to p53 to initiate its polyubiquitylation and finally to its degradation in cytoplasmic proteasomes and thereby keeps the cellular concentration of p53 normally rather low. (see **Figure 4**)[8; 12]

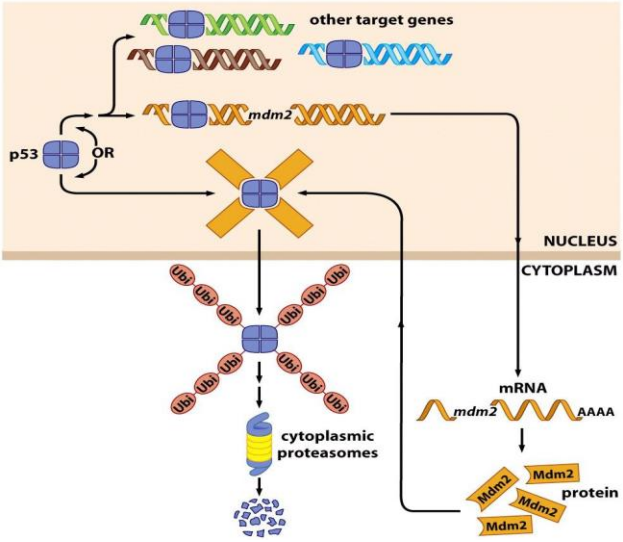
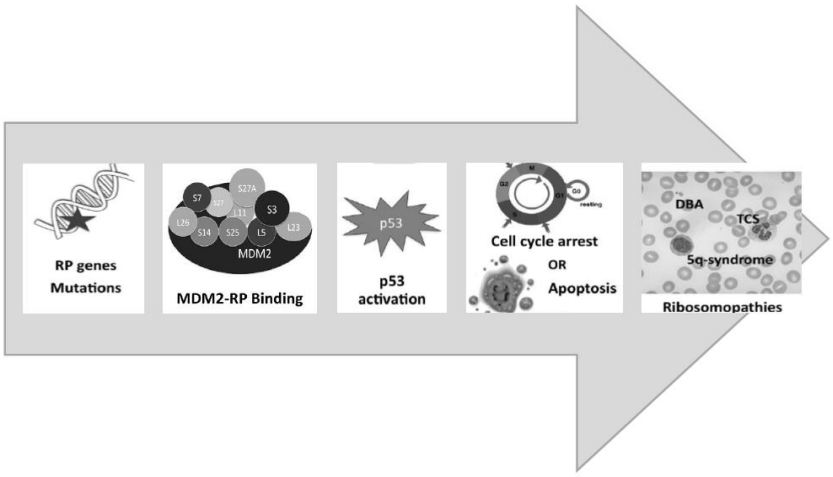


Figure 4: An overview of the role of MDM2 in p53 control.
In order to prevent overexpression of p53 and keep the p53 levels in a cell balanced, a negative feedback loop has to be activated. Upon an increase of the p53 concentration within a cell, MDM2 molecules bind to the transactivation domain of p53 so that its activity as a transcription factor (TF) is blocked. In addition, MDM2 marks p53 for polyubiquitylation, which triggers the export of excess p53 from the nucleus into the cytoplasm, where it finally becomes degraded by proteasomes.[4; 13]

Usually, the positive and the negative regulation of p53 are well balanced. So, if p53 levels increase, a negative feedback loop regulated by MDM2 (or HDM2) is activated, and if p53 levels drop, a positive feedback loop prevents MDM2 or HDM2 from binding and degrading p53, until the p53 concentration rises again, and so on.

However, there is evidence that ribosome dysfunction also activates the p53 feed-forward loop.[4] It has been found that MDM2 and respectively HDM2 can also bind RPs. If, however, the negative regulators of p53, MDM2 or HDM2 are occupied by RP, resulting from defects or dysregulations in ribosome biogenesis, then MDM2 (or HDM2) cannot bind p53 anymore. As a result, excess p53 is not degraded so that the concentrations keep increasing and hence also the pro-apoptotic activity of the TF. As a consequence, p53 triggers cell cycle arrest and apoptosis of not only damaged, but also normal healthy cells and causes specific clinical phenotypes of ribosomopathies like macrocytic anemia, such as for example in Diamond-Blackfan anemia (DBA), $5q^-$ myelodysplastic syndrome (MDS) and Treacher Collins syndrome (TCS). (see **Figure *5***)[4; 13]



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Hani Nakhoul, Jiangwei Ke, Xiang Zhou, Wenjuan Liao, Shelya X. Zeng and Hua Lu (2014)

Figure 5: The connection between p53 activation and pathophysiology in some ribosomopahties.
A mutation of a certain RP gene leads to excess RP, which is bound by MDM2 or HDM2 that initiates the activation of p53. p53 in turn causes cell cycle arrest and apoptosis, leading to different clinical phenotypes, such as DBA, MDS or TCS, generally classified as ribosomopathies.[13]

1.1.4 The Diversity of Ribosomopathies

The most studied disease of the category of ribosomopathies is by far DBA, which is therefore considered as a prototype of ribosome dysfunctions.[6] However, other syndromes that show sufficient overlaps with DBA to cause diagnostic problems are also associated with ribosomal pathophysiology.[1]

Because of their big variety and complexity, ribosomal dysfunctions can hardly be generalized. Not only significant differences in terms of signs and symptoms, but also presentation, tissue specificity and severity of defects between affected individuals, even within the same diagnosed syndromes, additionally complicate the identification of the disease causing factors.[6; 13] As a consequence, it is nearly impossible to find a general mechanism that can be said to apply to all of these diverse disorders. Since all of them have their very unique clinical phenotypes, it is assumed that they also show some differences in their disease mechanisms. However, ribosomal biogenesis and function play the most important role in every syndrome within this category.[5]

Common clinical features may include bone marrow failure and developmental abnormalities. Moreover, show patients of many subtypes of ribosomopathies an increased predisposition for developing cancer, especially leukemia, since RP deficiencies seem to have a significant effect on hematopoiesis, of which the exact correlation, however, is still unclear.[2; 13]

Additional ribosomopathies next to DBA are for example 5q$^-$ MDS, Treacher Collins syndrome TCS, Schwachmann-Diamond syndrome (SDS), Dyskeratosis congenita (DKC), Cartilage-hair hypoplasia (CHH) and many more. Although all of these diseases have mutations in RP genes, they show different clinical features (see **Table 1**).[4; 14]

Table 1: An overview of examples of ribosomopathies and their associated genes, clinical characteristics and diagnosis

Disease	Associated Genes	Clinical Characteristics	Diagnosis
DBA	RPS7, RPS10, RPS17, RPS19, RPS24, RPS26, RPL35A, RPL5, RPL11, RPL26, GATA1, etc.	Hypolpastic anemia, Erythrocyte aplasia, Short stature, Craniofacial anomalies, Thumb anomalies	RP gene sequencing, Elevated erythrocyte adenosine deaminase (eADA), Elevated haemoglobin fetal hemoglobin (HbgF) levels
5q$^-$ MDS	RPS14	Dysplastic bone marrow, Macrocytic anemia, Happloinsufficiency	Bone marrow aspiration Bone marrow biopsy
TCS	TCOF1	Craniofacial anomalies	Physical testing
SDS	SBDS	Exocrine pancreatic insufficiency, Defective hematopoiesis, Short stature	SBDS gene testing
DKC	DKC1, TERC, TERT, NOP10	Skin hyperpigmentation, Nail dystrophy, Mucosal leukoplagia	Telomere length analysis

CHH	RNase MRP	Hypoplastic anemia Short limb dwarfism, Skeletal abnormalities, Hypoplastic hair, Gastrointestinal dysfunction, Immunodeficieny	RMRP sequencing

4; 7

As shown in **Table 1**, are some overlaps within these syndromes, but often also big differences, especially in terms of their clinical phenotypes, since each disorder is associated with distinct aspects of ribosome biogenesis. How these ribosome defects and dysfunction exactly contribute to the different disease specific clinical characteristics and pathogenesis, is still not entirely clear.[14]

1.1.5 Diamond-Blackfan Anemia

DBA was first reported by Hugh W. Jospehs in 1936[15]. In 1938, Luis K. Diamond and Kenneth D. Blackfan, described the disease as a "congenital hypoplastic anemia" (lat. erythrogenesis imperfecta).[16]

The incidence of DBA is hard to be specified exactly, but it is estimated that there are between five and ten new cases per every million new-born children. The sex ratio is 1:1, so females and males are equally affected.[17] No racial or ethnic predisposition could been identified either.[18]

Typically, DBA is diagnosed during early infancy, but also adults have been diagnosed. These however, are a few exceptions, since the symptoms of most patients are relatively severe. With appropriate treatment, people who suffer from DBA, can have a long life expectancy, but often this is not the case. [17]

1.1.5.1 Clinical Characteristics

DBA is as a very rare, congenital hypoplastic anemia, which is typically diagnosed during the first 12 months of life.[2; 14] The majority of children with DBA starts to show

effects at the age of two months, ranging from birth to six years. Usually, the diagnosis is already made at three months of age, but this can also range widely from the time of birth to even adulthood.[17]

Common symptoms experienced by patients include pale skin, fatigue and dyspnea (shortness of breath or difficulty in breathing). These symptoms can mainly be associated with the anemia in DBA, resulting in a reduced oxygen supply of the body.[19]

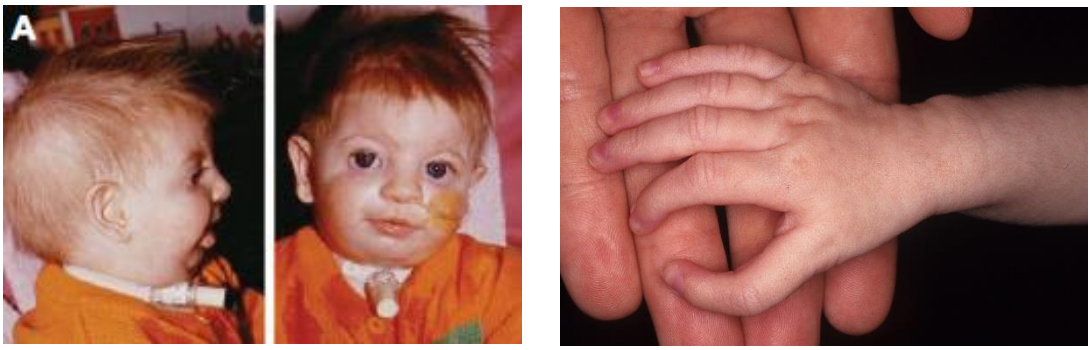
DBA is characterized as a bone marrow failure syndrome and a block in erythropoiesis, which leads to a decrease in the number of erythroid progenitor cells in the bone marrow. It is suggested that affected individuals probably also have a HSC defect.[2; 20]

Roughly 30% to 50% of patients show growth retardation or congenital abnormalities and are reported to have malformations, in particular of the face, heart and urogenital tract as well as of hands and fingers, with range in severity.[1; 4; 7; 13]
The most characteristic congenital anomalies are craniofacial (see

Adapted *from A. Narla and B. L. Ebert (2010)*
Figure *6*, A) and include cleft palate (opening of the roof of the mouth into the nose), microcephaly (significantly smaller head size), microtia (smaller and abnormal outer ear(s), sometimes also inner ear(s) and ear canal), epicanthus (skin fold covering the inner corner of the eye(s)), ptosis (drooping eyelid(s)), glycoma (increased pressure in eyeball(s)), etc.
Also the neck and shoulders can be malformed, causing for instance a short neck and underdeveloped, abnormally positioned shoulder blades.
In some patients the urinary tract, genital organs and kidneys are affected. Even a missing kidney that did not form during embryonic development can be caused by DBA.
About one out of five patients has a congenital malformation of the hands, especially of the fingers and thumbs. Examples for this are triphalangeal thumb (a thumb with two joints, basically looking like a finger) (see

Adapted *from A. Narla and B. L. Ebert (2010)*

Figure *6*, B), duplex thumb (two thumbs on the same hand or a divided thumb), hypoplastic thumb (an underdeveloped thumb), fused or webbed fingers, and many more.[17]



Adapted from A. Narla and B. L. Ebert (2010)

Figure 6: Examples for physical abnormalities of children with DBA. A) Craniofacial anomalies, B) Triphalangeal thumb.
(A) A boy with DBA, who has characteristic craniofacial anomalies, involving the skull and the eyes. (B) A typical case of a triaphalangeal thumb with one extra joint, making it look like a finger.

Apart from these clinical features, which are directly related to DBA, have patients also a significantly higher risk for cancer. Moreover, neoplasia starts much earlier in people with DBA than in the normal healthy population.[21]

It seems to be against all odds that an upregulation in an important TS as p53 makes DBA a cancer predisposition syndrome. But up to one fifth of patients with DBA develops cancer during their forties, especially lymphomas but also several types of solid tumors. A study showed that a mutation in RPL11, which normally serves as a positive regulator of p53 to inhibit over-proliferation and tumor formation, leads to the loss of its ability to activate p53. Furthermore, a mutated RPL11 results in an increase of MYC levels, which promotes tumor growth. It is assumed that both factors contribute to malignancy in DBA.[22; 23]

1.1.5.2 Genetics

There is a wide range of disease associated genes in DBA and all of them display slightly different features. At least ten RP genes are involved in DBA. DBA1 to DBA10 are known types of the syndrome.[18]

More than half of the patients have a mutation in a RP protein gene.[1]
Analyzed genes linked to the pathophysiology in DBA are RPS7, RPS10, RPS17, RPS19, RPS24, RPS26 of the small ribosomal subunit and RPL5, RPL11, RPL26, RPL35A of the large subunit.[18]
Most mutations lead to haploinsufficiency of the protein corresponding to the involved gene.[1]

About 25% of affected individuals carry a mutation in the RPS19 gene, which include mainly nonsense and missense mutations, but also splice site mutations, as well as frameshifts and deletions, leading to clinical features that can be associated with the erythropoietic and embryogenetic function of RPS19.[24]

But not all cases can be correlated to mutated RP genes. GATA1, a transcription factor for erythroid development, has also been identified as one of the disease causing genes.[25; 26]

In exome sequencing of siblings, diagnosed with DBA, GATA1 has been specifically linked to an X-linked or autosomal recessive inheritable form of DBA, since the parents have completely normal hematological findings.[26]

Pedigrees reveal that about 45% of all cases have a family history in DBA and usually follow an autosomal dominant inheritance pattern. The remaining 55%, however, arise sporadically.[27; 28] Such de novo mutations make up the majority of genetic causes of the syndrome, but incomplete penetrance and variable expressivity, despite the inheritance of the exactly identical genetic mutation, make even familial cases hard to ascertain in disease pedigrees, especially because the mechanism behind this is not entirely understood yet, suggesting that additional pathways might be involved.[14; 29]

So far, only heterozygous mutations have been reported in relation to DBA, which is probably because a homozygous mutation is lethal to the embryo.[24] Due to the heterozygous manifestation of the disease, only one allele of the gene is affected so that the second copy is still normal. This means that patients are expected to technically have 50% of their functional RP, which is apparently already enough limitation to provoke severe abnormalities and defects.

1.1.5.3 Mechanism of Disease

The exact mechanism of DBA is not fully known yet, but there are some potential explanations, which involve a defect in ribosome assembly or stress signals to activate p53. Especially the interaction of RP with MDM2, or in humans with HDM2, plays a significant role in the pathophysiology of DBA, since these are important negative regulators to control p53 levels and activity.[30] It was found that RPL5, RPL11 and also RPL23, RPs of the large 60S ribosomal subunit, are able to bind to MDM2 and thereby prevent the negative feedback loop of the pro-apoptotic TS p53, which then causes hypoplastic anemia. (see **Figure *7***)[31-34]

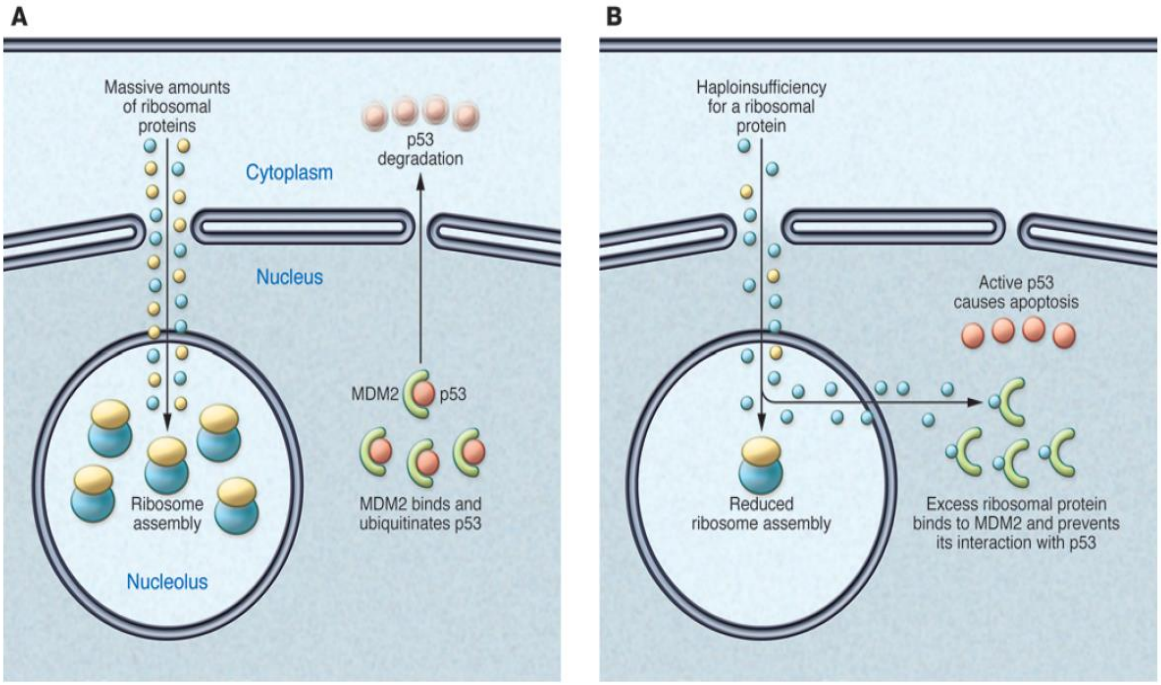


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Figure 7: A model of the correlation between ribosome biogenesis and RP haploinsufficiency in DBA. A) Normal ribosome biogenesis. B) Haploinsufficiency for a RP. (A) In normal ribosome biogenesis, erythroblasts produce sufficient RP for protein synthesis and the cellular p53 concentration is kept in balance by a negative feedback loop, regulated by MDM2, which induces the degradation of excess p53. (B) However, if there is a haploinsufficiency for a RP, less functional ribosome complexes can form, resulting in an excess of another unbound RP, which then binds to MDM2 and thereby prevents its interaction with p53. p53 can accumulate and induce increased cell cycle arrest and apoptosis of red blood cells, which results in the characteristic hypoplastic anemia of DBA.[34]

1.1.5.4 Diagnosis

The classic symptoms of DBA alone are diagnostically not conclusive enough, as these could also be clinical phenotypes of other ribosomopathies or non-congenital anemias, if no physical anomalies are present.

There is a number of clinical tests that help to make a definite diagnosis:
This can be for instance a *Complete Blood Count (CBC)* to determine the blood components and their concentrations. Especially relevant to diagnose DBA are the amount of erythrocytes, leukocytes (including the percentage of lymphocytes, neutrophils, eosinophils, basophils, and monocytes), platelets, as well as total hemoglobin. In addition, the hematocrit (fraction of blood made of erythrocytes), the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) of erythrocytes determined by a CBC can give important information for making a diagnosis. Erythrocytes of patients with DBA typically have a higher MCV, which also defines the phenotype of macrocytosis.
A *Reticulocyte Count* for the number of precursors, progenitors and immature erythrocytes, is a good method to investigate the bone marrow activity and enables quantification of erythropoiesis of the last 24 to 48 hours. The reticulocyte count in DBA patients is lower than that of healthy people and typically lies under 1%.
Antibodies that target specifically erythrocytes and destroy them can be disease and in particular hemolytic anemia causing, but are usually not present in patients with DBA. This is commonly analyzed via a *Coombs Test*.
In up to 85% of DBA patients, the *erythrocyte adenosine deaminase (eADA) levels* are elevated, provided that the patient has not received blood transfusions within less than three months prior to the test.
Very characteristic for DBA are elevated *fetal hemoglobin (HgbF) levels*. It has been found that patients with DBA remain a significantly high percentage of HgbF after birth, unlike in healthy people, where at least 95% of the total hemoglobin is replaced by adult hemoglobin (HgbA).
To determine the activity of the bone marrow in the production of blood cells and their health, a *bone marrow aspiration* or *bone marrow biopsy* is done. In DBA, this activity is significantly decreased.[17]

Since DBA is a genetic disease, *genetic testing* is probably the most reliable method to diagnose DBA, provided that the patient is among the about 60%, who have a mutation in a RP gene, or a non-RP gene, such as GATA1, which is known to be associated with the syndrome. For the remaining percentage of patients, the disease causing genes have not been identified yet.[17]

1.1.5.5 Current Available Treatment Options

At present, there exist only a few possible treatment methods, which include the administration of steroids and blood transfusions.

Corticosteroid therapy induces the production of erythrocytes in the bone marrow. The initial daily dose of steroids administered to DBA patients is very high and thus cannot be taken for too long periods of time, as steroids also provoke rather severe side effects. So, after about two to four weeks of the high starting dose, typically of two milligrams per one kilogram of bodyweight, the dose must be steadily lowered in order to slowly wean the medication. A long term corticosteroid treatment has to be at a much lower dose, adapted to each individual to enable them a life with very little to no side effects. Even though with roughly 80% a very high percentage of patients initially responds to corticosteroids, are at least 15% non-responsive to the treatment. Some patients become resistant after an initial response.[35]

Short term side effects, like upset stomach, increased blood sugar, increased risk for infections and high blood pressure, or long term side effects, such as growth retardation, brittle bones, muscle weakness and diabetes, become unacceptable in some responsive patients so that the treatment has to be discontinued completely .[17] Especially growth retardation is a very critical side effect of corticosteroids, as the syndrome itself causes short stature, therefore the growth in particular during infancy must me tightly surveilled so that one can interfere with the negative effects of the treatment as early as possible.[35]

Nonetheless, corticosteroids remain the primary treatment method in DBA. The exact drug mechanism still has to be investigated, but currently steroid responsiveness cannot be predicted.[30]

For patients who are non-responsive to steroids or must stop the treatment, it is recommended to have *red blood cell transfusions* when needed or as a chronic blood transfusion treatment, where they receive transfusion every few weeks to increase dangerously low hemoglobin levels and maintain them over the long haul.
As all transplants, blood transfusions often provoke graft-versus-host reactions. Direct related family members, who are usually the best hits for donors, are not used for blood transfusions to treat DBA, as the recipient could develop antibodies against the blood of immediate family members, which would exclude them from being potential HSC donors, as this would lead to a strong immune response and severe complications.[17]
A patient who is dependent on chronic blood transfusion therapy is at a high risk to have an iron overload. Since people with DBA cannot use iron that is released from the breakdown of erythrocytes by macrophages to build new red blood cells to the same extent as healthy people can, excess iron will accumulate in tissues and organs and damages them, if not removed. The human body has no mechanism to degrade or excrete excess iron, therefore the iron levels of the patients who have to receive multiple blood transfusions must be regularly monitored.[17; 35]

To treat or even prevent iron overload, a chelation therapy is an important part of the treatment of DBA additional to blood transfusion therapy and currently the only way to remove excess iron from the body. However, a chelation therapy can be harmful, if initiated too early and might lead to toxicity. Delaying the treatment, on the other hand is also risky since intervention of already progressed damage of iron overload cannot rescue the tissue and especially, if organ dysfunction and hence increased morbidity have already commenced.[17; 35]

For that reason, several tests to measure iron levels help in administering chelation medication at the right point of time.
One guide for the initiation of a chelation therapy are *ferritin levels*. By means of a blood test (serum ferritin) the iron balance in the body can be determined, which

should be not higher than 1500ng/mL blood. Another measure is a *hepatic iron concentration* as high as 7mg/g (dry weight), which is equal to red blood cell transfusion of about 200mL/kg body weight.
There are also some non-blood tests to determine the iron levels in the body, as the *SQUID test*, a certain type of x-ray which uses magnets for measuring a body's iron content or the *T2* MRI and FerriScan MRI.* T2* MRI measures the iron concentration in the heart and FerriScan MRI in the liver. A combination of both gives a very accurate result for the iron levels in the body, however, only selected hospitals have the ability to use this method.
Instead often a *liver biopsy* can help, as it is also a very accurate although invasive method to measure the amount of iron in the liver.

In many patients, DBA is caused by a haploid RP gene deficiency. Enhancing the expression of the deficient RP via *gene therapy* is a potential cure for the syndrome.[36]

Currently, the only really curative approach of DBA is *HSC transplant*, but this a very risky treatment and thus extremely controversial. Depending on the donor source, HSCs from cord or peripheral blood or from the bone marrow of a healthy donor are used to replace the defective marrow of the recipient. Most ideal for HSC transplants are human leukocyte antigen (HLA)-matched related donors, i.e. allogeneic siblings, where the overall survival is at 77% and even higher under the age of 10 with about 94%, according to the Diamond Blackfan Anemia Registry (DBAR). HSC transplant is curative if it is successful, but it is a very dangerous medical treatment and there is no 100% guarantee that the HSCs will engraft without major or even lethal complications.[17; 37]

1.2 The Zebrafish Disease Model

Danio rerio, better known as zebrafish, has gained increased popularity as a model organism in scientific research since the last few decades and many of its advantages help to overcome the restrictions of other disease models.[38]

1.2.1 Animal Characteristics

The zebrafish belongs to the family of Cyprinidae of the order Cypriniformes and is a small tropical vertebrate freshwater fish, native to the Himalayan region of south-east Asia.[39]

Adult Danio rerios are typically 2.5cm to 4cm long, where sexually mature females usually have a rounder belly than males, due to the eggs they carry. Apart from that are female zebrafish generally less pink and of darker color than males. (see **. The** stripes extend onto the end of the caudal fin rays, whereas the anal fin is distinctively striped.[39; 40]

Figure 8: Visual sex determination of Danio rerio.
(A) A slender male zebrafish with pink and yellow tinge.
(B) A female zebrafish with a round belly and less pink and yellow tinge.) The eggs and larvae are transparent and only as the fish mature to adults, they develop their characteristic longitudinal blue stripes on the otherwise yellow to golden colored scales. The stripes extend onto the end of the caudal fin rays, whereas the anal fin is distinctively striped.[39; 40]

Figure 8: Visual sex determination of Danio rerio.
(A) A slender male zebrafish with pink and yellow tinge.
(B) A female zebrafish with a round belly and less pink and yellow tinge.

Zebrafish can live up to roughly six years, but there average life span in captivity is about three years, though under optimal conditions, the fish can indeed attain the maximum life expectation of 66 months.[41] Danio rerio prefer water with a pH range of 6.0 to 8.0 and a water hardness between 5 to 19 dH. Since zebrafish belong to the group of tropical fish, they best survive for long terms at temperatures above 18°C, but not warmer than 24°C.[39]

The zebrafish belongs to the category of vertebrates and mostly has the same major tissues and organs as mammals. Zebrafish muscle tissue, blood, eyes, kidney etc.

are very similar to those of humans and also function in the same manner. (see **Figure 9**)[40; 42]

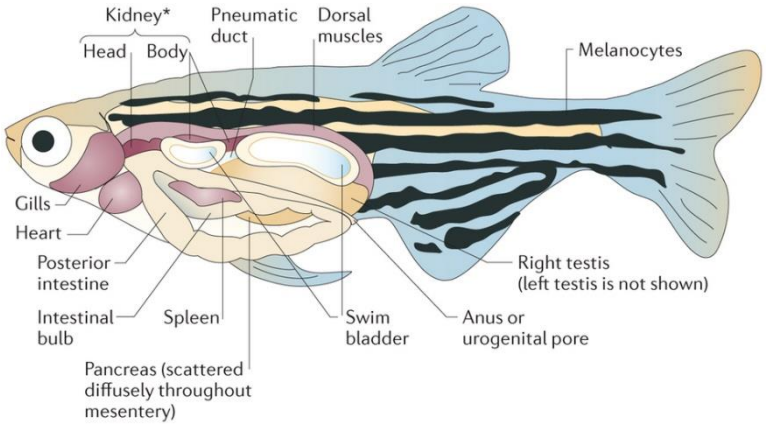

Figure 9: A schematic of the anatomy of Danio rerio. An adult zebrafish is depicted with its general anatomical structures as labeled. Zebrafish share most of the main organs, as for example heart, brain, kidney, pancreas, spleen, testis or ovaries, with mammals.[43]

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After the complete zebrafish genome has been sequenced as part of the zebrafish genome project in 2013, evidence for over 26,000 protein-coding genes could be provided, so that the zebrafish genome has become an important resource for scientific research.[40]

Danio rerio have, unlike most other vertebrates, an additional round of whole genome duplication (WGD), the so-termed teleost-specific genome duplication (TSD), as zebrafish belong to the teleostei infraclass (ray-finned fish). The zebrafish genome contains more species-specific genes than most other vertebrates, including human or mouse.[44; 45]

Also on the genetic level are zebrafish highly conserved with humans as well as other mammals. 71.4% of human genes have one or more zebrafish orthologue(s) and 69% of zebrafish genes also have at least one human orthologous gene sequence. 84% of genes that have been identified to be associated with known human diseases have a counterpart in zebrafish.[45]

1.2.2 Embryonic Development

Adult female zebrafish lay 200 to 300 embryos per week. At optimal conditions (28.5°C and 5-10 embryos/mL water) during early stages, the embryos will develop into young zebrafish larvae within an average of 72 hours post fertilization (hpf).[46] The stages of embryonic development in Danio rerio can be grouped into distinct periods, starting with the zygote and then going from cleavage to blastula, gastrula, segmentation, pharyngula and finally to hatching of the larva. (see **Table 2**)[47]

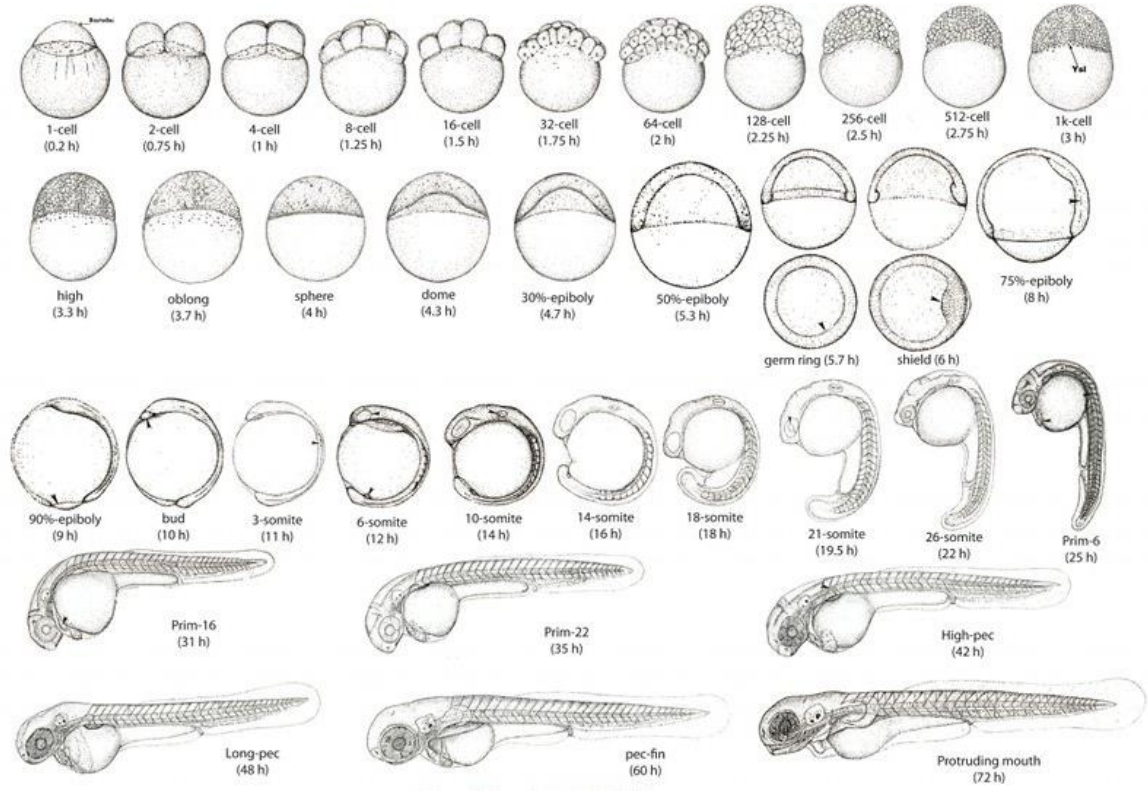
Table 2: The seven periods of embryonic development in Danio rerio.

Period	hpf	Description
Zygote	0	Fertilized egg until the first completed cell cycle
Cleavage	0.75	Synchronous cell cycles 2 to 7
Blastula	2.25	Metasynchronous cell cycles 8 and 9 pass into slower asynchronous cell cycles at the transition to midblastula; epiboly starts
Gastrula	5.25	Involution, convergence and extension form epiblast, hypoblast, embryonic axis through the end of epiboly
Segmentation	10	Development of somites, neuromeres etc.; primary organs and tail form; motion starts
Pharyngula	24	Body axis straightens out; fins form; circulation, pigmentation starts

Hatching	48	Complete morphogenesis of main organs and cartilage in head and fins; asynchronous hatching
Larva	72	Swim bladder blows up; survival instincts become active

© *Adapted from C. B. Kimmel, W. W. Ballard, S. R. Kimmel, B. Ullmann, T. F Schilling (1995)*

These developmental periods, named after the main morphological changes that the embryos undergo during the first three days after fertilization, are further divided into the single stages of embryogenesis. (see **Figure 10**).[47]



© *Systems BioImaging Lab, ECE, UCSB*

Figure 10: A depiction of the developmental stages of zebrafish embryos.
Within just 72 hpf, a zebrafish embryo undergoes multiple morphological changes, from a zygote right after fertilization to an early larva that has all of its primary organ systems as well as active food-seeking and survival instincts. During the developmental stages, the embryo is provided with nutrients from its yolk sac, which becomes increasingly smaller in the course of embryogenesis.

1.2.3 Benefits of the Zebrafish Model

The use of Danio rerio as a model organism in scientific research for developmental as well as disease studies, started in the sixties of the last century and since then gained increased popularity among scientists for a lot of reasons. The zebrafish has been a useful tool in many studies to investigate and understand a number of biological processes and mechanisms in evolutionary or embryonic development, but also in complex diseases, like cancer.[40; 42]

The physiological and genetic similarities to humans, which make zebrafish an ideal model organism to study development and disease, are not the only reasons why these fish now belong to the most favored animal model systems in scientific research.

Zebrafish are small, yet robust animals and thus relatively easy to handle. A big advantage in zebrafish husbandry is that the fish do not have to be kept in isolation. In general, they are much more economic and easier to raise and maintain than many other model organisms, enabling to keep thousands of fish in a laboratory aquaculture at affordable costs.[40; 42]

Another important benefit of the zebrafish model is their high fecundity and fast reproduction rate. As zebrafish females produce several hundreds of embryos in an interval of just seven days and mating is triggered by sun rise, which is easily inducible in aquacultures, scientists can obtain a steady supply of big numbers of fertilized embryos and thus also perform high-throughput studies. The fact that the offspring of zebrafish develop externally and are practically transparent, makes it particularly easy to examine them in all of their developmental stages. Additionally, Danio rerio embryos grow extremely fast so that approximately one month of human embryonic development corresponds to only one day of zebrafish development.
One of the most unique features of zebrafish, which is of special interest in regenerative medicine, is their ability to repair a variety of its tissues and organs including fins, retina, spinal cord and even the heart.[48]

Hematopoiesis is also highly conserved in zebrafish and they develop erythrocytes within 24 hpf, which makes them a great model system to study general hematologic

development and functions as well as different blood disorders, like for instance DBA.[38]

1.2.4 Disease Modeling of DBA in Zebrafish

In several studies, zebrafish served as an in vivo model system for RP deficiency, since hematopoietic regulation is highly conserved with humans and their whole genome has been already sequenced. For this purpose, many Danio rerio strains, mutant in a RP gene, have been generated. Another way to obtain a transgenic disease model are targeted knockouts of the RP genes of interest with morpholinos.[49]

In the case of DBA, it was found that the *rps29*$^{-/-}$ zebrafish can be associated with DBA families.[50] By downregulation of RP genes, especially *rps29*, many disease specific phenotypes of DBA, including hematopoietic defects and p53 activation could be modelled in zebrafish. Even though no homozygous patient with DBA has ever been identified so far, most likely because a homozygous form of the syndrome would be lethal, homozygous mutant *rps29*$^{-/-}$ fish survive for up to five days, which is probably due to the contribution of maternal RNA and proteins in the early larva stage.[2] In order to obtain a steady supply of mutant zebrafish embryos for scientific studies, heterozygous strains are in-crossed. According to Mendelian genetics, this should technically lead to 25% of mutant progeny.

RPS mutant zebrafish typically have growth retardation compared to wildtype zebrafish as well as a characteristic head phenotype and most importantly, a lack of hemoglobin, which represents basically the major symptoms of DBA patients.[2]
It has been shown that with the help of p53 mutant fish, these phenotypes can be rescued. This proves that p53 activation plays a big role in RP deficiency. (see **Figure 5** and **Figure 7**) But a mutation in p53 is not able to rescue the lethality of *rps29*$^{-/-}$ homozygous mutants, as this does have no influence on the fact that the embryos are not capable of producing RP of the knocked out gene, once the maternal RNA and proteins are lost.[2; 13] A different approach to find new treatment methods for patients, who suffer from DBA, is to discover new or improve already existing and approved drugs. Again, zebrafish are an important and useful tool for that, for example in in vivo drug screens.[51]

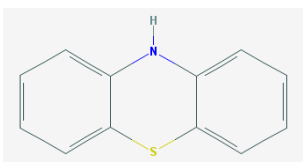
Both approaches and probably also others have a great potential to rescue RP deficiencies, however there is still a lot to investigate.

1.3 Aim of the project

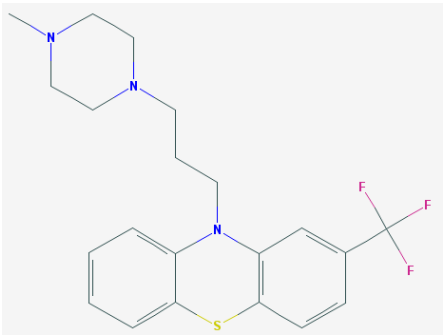
The goal of the project was to investigate novel drug candidates to rescue ribosomal protein deficiencies, with the help of an *rps29* mutant zebrafish model system for DBA.

In previous studies, it has been found that phenothiazine (PTZ) derivatives are able to rescue hemoglobin in RP deficiencies. Fluphenazine (FLU) rescues hemoglobin in zebrafish models, Trifluoperazine (TFP) rescues hemoglobin in mouse and human cell model systems.[3; 52]

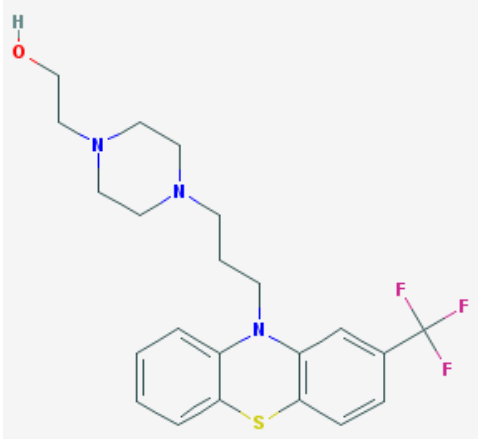
The problem with these compounds is that they are primarily used as antipsychotics to treat schizophrenia and thus cross the blood-brain barrier (BBB).[3] So, the aim was to identify phenothiazine derivatives that do not cross the BBB, but are still able to rescue hemoglobin in DBA and potentially also in other ribosomopathies.



Phenothiazine
(PTZ)



Trifluoperazine
(TFP)



Fluphenazine
(FLU)

Figure 11: Chemical structures of PTZ, TFP and FLU
Specific modifications of the basic structure of PTZ with additional side chains, result in TFP and FLU.

2 Materials and methods

2.1 In vivo drug treatment

For this project mainly *rps29*$^{-/-}$ zebrafish were used as an in vivo disease model for DBA. In order to obtain *rps29* null zebrafish embryos, AB x rps29 zebrafish were genotyped and *rps29*$^{+/-}$ zebrafish were incrossed, since a homozygous mutation of the *rps29* gene is lethal in zebrafish.[2]

2.1.1 Genotyping of *rps29*$^{+/-}$ zebrafish

AB x rps29 zebrafish were fin-clipped in order to genotype them. For that, the adult zebrafish were anaesthetized with a tricane (ethyl 3-aminobenzoate methanosulfoate)-fish water (E3) solution. To use tricane as an anaesthetic for zebrafish, 4mL of the tricane were diluted in 100mL of fresh tank water. A fish was placed into a small dish containing the diluted tricane solution and after approximately 1-2 minutes, when the movement of the fish stopped, the fish was transferred onto a plastic board with a spoon and a small piece of its tail fin was cut off via a razor blade. The fin piece was brought via a forceps into a well of a 96-well PCR plate on ice, which contained 75µL of Hot Sodium Hydroxide and Tris(hydroxymethyl)-aminomethane (Tris) (HotSHOT) solution for DNA preparation. The fish had to be placed back into fresh tank water for approximately 3-5 minutes, so that it recovered fully, before it could be transferred into an isolated tank, which was labeled accordingly to the well of the 96-well PCR plate that contained the fin piece of the corresponding fish. This procedure was repeated with all the zebrafish that had to be genotyped.

Afterwards, the PCR plate with the fin pieces in HotSHOT alkaline lysis reagent was sealed and placed into a thermocycler, programmed for 60 minutes of heating at 95°C and then cooling down to 4°C. The samples were neutralized with 75µL of neutralization buffer (equal amount as HotSHOT) and diluted in a 1:10 ratio with United States Pharmacopeia (USP) Sterile Purified Water. For genotyping, a tail clip PCR Master Mix by Roche was prepared, which contained 1µL of 10µM forward primer (Mutant rps29 15) and 1µL of 10µM reverse primer (Mutant rps29 113) per

10µL of master mix solution. To the 10µL of master mix solution in each well of a 96-well PCR plate, 2µL of the 1:10 diluted DNA were added and the sealed plate was centrifuged at 2000rpm for 1 minute. The plate was placed into the thermocycler set for 35 cycles of 3:00 minutes initiation phase at 94°C, 0:30 minutes denaturation phase also at 94°C, 0:30 minutes annealing phase at 55°C and 0:45 minutes elongation phase at 72°C, and a final cool down after the last cycle to 4°C.

After DNA amplification, the samples were analyzed via gel electrophoresis. A 2% agarose gel was prepared with 1X Tris-acetate-Ethylenediaminetetraacetic acid (EDTA) (TAE) buffer. As an intercalating agent, 5µL of ethidium bromide (EtBr) were used. 1.2µL of 10X loading dye were added to each 12µL of DNA sample, before 8µL of it could be loaded into the agarose gel. In addition, 6µL of Quick-Load® Purple Low Molecular Weight DNA Ladder (New England Biolabs), a pre-mixed, ready-to-use molecular marker containing bromophenol blue as a tracking dye, were loaded (see **Figure 12**). The gel was run at 120 volts for 30 minutes and analyzed. A visible band at 200bp indicated that the DNA sample came from an *rps29* mutant zebrafish. All fish with the mutation could be kept for further experiments. The fish without the mutant *rps29* gene had to be sacrificed in ice water and stored in a carcass freezer.

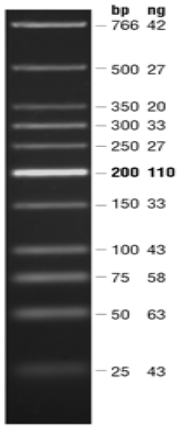


Figure 12: Quick-Load® Purple Low Molecular Weight DNA Ladder visualized on an agarose gel
The Quick-Load® Purple Low Molecular Weight DNA Ladder contains a plasmid which is digested by restriction enzymes into DNA fragments ranging from 25-766bp to yield 11 bands in total. The band at 200bp (i.e. 110ng) serves as a reference and therefore has an increased intensity.[53]

2.1.2 Breeding of *rps29*$^{-/-}$ zebrafish embryos

To produce *rps29*$^{-/-}$ offspring, *rps29*$^{+/-}$ mutant zebrafish were incrossed via pairwise breeding. After about seven days of recovery, the genotyped *rps29*$^{+/-}$ adult zebrafish were ready for breeding. First, the *rps29* mutant zebrafish had to be separated into male (smaller, flat, lighter, bright yellow and pink stripes) and female (larger, round belly, darker) fish. Always one male and one female zebrafish were placed into an isolated tank with a divider that still kept the male separated from the female fish. In addition, an inserted tank with a grid-like bottom separated the eggs from the adult fish to prevent them from eating up the eggs, a peculiar behavior which zebrafish commonly show in captivity.[46]

The fish remained in this breeding set-up overnight and the next morning, the dividers were pulled to let them breed. Since zebrafish are freshwater fish, the tank water was changed beforehand. Moreover, to provide ideal breeding conditions, a slope was created by tilting the inserted tank with the help of the divider and breeding grass was added, as zebrafish in their natural habitat typically lay the eggs in shallow water at the waterfront. Zebrafish have photoperiodic breeding habits and produce embryos shortly after sunrise. The circadian rhythm of the fish was controlled by artificial light that was set by an automatic timer.[46]

So, approximately after 20-30 minutes, the fish started to mate and a few minutes later the eggs could be found on the bottom of the tank. The fish were then put back into their original tank on the system and they were allowed to rest for a minimum of seven days, before they could be incrossed again. The embryos were collected in petri dishes via a tea strainer and placed into an incubator at 28.5°C until needed for further use.

2.1.3 Drug treatment of *rps29*$^{-/-}$ zebrafish embryos

Unfertilized eggs and dead embryos were sorted out from the progeny of the in-crossed zebrafish. Always 20 fertilized embryos were transferred with some E3 into a well of a 24-well plate and incubated at 28.5°C.

As soon as the embryos reached the developmental stage of 50% epiboly, where the blastoderm covered half of the yolk, the E3 was removed from each well and the embryos were treated with 600µL of drug-E3 solution in different concentrations, always in triplets. The plate was covered in aluminum foil, in case the chemicals were light sensitive, and incubated again at 28.5°C overnight (o/n).

The next day, the E3-drug solution was removed via a transfer pipette and the chorions of the zebrafish embryos were degraded via treatment with 500µL of 1X Pronase for a maximum of 10 minutes. After that, the embryos were washed at least seven times with fresh E3 and the drug treatment was repeated in the same manner as the first time. Once again, the embryos were incubated at 28.5°C until they reached the stage of 48 hpf.

In this experiment, different PTZ derivatives were used as drugs to rescue hemoglobin in the RP deficient zebrafish disease model. In addition, the positive control FLU and the negative control dimethyl sulfoxide (DMSO), as well as two PTZ controls, were used as references, to be able to measure the efficacy of the investigated chemicals (see **Table 3**).

All drugs were tested in concentrations ranging from 0.01 to 50µM in order to determine toxic and effective doses of each chemical.

Table 3: List of drugs and controls used for in vivo drug treatments of zebrafish embryos

Drugs	Phenothiazine controls	Positive control	Negative control
083	079	Fluphenazine	Dimethyl sulfoxide
084	085		
086			
087-2			
087-3			
088-2			
088-3			
089			

2.1.4 Staining for hemoglobin

When the drug treated embryos reached a stage of 48 hpf, they were ready to be stained for their hemoglobin levels. The embryos of the triplets that were treated with the same drug were pooled in one well of the 24-well plate or alternatively in an Eppendorf tube and the remaining E3-drug solution was removed via a thin transfer pipette. 500µL of staining solution, containing 2mL of o-dianisidine, 2mL of distilled water (dH_2O) (Milli-Q by Millipore), 16.7µL of 3M sodium acetate (NaOAc) with a pH of 4.5 and 100µL of 30% hydrogen peroxide (H_2O_2), were pipetted onto the embryos and incubated on a shaker at room temperature (RT) for 30 minutes.

After that, the o-dianisidine staining solution was neutralized and the embryos were washed three times with 500µL of 1X phosphate buffered saline-Tris (PBS-T). Finally, the drug treated and stained embryos were fixed in 4% paraformaldehyde (PFA).

2.1.5 Scoring

The in PFA fixed embryos were washed three times with PBS-T. Then they were scored according to the amount of hemoglobin in their yolk sacs. First, all the wild type and heterozygous mutant embryos were sorted out and then, the homozygous mutants were scored into high, medium and low hemoglobin levels by eye (see **Figure 13**). In order to avoid biased results, the samples were blinded before scoring.

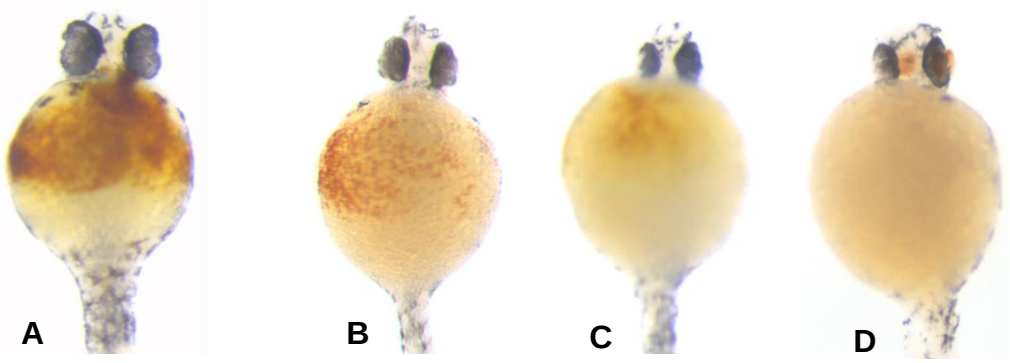


Figure 13: Drug treated and o-dianisidine stained wild type and mutant embryos with high, medium and low hemoglobin levels in their yolk sacs
(A) wild type (rps29$^{+/+}$) embryo with normal high hemoglobin levels **(B-D)** homozygous mutant (rps29$^{-/-}$) embryos
(B) high hemoglobin **(C)** medium hemoglobin **(D)** low to no hemoglobin

2.1.6 Genotyping of *rps29*$^{-/-}$ zebrafish embryos

In order to confirm that the embryos, which had high (and medium) hemoglobin levels, were really homozygous mutants, they were genotyped, as they were only scored by eye and in many cases they were not easily distinguishable from heterozygous mutants. For that always one mutant embryo was placed into a well of a 96-well PCR plate, the remaining PBS-T was removed and 25µL of HotSHOT alkaline lysis buffer were added per well. In addition, two wild type embryos served as a control, if all of the primers were working. The PCR plate with the samples was then

put into the thermocycler, where they were heated up to 95°C for 60 minutes and afterwards cooled down to 4°C. After the initial HotSHOT phase, the samples were neutralized with 25µL of neutralization buffer.

Two different PCR master mixes (Roche) were prepared, one that contained primers for mutant *rps29* genotypes (10µM Mutant rps29 15 and 10µM Mutant rps29 113) and one with primers for wild type *rps29* genotypes (10µM WT rps29 3039-c and 10µM WT rps29 3039-3). Always 1µL of each primer was used per 10µL of master mix solution. 2µL of the DNA samples were added to 10µL of each master mix and the PCR plates were centrifuged at 1000rpm for 1 minute, before they were placed into the thermocycler. The PCR and gel electrophoresis were the same as in genotyping of adult zebrafish via fin clipping (see **2.1.1 Genotyping of *rps29*$^{+/-}$ zebrafish**).

A band at 200bp with the mutant master mix only, indicated that the embryo had a homozygous mutation of *rps29* and therefore the *rps29*$^{-/-}$ genotype, which we were looking for. But, if there was also a band visible with the wild type master mix, the embryo carried a heterozygous mutation, so that its genotype was *rps29*$^{+/-}$. If there was only a band with the wild type primers and no band with the mutant primers, the DNA sample came from a wild type *rps29*$^{+/+}$ embryo.

2.1.7 Protein assay of drug treated zebrafish

For the protein assay, the offspring of incrossed *rps29*$^{+/-}$ zebrafish were drug treated with the same method as mentioned above (see **2.1.3 Drug treatment of *rps29*$^{-/-}$ zebrafish embryos**), but instead of staining them for hemoglobin, they were used to obtain protein samples for Western blotting.

DMSO still served as a negative and FLU as a positive control. The PTZ derivatives tested with the protein assay were 083, 087-2 and 087-3, each at a concentration of 1µM.

2.1.7.1 Sample preparation

rps29$^{+/-}$ zebrafish were incrossed via pairwise breeding and the progeny was drug treated, by following the normal drug treatment procedure (see **2.1.3 Drug treatment of *rps29*$^{-/-}$ zebrafish embryos**), so always 20 embryos were transferred into a well of a 24-well plate and at 50% epiboly they were treated with 600µL of drug-E3 solution. Next to the negative control DMSO and the positive control FLU (10µM), the PTZ derivatives 083 (1µM), 087-2 (1µM) and 087-3 (1µM) were used. The embryos were incubated at 28°C o/n and about 50 to 55 hpf, they were separated into wild type and mutant zebrafish phenotypes and transferred into separate plates, with 20 to 40 embryos per well. The chorions of the embryos were removed with 500µL of 1X Pronase and the wells were cleaned seven times with fresh E3, before the second drug treatment could follow.

The plate with the wild type embryos was incubated at 19°C o/n and the plate with the mutant embryos at 32°C o/n, in order to stage match them, as mutant zebrafish have a slowed down development compared to wild type zebrafish.

The next morning, the wild type and mutant embryos should have reached the same developmental stage. It was double-checked, if wild type and mutant embryos were properly separated and then they were transferred into special microcentrifuge tubes that fit a small pestle. The remaining drug-E3 solution was removed and 1mL of deyolking buffer (on ice), containg 1.607g of sodium chloride (NaCl), 0.067g of potassium chloride (KCl) and 0.053g sodium hydrogen carbonate ($NaHCO_3$) per 500mL of dH_2O (Milli-Q) and a protease inhibitor (1:100), was added to each tube. The mixture was gently pipetted up and down to disrupt the yolks of the zebrafish embryos and the samples were centrifuged at 4°C with 300*g for 1 minute.

Each pellet was washed with 1mL of cold washing buffer that contained 3.214g of NaCl, 0.130g of KCl, 0.198g of calcium chloride dihydrate ($CaCl_2.2H_2O$) and 0.788g of Tris-Hydrochloride (Tris-HCl) per 500mL of dH_2O (Milli-Q) and a protease inhibitor (1:100) and had a pH of 8.5. The pellet was resuspended and the samples were centrifuged again at 4°C with 300*g for 1 minute. This washing step was repeated once.After the second wash and spin down, the supernatant was removed and 50-100µL of Radio-Immunoprecipitation Assay (RIPA) buffer (Thermo Fisher Scientific)

with phosphatase inhibitors and a protease inhibitor (1:100) were added to each sample.

The embryos were dissociated with a pestle until a homogenous solution was obtained and the samples were centrifuged at 4°C with maximum speed for 10 minutes to get rid of the debris.

The supernatant of each wild type and mutant sample was collected, transferred into fresh Eppendorf tubes and stored at -80°C for further use.

2.1.7.2 Protein quantification

The protein of the wild type and mutant samples was quantified according to the Bio-Rad Quantification Protocol with the Bio-Rad Detergent Compatible™ Protein Microassay Kit II.

First, 20µL of Protein Assay Reagent S were added to 1mL of Protein Assay Reagent A to make Reagent A'. Then a standard set of different bovine serum albumin (BSA) concentrations was prepared. 15µL of 10mg/mL BSA stock were serially diluted with 15µL of Dulbecco's phosphate-buffered saline (DPBS) to produce a 5µg, a 2.5µg, a 1.25µg and a 0.625µg BSA standard. 5µL of each standard and also of each wild type and mutant sample were pipetted in duplicates into a flat bottom 96-well plate. 25µL of Reagent A' were added to each well including to a duplicate of empty wells, which served as a blank. 200µL of Protein Assay Reagent B were added to each well. The plate was incubated for 5 minutes in order to let the reagents react. The protein samples were read at 750nm in a microplate reader.

The raw data from this reading were used to make a standard curve from the BSA standard sets, which was needed to calculate the amount of protein present in the samples from the wild type and mutant zebrafish embryos.

2.1.7.3 Western blotting

The protein samples of the drug treated wild type and mutant embryos were analyzed via Western blotting. For that, the Invitrogen Western Blotting Kit by Thermo Fisher Scientific was used.

A 15-well 4-20% Tris-Glycine Midi Protein Gel (Invitrogen™ Novex™ by Thermo Fisher Scientific) was placed into a mini gel tank, which was filled with 1X sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) running buffer. 6µL of Full Range Amersham™ ECL™ Rainbow™ Molecular Weight Marker (GE Healthcare Life Sciences) (see **Figure 14**) were loaded into the gel. 5X loading dye was added to the protein samples, before they were loaded into the mini gel, according to the volume calculated from the amount of protein present in each sample. The mini gel was run at 225 volts for 40 minutes and subsequently analyzed with the iBlot® Dry Blotting System (Invitrogen™ by Thermo Fisher Scientific).
The mini gel, was placed into the transfer device on top of a bottom plate with a cathode. The gel was followed by an in dH_2O (Milli-Q) wetted filter membrane, a top plate with the anode and a sponge with two conductors that closed the current between cathode, anode and transfer device. After seven minutes, the negatively charged proteins from the gel were transferred onto the filter membrane. The membrane was cut below the purple band (52kb), and blocked with 5% BSA in 1X Tris-buffered saline-Tween 20 (TBS-T) on the shaker at RT for 60 minutes.

After that the remaining BSA was poured off, the primary antibodies P-p70S6K T389 (rabbit) and P-RPS6 235/256 (rabbit) in 5% BSA in 1X TBS-T were added to the corresponding filter membrane in a 1:1000 dilution and the blots were incubated on the shaker at 4°C o/n.

The next day, the unbound primary antibodies were washed off from the blots by washing three times quick with 1X TBS-T and incubating on the shaker at RT for 10 minutes. This was repeated two more times, before the secondary antibody (anti-rabbit) in 5% milk in 1X TBS-T was added to the blots in a 1:2000 ratio. After 45-60 minutes of incubation time on the shaker at RT, the unbound secondary antibody was washed off again with 1X TBS-T. 1mL of Detection Reagent A - Luminol Enhancer Solution and Detection Reagent B - Peroxide Solution (Amersham™ ECL™ Prime Western Blotting Detection Reagents by GE Healthcare) was added onto each blot in a 1:1 ratio. In contrast to P-RPS6, which was detectable by the regular detection solutions, ultrasensitive detection solutions had to be used for P-p70S6K. The excess detection solutions were poured off and the blots were transferred into

a cassette. Via the chemiluminescence reaction of the detection reagents the protein levels were transferred onto x-ray films, which were developed by an imaging machine in the dark room.

After the detection, the blots were washed with 1X TBS-T and stripped with 10mL of Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific) for 15 minutes. The blots were washed again with 1X TBS-T and re-blocked with 5% BSA in 1X TBS-T o/n.

The same procedure was repeated two more times with the primary antibodies total p70S6K (rabbit) and total RPS6 (mouse), both at a dilution of 1:1000. The second antibody for total p70S6K was anti-rabbit, while the second antibody for total RPS6 was anti-mouse. In the final step, GAPDH (rabbit) was used as a primary antibody and anti-rabbit as a secondary antibody.

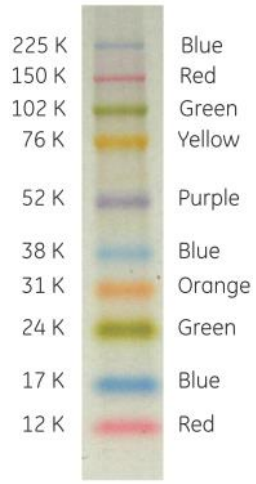


Figure 14: Full Range Amersham™ ECL™ Rainbow™ Molecular Weight Marker
Typical results of the ladder in a 4-20% Tris-Glycine gradient SDS-PAGE gel.

P-p70S6K and total p70S6K should be at ~70 K, P-RPS6 and total RPS6 at ~31 K and GAPDH at ~37 K.

2.2 Behavior assay

The compounds which gave the most promising results in the in vivo drug treatments of *rps29*$^{-/-}$ zebrafish embryos were also tested in a behavior assay with wild type zebrafish larvae in order to check, if the chemicals cross the BBB, which would be indicated by alterations of the fish behavior upon treatment with the selected PTZ derivatives.

2.2.1 Incrossing and breeding of wild type AB zebrafish

For the behavior assay, 6-day old wild type AB zebrafish larvae were used, since a homozygous *rps29* mutation is lethal in zebrafish after a couple of days, so that the mutant fish would not reach the larval stage.

Adult wild type AB zebrafish were incrossed in the iSpawn® (by the Zon Laboratory, Boston Children's Hospital) to efficiently produce a large number of zebrafish offspring in a rather short period of time. Male and female AB zebrafish were placed into the iSpawn and let breed o/n. The eggs were collected and dead embryos as well as unfertilized eggs were cleaned out.

The AB offspring were incubated at 28.5°C until 6 dpf, to make sure that the BBB was fully developed in the fish, before the behavior assay was done. At about 72 hpf when the larvae hatched, the water had to be exchanged to clean out chorion residues and dead fish.

On day 6, the zebrafish larvae were transferred into 96-deep-well plates. For that, always a single fish was sucked up carefully with a pipette in 100µL of E3 and pipetted into a well of a 96-deep-well plate. After that each fish was treated with 200µL of drug-E3 solution with the final concentrations 0.01µM, 0.1µM, 1µM and 5µM. The drugs 083, 088-2, 088-3 and 089, as well as four controls were tested at 0.1µM

each. FLU served as a negative control, Chk2 inhibitor as a positive control that should not influence the behavior of the zebrafish larvae, and 079 as well as 085 were PTZ controls that had similar chemical structures like the test compounds. In addition, a DMSO control had to be included in every plate, which served as a reference chemical to be able to compare the drugs and to check, if there were any problems with the experimental set up in general.

A total of eight fish were used per drug and dose, and three replicates were produced in order to obtain statistically relevant results.

2.2.2 Measurement of swimming activity

Once the drugs were administered to all the zebrafish larvae, the plates were placed into specifically designed boxes (in cooperation with the Schier Laboratory, Harvard University) and the activity measurement of the fish could start.

The specially built experimental set up consisted of six individual boxes, which were connected to a computer. The plates were placed into the boxes below a board on the ceiling containing many small lamps. By means of the special computer program (by Dr. Summer Thyme), the lamps and the cameras, to track the fish' swimming activity, were controlled automatically.

First, the light was turned on, but dimmed slightly before it was automatically turned on again at a set time. This was the time point at which the measurement of the fish' movement in the wells of the 96-deep-well plates started. The fish were observed by the cameras for a total of 120 minutes and the computer program recorded the amount of movement of each fish individually. The swimming activity was measured in bouts per unit of time (minutes). So, the computer counted every bout the fish made in one minute, based on the information the cameras recorded. All the collected raw data were then processed and summarized with a special statistics program (by Dr. Summer Thyme).

2.3 In vitro drug treatment of human cell models

To confirm the findings of the in vivo drug treatment and behavior assay in zebrafish, the most promising PTZ derivative 088-2 was tested in an in vitro human cell model.

2.3.1 Virus titering

The titer of lentivirus (tagged with a Green Fluorescence Protein (GFP)) was determined.

The day before titering, 50,000 Human Embryonic Kidney (HEK) 293T cells were seeded per well of a 24-well plate. 5 wells were plated per virus to be titered. The cells in one of the wells were trypsinized and counted. Titering media was prepared from Gibco® Dulbecco's Modified Eagles Medium (DMEM) (Life Technologies™), 10% FBS and hexadimethrine bromide (Polybrene®) (1000X stock) (Sigma Aldrich).

The cryoviral designated for viral titering was thawed and diluted as following:

Sample (a): 5µL of virus were diluted in 1mL of titering media

Sample (b): 200µL of sample (a) were aliquoted into an Eppendorf tube (corresponds to 1µL of virus).

Sample (c): 50µL of sample (a) were diluted with 150µL of titering media (corresponds to 0.25µL of virus).

Sample (d) 10µL of sample (a) were diluted with 190µL of titering media (corresponds to 0.05µL of virus).

Sample (e): 10µL of sample (a) were diluted with 990µL of tittering media (corresponds to 0.01µL of virus). These dilutions covered the virus thousand-fold.

The media from the wells (a to e) containing HEK 293T cells was aspirated and each well was infected with 200µL of the corresponding viral dilution. The plate was incubated at 37°C o/n. 24h after infection, 1(-1.5mL) of fresh 293T media was added and the cells were allowed to grow. After 2(-3) days, the infected cells and uninfected cells as a negative control were trypsinized with 150µL of trypsin and quenched with

250µL of 293T media. The cells were transferred into a Fluorescence Activated Cell Sorting (FACS) tube to proceed with the flow cytometric analysis of GFP^+ cells.

Equation 1: Formula to calculate the virus titer

$$\frac{(\text{number of cells on day of titering} * \text{percentage of } GFP^+ \text{ cells}) * 1000}{\text{volume of virus}} = \text{virus particles/mL}$$

2.3.2 Thawing of $CD34^+$ cells

Human $CD34^+$ cells were purchased from Fred Hutchinson Cancer Research Center (FHCRC), Seattle. The cells were thawed according to the FHCRC Thawing Protocol.

The cell vials were thawed rapidly in 37°C, transferred into a 50mL Falcon tube and serially diluted with an equal amount of thawing media (at RT) containing 1X PBS with 1% fetal bovine serum (FBS) by dripping the media very slowly on the inner wall of the tube until the total volume reached 25mL. Before adding more thawing media, each time the cells were allowed to rest for 3 minutes, in order to equilibrate. Then, the cells were pelleted at 1000rpm for 10 minutes at RT, the supernatant was removed and the cells were resuspended in 25mL of thawing media. The cells were pelleted again at 1000rpm for 10 minutes at RT, the supernatant was removed and the cells were resuspended in 10mL of filtered expansion media, which contained StemSpan® SFEM media, 2% Penicillin-Streptomycin (Pen Strep) and 100X StemSpan® CC100 1X cytokines. The cells were counted with a hemocytometer (1:2 dilution of cell suspension in 0.4% trypan blue stain) and placed at 2-5x10^5 cells/mL into a 25cm² cell culture flask.

2.3.3 Lentivirus infection of human peripheral blood mononuclear $CD34^+$ cells

The day before virus infection, a Retronectin® plate was prepared. Non-tissue culture treated flat bottom 96-well plates were coated with Retronectin® at 10µg/cm²

(3.2µg/well). So, 3.2µL of 1mg/mL Retronectin® stock were added to 100µL of sterile DPBS. 100µL were aliquoted into each well of the 96-well plate. The plate was sealed with Parafilm M® and stored at 4°C o/n.

$CD34^+$ cells were counted to calculate how many wells would be used to seed 10^5 cells/well and how much infection media would be needed.

200µL of a 50 multiplicity of infection (MOI) virus stock were thawed on ice. Only 70% of the total cell number were used, the remaining 30% served as a control. 150µL of fresh expansion media with protamine sulfate (1000X) were used per well. So, for the cell count of $3.275*10^5$ cells/mL (volume 20mL), 46 wells were used to cover 70% of the total cell number, 6.9mL of media and 6.9µL of protamine sulfate were needed.

The cells were split into two tubes, one containing the 70% of the cells that will be infected and one containing the 30% for control. The cells were spun down at 1000rpm for 5 minutes. The supernatant was removed and the 70% of the cells were resuspended in infection media, which contained 9.6mL of pre-warmed expansion media, 9.6µL of 1000X protamine sulfate and 200µL of 50 MOI virus. The control cells were resusended in regular expansion media and placed back into the cell culture flask at about $2*10^5$ cells/mL.

The supernatant PBS of the Retronectin® coated plate was removed (Retronectin® was now bound to the plate surface) and 150µL of the cells in the infection media were aliquoted into the inner wells of the well plate. The outer wells were filled with PBS in order to prevent evaporation of the samples. The plate was sealed and centrifuged at 2,400rpm at 30°C for 60 minutes. Then, the plate was incubated at 37°C o/n. About 24h after the infection, the cells were removed from the plate and transferred into a 15mL Falcon tube. The cells were spun down at 1000rpm for 5 minutes and the pellet was resuspended in fresh, pre-warmed expansion media. The infected and also the control cells were counted analyzed via FACS.

2.3.4 Drug treatment of shRPS19 CD34^{+} cells

200,000 to 500,000 cells were used for each drug treatment. 1mL of cell suspension was added per well of a 24-well plate and 1µL of drug was added. In total, 8 drug treatments were done, 4 treatments of the infected shRPS19 CD34^{+} cells and 4 treatments of the control cells. DMSO was used as a negative control, TFP at 1µM served as a positive control and the drug 088-2, was tested at 1µM and 5µM. The drug treated cells were incubated at 37°C o/n.

2.3.5 Cell lysis and protein harvest

The cell lysis and protein harvest was carried out by following the protocol for the Procedure for Lysis and Suspension-cultured Mammalian Cells by Thermo Fisher Scientific.

24h after the drug treatment, the shRPS19 and control cells were collected in Eppendorf tubes and pelleted by centrifugation at 2500*g for 5 minutes. The supernatant was discarded and the cells were resuspended and washed in 1mL of DPBS. The cells were pelleted again and resuspended in 40µL of cold RIPA buffer (Thermo Fisher Scientific) with phosphatase and protease inhibitors. The mixture was gently shaken on ice for 15 minutes and then centrifuged at 14,000*g for 15 minutes to pellet the cell debris. The supernatant was collected in new tubes and stored at -80°C for further use.

2.3.6 Protein quantification

See 2.3.6.1 Protein quantification - Bio-Rad Quantification Protocol with the Bio-Rad Detergent Compatible™ Protein Microassay Kit II.

2.3.7 Western blotting

See 2.3.7.1 Invitrogen Western Blotting Kit by Thermo Fisher Scientific.

A 10% Tris-Glycine Midi Protein Gel (Invitrogen™ Novex™ by Thermo Fisher Scientific) was used. After the transfer, the membrane was cut below 52kb. The membranes were blocked with 5% BSA in 1X TBS-T for 60 minutes and immunostained

with the primary antibodies total p53 (mouse) or P-RPS6 235/256 (rabbit), total RPS6 (mouse) and GAPDH (rabbit).

3 Results

3.1 Effects of novel phenothiazine derivatives on hemoglobin levels in *rps29*$^{-/-}$ zebrafish embryos

The PTZ derivatives 083, 084, 086, 087-2, 087-3, 088-2, 088-3 and 089 were tested along with the negative control DMSO, the positive control FLU and the two PTZ controls 079 and 085 in the in vivo *rps29*$^{-/-}$ zebrafish model system, to see, whether the compounds would be able to rescue hemoglobin.

rps29$^{-/-}$ zebrafish embryos were drug treated twice and stained for their hemoglobin levels. The embryos were scored into high, medium and low hemoglobin and genotyped.

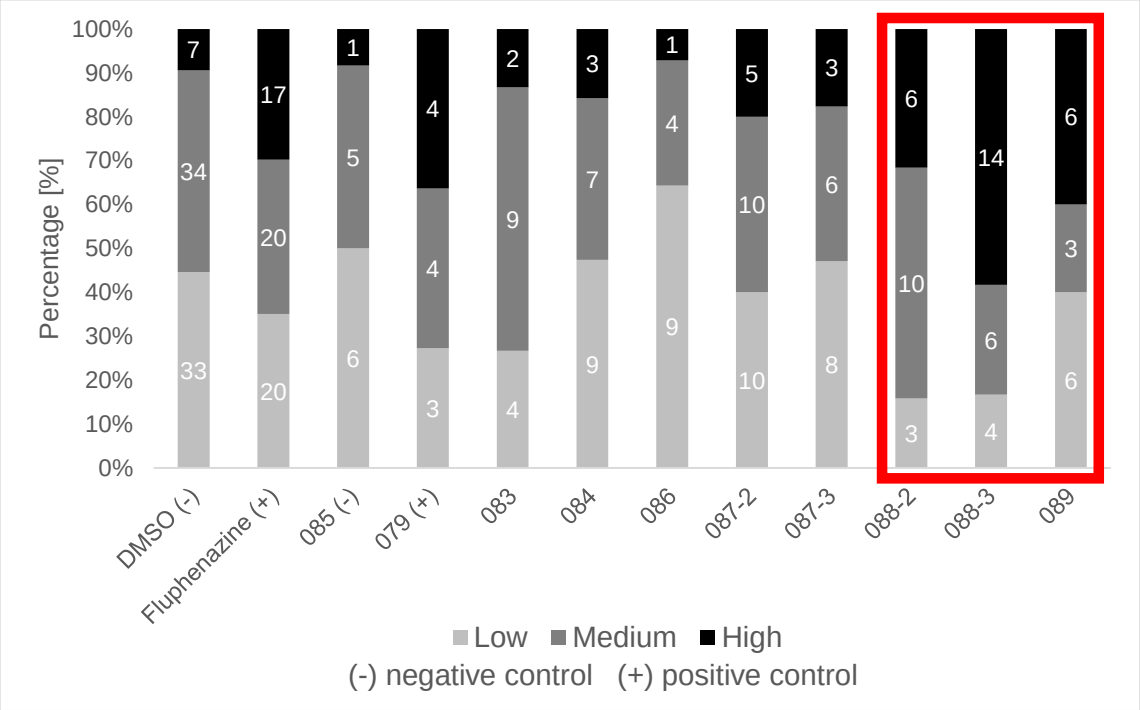
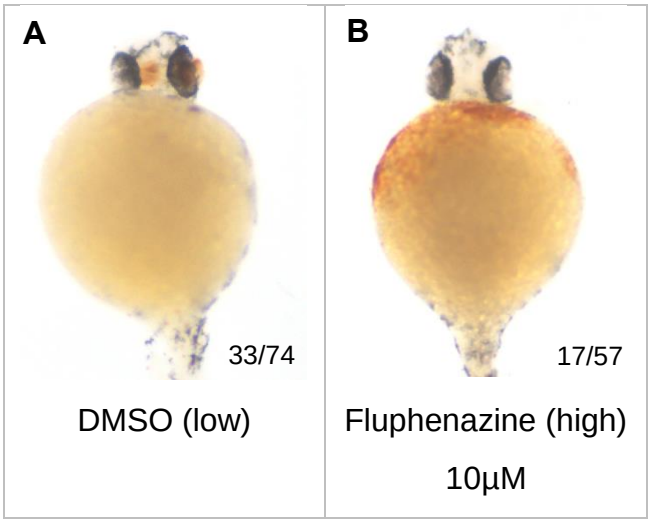


Figure 15: Results of the drug treatments at a dose of 0.05µM
Summary of the efficacy of all PTZ derivatives at 0.05µM. DMSO served as a negative and FLU (10µM) as a positive control. The PTZ control 085 was practically ineffective, but 079 had a positive effect on the hemoglobin level and therefore served as another positive control. The drugs that are highlighted in the red box were most effective. 31.6% of the embryos treated with 088-2, 58.3% of the embryos treated with 088-3 and 40% of the embryos treated with 089 had high hemoglobin levels.

Negative control **Positive control**



Drugs

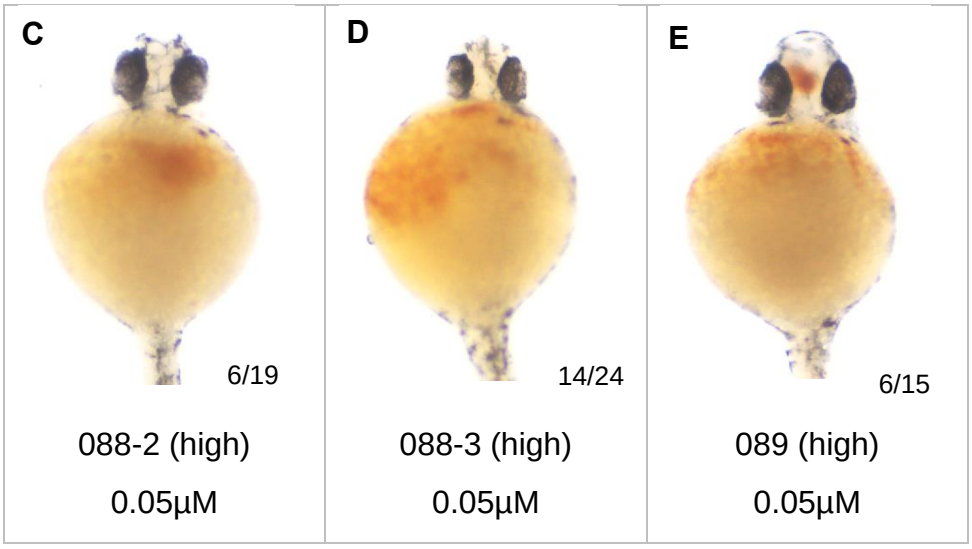


Figure 16: Hemoglobin levels in rsp29$^{-/-}$ zebrafish embryos after drug treatment with the most effective PTZ derivatives at 0.05µM

(A) rps29$^{-/-}$ embryo with low hemoglobin level after drug treatment with the negative control DMSO **(B)** rps29$^{-/-}$ embryo with high hemoglobin level after drug treatment with the positive control FLU (10µM) **(C-E)** results of rps29$^{-/-}$ embryos treated with the most effective PTZ derivatives **(C)** rps29$^{-/-}$ embryo with high hemoglobin level after drug treatment with 0.05µM 088-2 **(D)** rps29$^{-/-}$ embryo with high hemoglobin level after drug treatment with 0.05µM 088-3 **(E)** rps29$^{-/-}$ embryo with high hemoglobin level after drug treatment with 0.05µM 089

The less effective, but still not completely ineffective PTZ derivatives 083, 087-2 and 087-3 were tested in a protein assay at a concentration of 1µM, in order to check, if the chemicals changed the levels of RPS6. Protein was collected from wild type and mutant zebrafish embryos that were treated with these selected chemicals and immunostained for P-RPS6, total RPS6 as well as GAPDH via Western blotting.

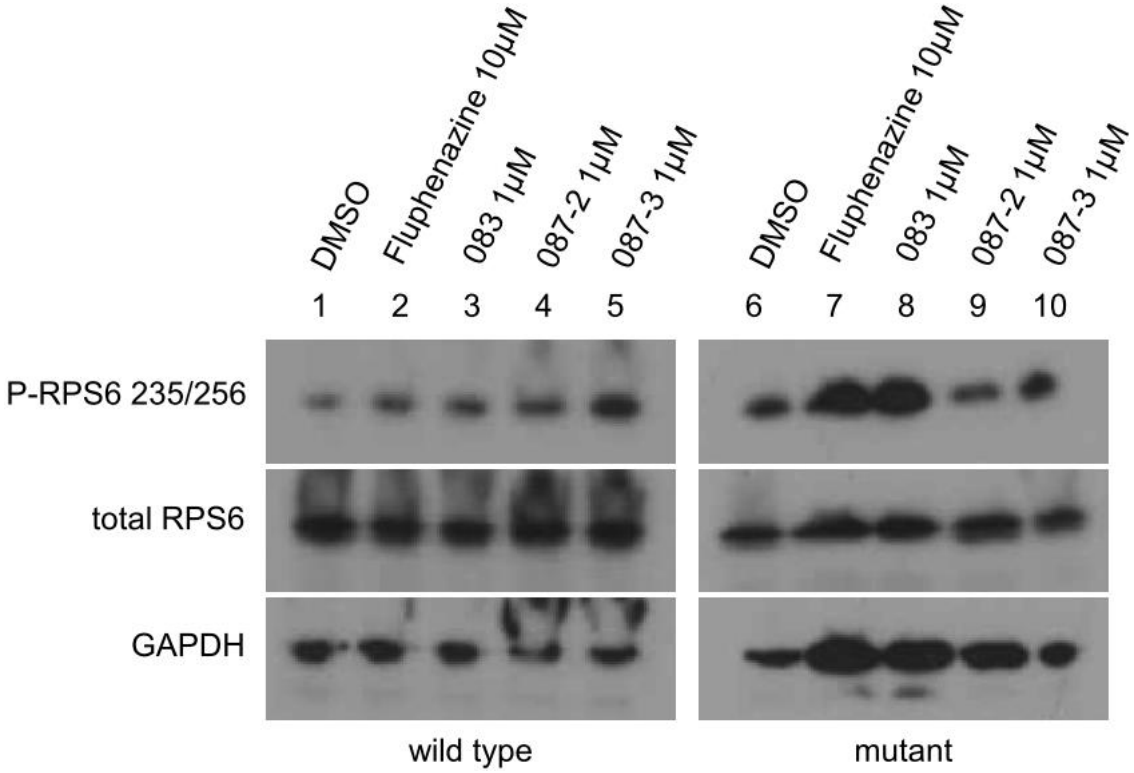


Figure 17: Results of the protein assay of drug treated embryos with selected PTZ derivatives

Protein of wild type embryos (on the left) and mutant embryos (on the right) treated with DMSO (negative control), FLU 10µM (positive control), 083 1µM, 087-2 1µM and 087-3 1µM in Western blots for P-RPS6 235/256, total RPS6 and GAPDH. In wild type embryos P-RPS6, total RPS6 and GAPDH levels were more or less unchanged with any of the PTZ derivatives. In mutant embryos P-RPS6, but also GAPDH levels seemed to be slightly elevated in those embryos, which were treated with the positive control FLU or the drug 083.

3.2 Effects of selected compounds on larval zebrafish behavior

The most effective drugs from **Figure 15** were tested in the behavior assay. 6-day old zebrafish larvae were treated with the selected PTZ derivatives at the following concentrations: 0.01µM, 0.1µM, 1µM and 5µM. The fish' swimming activity was tracked for 120 minutes in order to measure any behavioral changes caused by the administered drugs, which would indicate that they cross the BBB. The degree of movement of the zebrafish larvae was measured in the number of bouts per unit of time (minute).

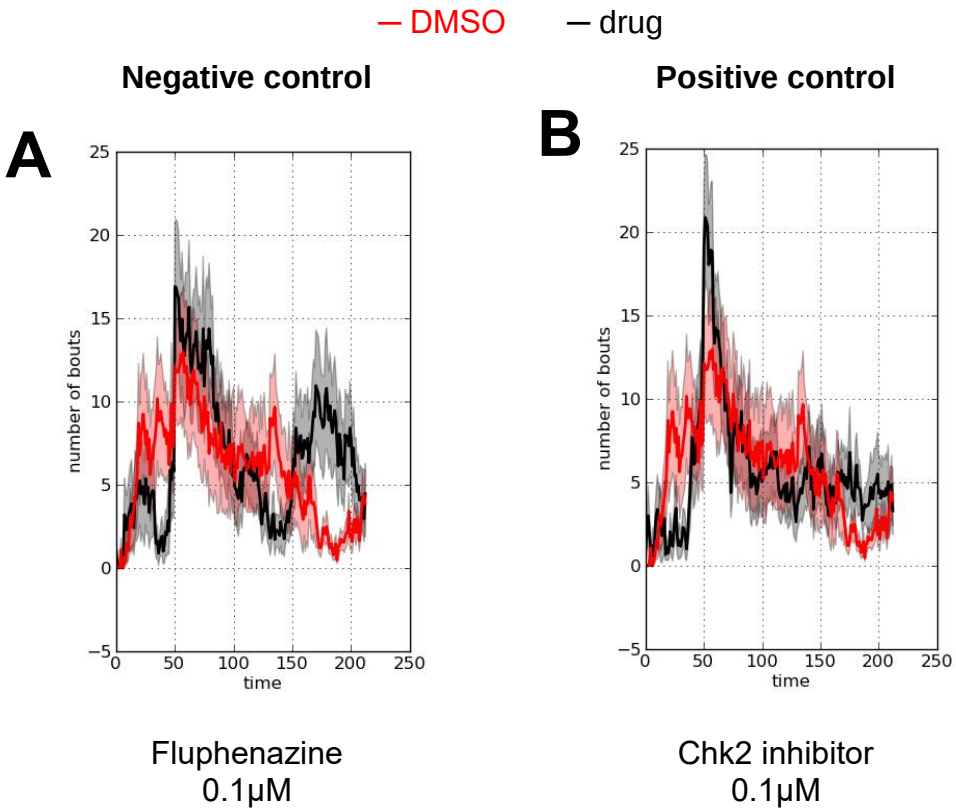


Figure 18: Effects of the negative and the positive control on the behavior of zebrafish larvae

(A) Results of the analysis of the effect of the negative control FLU on the fish' swimming activity at a concentration of 0.1µM; **(B)** Results of the analysis of the effect of the positive control Chk2 inhibitor on the fish' swimming activity at a concentration at 0.1µM;
The zebrafish larvae treated with the negative control FLU showed changes is their behavior compared to the reference chemical DMSO, while the larvae treated with the positive control Chk2 inhibitor had a very similar pattern of movement as DMSO.

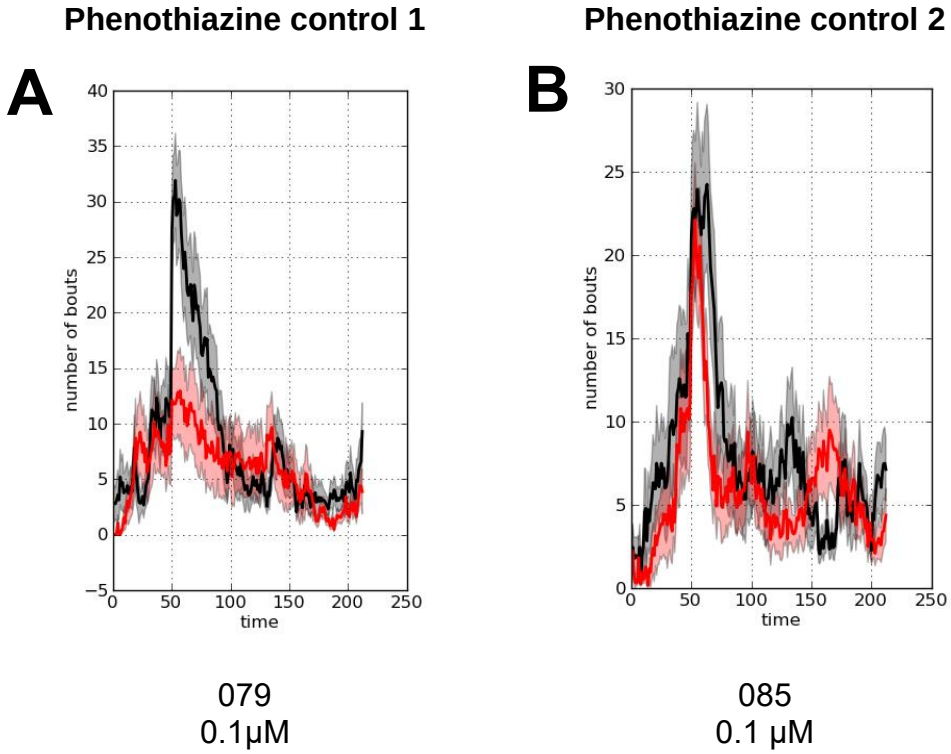


Figure 19: Effects of the PTZ controls on the behavior of zebrafish larvae
(A) Results of the analysis of the effects of PTZ control 079 on the fish' swimming activity at a concentration of 0.1µM; **(B)** Results of the analysis of the effects of PTZ control 085 on the fish' swimming activity at a concentration of 0.1µM;
The zebrafish larvae treated with the PTZ control 079 showed changes is their behavior compared to the reference chemical DMSO, while the larvae treated with the PTZ control 085 had a similar pattern of movement as DMSO.

These four controls (FLU, Chk2 inhibitor, 079 and 085) as well as the reference chemical DMSO were used to determine the effects of the tested PTZ derivatives on the behavior of 6-day old zebrafish larvae and to identify possible drug candidates to rescue RP deficiencies.

So, the PTZ derivatives that gave the best results in the drug treatment of *rps29*$^{-/-}$ zebrafish embryos, 089, 088-3, and 088-2, where compared to the results of the control chemicals, as well as the less effective PTZ derivative 083, and observed for their effects on the movement of zebrafish larvae, with the aim to find a compound that does not cause any abnormal behavior in the fish.

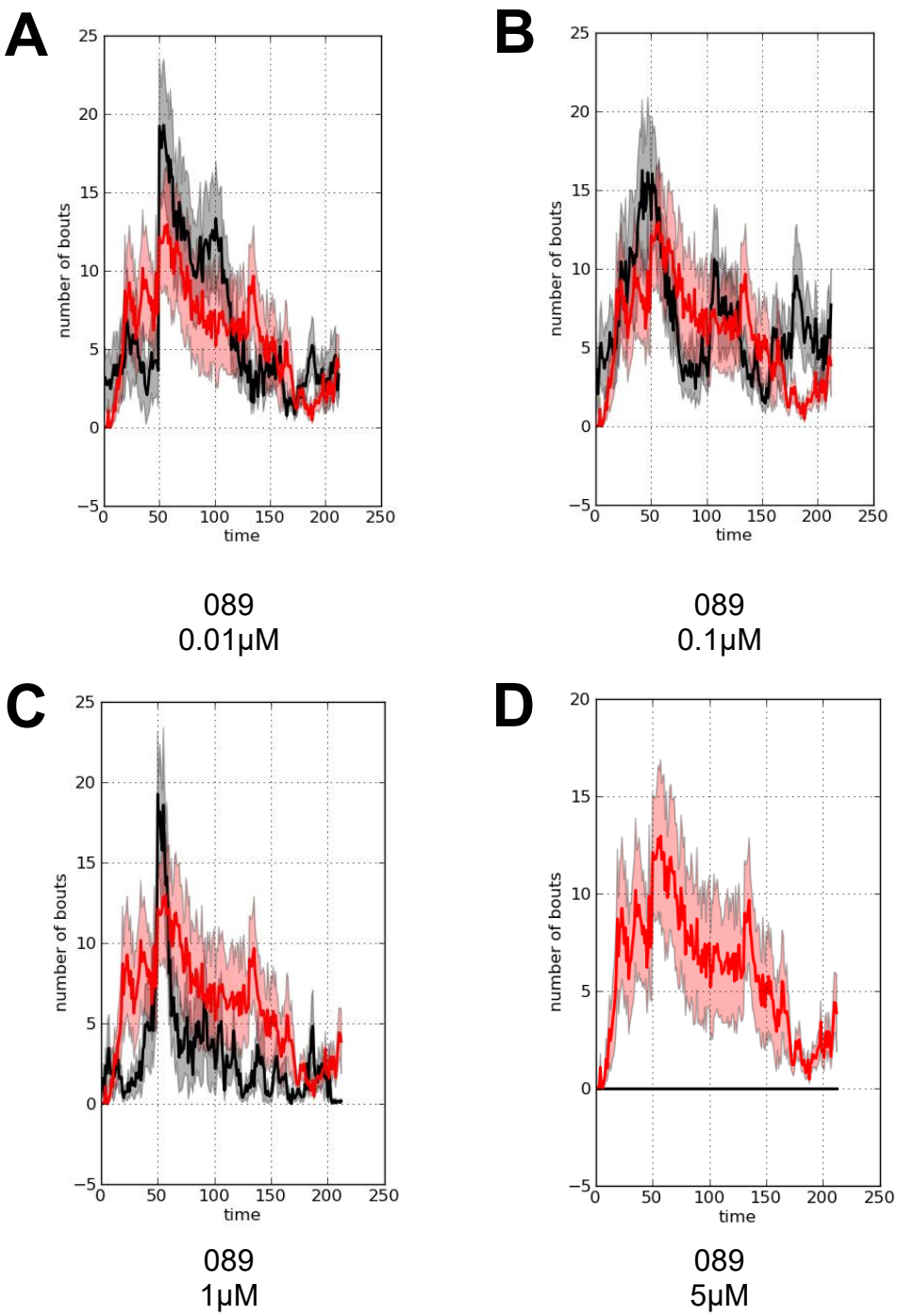


Figure 20: Effects of drug 089 on the behavior of zebrafish larvae
Results of the analysis of PTZ derivative 089 on the fish' swimming activity at a concentration of 0.01µM **(A)**, 0.1µM **(B)**, 1µM **(C)** and 5µM **(D)**. At a very low concentration of 0.01µM, there was no significant difference observed between the embryos treated with the drug 089 and DMSO. With increasing concentration, the fish' swimming activity differed increasingly from the control fish. The movement of the fish treated with 089 was generally more erratic than of the control fish. At a drug concentration of 5µM, the zebrafish were not moving at all.

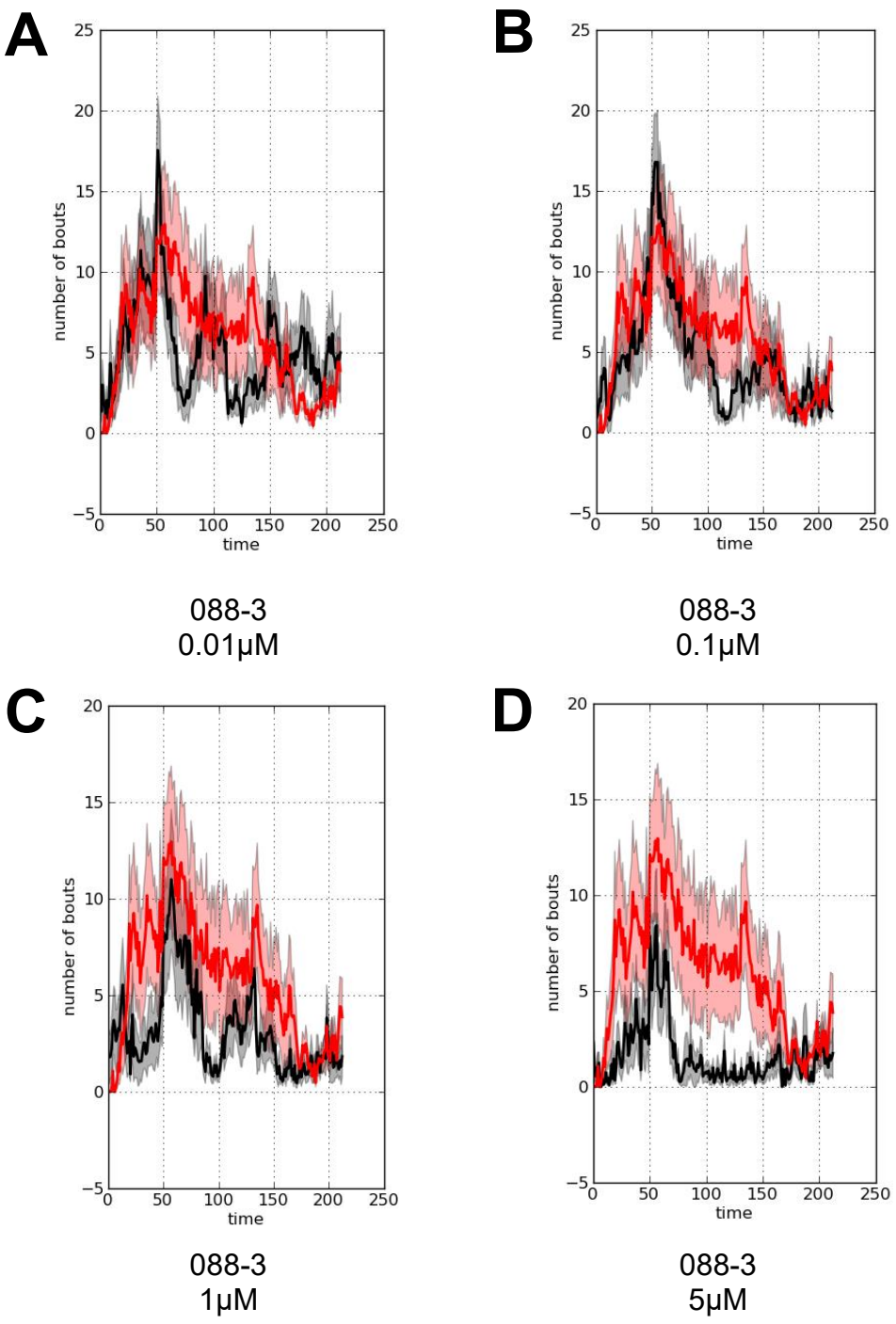


Figure 21: Effects of drug 088-3 on the behavior of zebrafish larvae
Results of the analysis of PTZ derivative 088-3 on the fish' swimming activity at a concentration of 0.01µM **(A)**, 0.1µM **(B)**, 1µM **(C)** and 5µM **(D)**. Some significant differences in the pattern of movement could be observed between the drug treated and the control fish. The activity of the drug treated fish was characterized by high increases of bouts per minute, followed by high decreases, indicating erratic movements. At 5µM, the fish' swimming activity decreased over time to less than 5 bouts per minute.

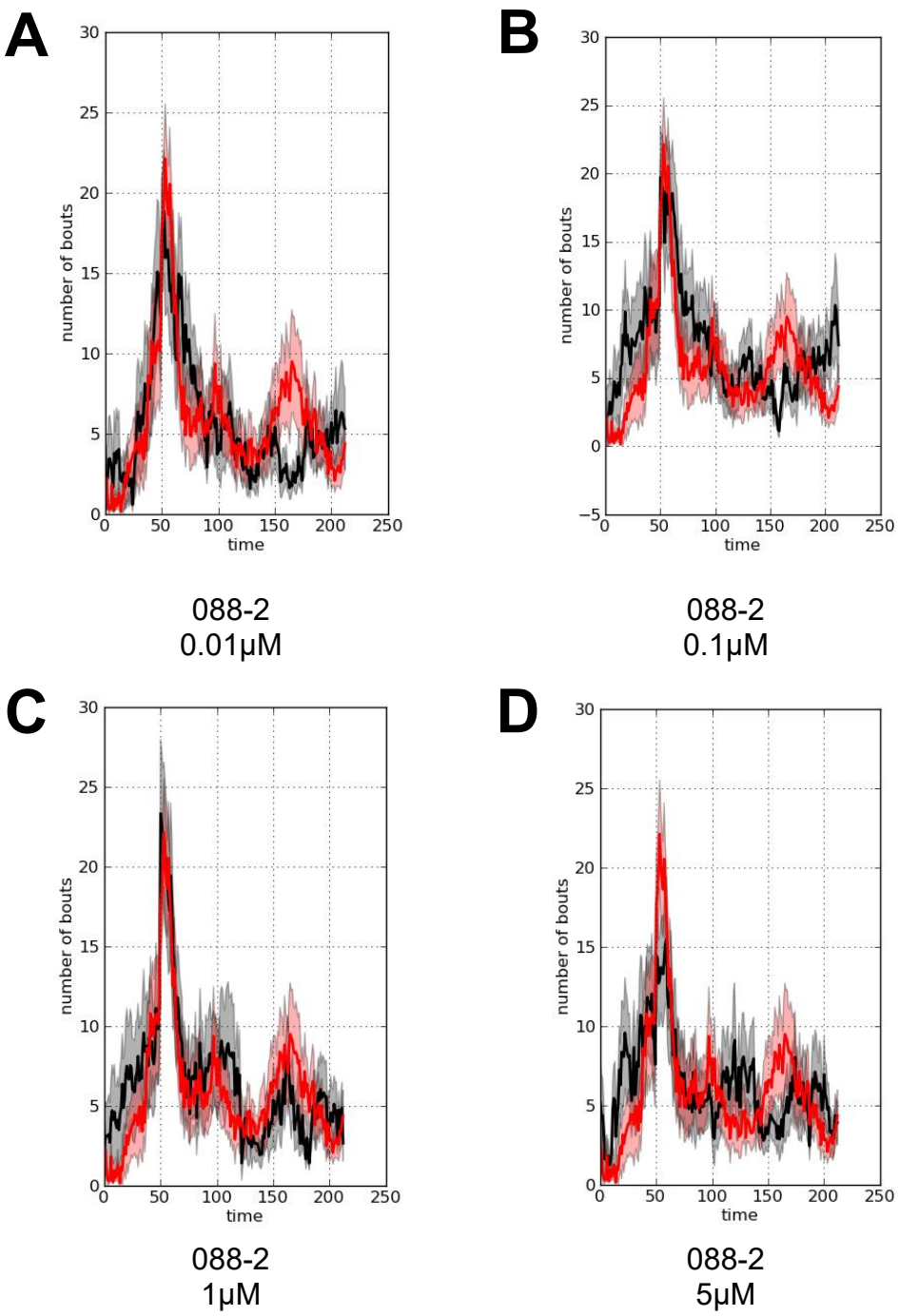


Figure 22: Effects of drug 088-2 on the behavior of zebrafish larvae
Results of the analysis of PTZ derivative 088-2 on the fish' swimming activity at a concentration of 0.01µM **(A)**, 0.1µM **(B)**, 1µM **(C)** and 5µM **(D)**. Minimal differences in the pattern of movement could be observed between the drug treated and the control fish. At 1µM, the fish' swimming activity of the drug treated and the control fish was almost identical.

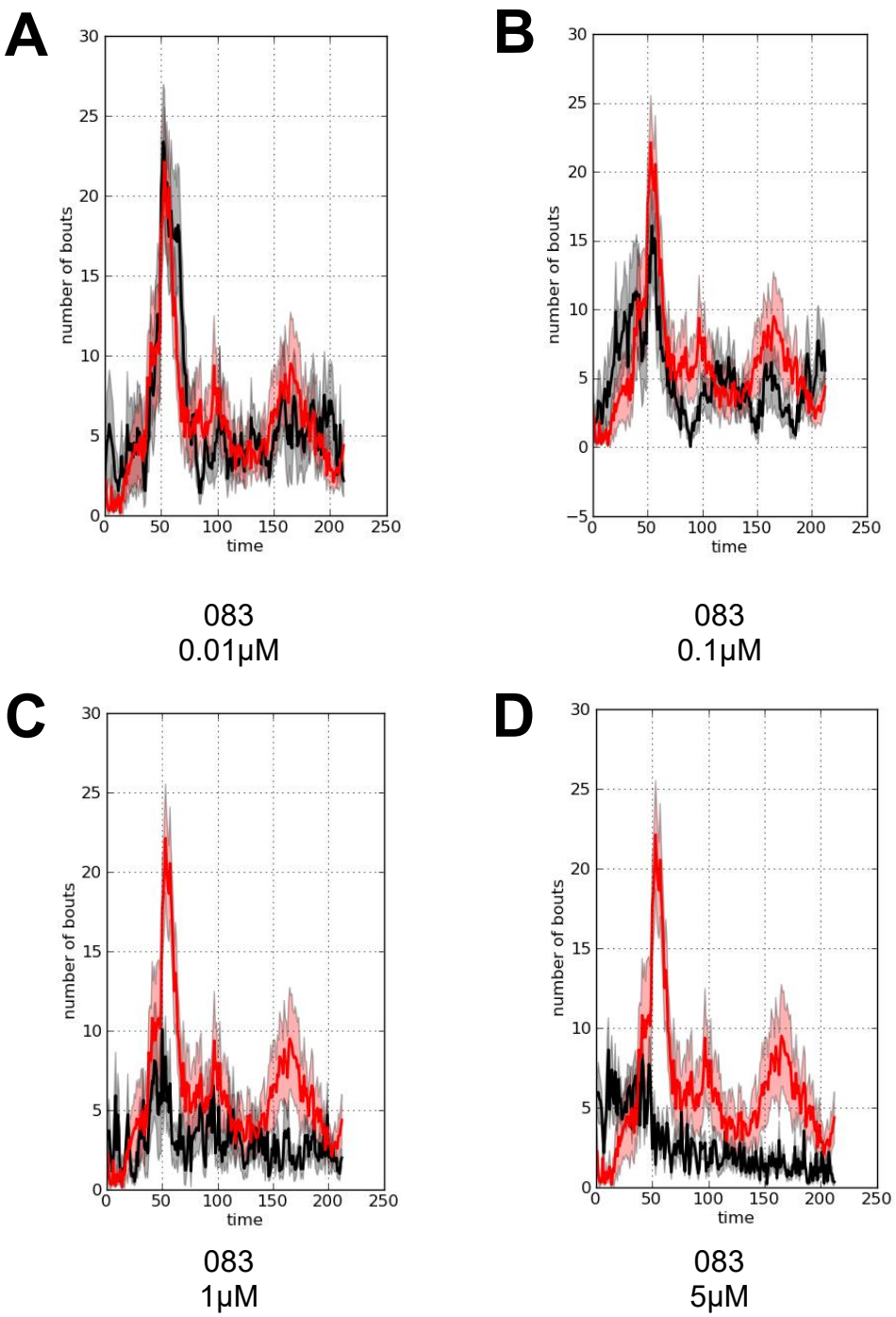


Figure 23: Effects of drug 083 on the behavior of zebrafish larvae
Results of the analysis of PTZ derivative 083 on the fish' swimming activity at a concentration of 0.01µM **(A)**, 0.1µM **(B)**, 1µM **(C)** and 5µM **(D)**. At a very low concentration of 0.01µM, there was no significant difference observed between the embryos treated with the drug 083 and DMSO. The activity of the drug treated fish slowed down with increased concentration over time. Also, with a dose of 1µM and 5µM the movement was more erratic.

3.3 Confirmation of the efficacy of selected compounds in the human shRPS19 CD34⁺ cell model

The PTZ derivative 088-2, which gave the best results in the drug treatment and the behavior assay was also tested in an in vitro human cell model with shRPS19 CD34⁺ cells. Wild type and shRPS19 CD34⁺ cells were treated with 088-2 at a concentration of 1µM and 5µM. DMSO was used as a negative control and Trifluoperazine as a positive control. The protein of the drug treated cells were harvested and immunostained for P-RPS6 235/256, total RPS6 and GAPDH by Western blotting.

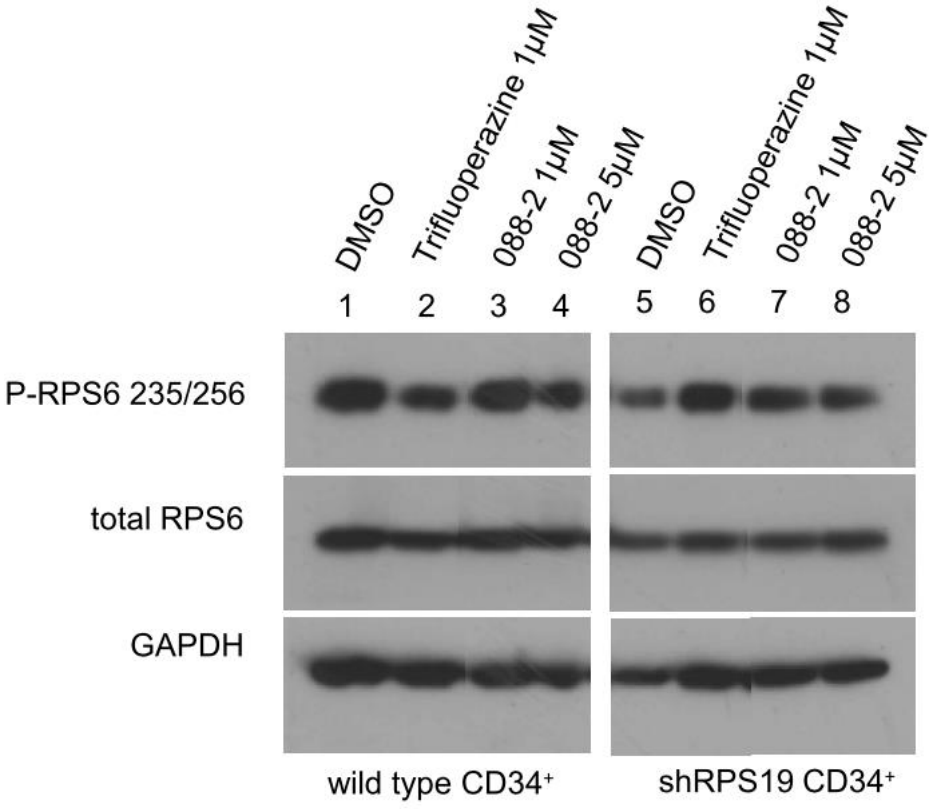


Figure 24: Results of the protein assay of drug treated CD34⁺ cells with the PTZ derivative 088-2
Protein of wild type CD34⁺ cells and shRPS19 CD34⁺ cells, treated with DMSO (negative control) (**1** and **5**), TFP 1µM (positive control) (**2** and **6**), 088-2 at 1µM (**3** and **7**) and 5µM (**4** and **8**) in Western blots for P-RPS6 235/256, total RPS6 and GAPDH. P-RPS6 levels in TFP and 088-2 treated shRPS19 cells were almost as high as in the wild type cells, compared to the DMSO control (**5**). Total RPS6 and GAPDH levels of the shRPS19 cells were lower as in the wild type cells, but more or less the same as in the DMSO control (**5**).

4 Discussion

On average, 50 - 60% of naturally set-up fish should produce progeny, with high variabilities (depending on feeding, medical condition, age, stress, set-up, etc.).[46] According to Mendelian genetics, incrossing of heterozygous *rps29* mutant zebrafish would technically give 25% of *rps29*$^{-/-}$ mutant zebrafish, but this does not really correspond to reality. This can probably be mostly be due to the fact that many embryos die very early and since a homozygous mutation is in many cases lethal, as it is the case in *rps29*$^{-/-}$ zebrafish. Homozygous *rps29*$^{-/-}$. As a result the number of mutant embryos was not always as high as expected.

In the in vivo drug screen of the PTZ derivatives 083, 084, 086, 087-2, 087-3, 088-2, 088-3 and 089 the last three were percentage-wise the most effective in terms of rescuing hemoglobin levels of homozygous mutant *rps29* embryos (see **Figure 15**). Each drug was tested triplets and the experiments were repeated three times, in order to obtain statistically relevant results. The total number of embryos per drug treatment varies, due to the fact that the homozygous mutant embryos were separated from the heterozygous mutant and wild type embryos post drug treatment and staining. Since the embryos were sorted by eye, it was not possible to distinguish *rps29*$^{-/-}$ zebrafish from *rps29*$^{+/-}$ and *rps29*$^{+/+}$ zebrafish earlier. Also some embryos always die during the first few hpf, either over the course of the drug treatment procedure or naturally. In order to optimize the statistical values of the drug treatment, the experiment would need to be scaled up and automatized. Moreover, even though the samples with the different PTZ derivatives and the controls were blinded, it is not possible to eliminate every source of bias, if the embryos are sorted and scored by eye. On the other hand it is advantageous, if all the chemicals are screened by the same person, in order to reduce deviations from the operating procedure as much as possible. Another factor that is almost not controllable, is the difference from batch to batch of zebrafish progeny. Often, there are batches which are not completely healthy, consequently, the embryos are not as robust and might tolerate lower drug concentrations or there is a high percentage of death embryos.

The drugs that were not as effective as 088-2, 088-3 and 089 were tested in a protein assay, to check, if the compounds change total RPS6 levels. The positive control FLU and 083 seemed to slightly increase P-RPS6, compared to DMSO and 087-2 or 087-3, however, since also GAPDH levels were elevated in those two samples, it is assumed that a different amount of protein was loaded. In order to be able to conclude anything from this, the protein would have to be repeated at least one more time.

Since 088-2, 088-3 and 089 gave the most promising results in the drug screen, these compounds were also tested in the behavior assay. Interestingly, 088-2 was the only drug, which seemed to have no influence on the zebrafish larvaes swimming activity. The other drugs caused rather erratic movements and with increasing dose most fish slowed down significantly over time, compared to the control fish. At a dose of 5µM 089 was already toxic, since the drug treated larvae did not move at all. At a concentration of 10µM 089 was also toxic in the in vivo drug treatment of the *rps29* mutant embryos. Also 088-3 and 083 reduced the activity of the zebrafish larvae. The controls seemed to be working as expected, but the dose of 0.1µM might actually be a little too low, especially for FLU, which is usually works best at 10µM. Generally, lower drug concentrations were used in the behavior assay than in the drug screen, as it was noticed that zebrafish are less robust and more sensitive to the drugs at 6 dpf.

DMSO always served as a reference chemical for all other controls and drugs, since it was used as a vehicle for all of the chemicals. DMSO does not cross the BBB, and thus also does not lead to any changes in the fish' swimming activity. The same applies to the Chk2 inhibitor, which also cannot cross the BBB and thereby influence the fish' movement FLU on the other hand, is known to be able to rescue hemoglobin in mutant zebrafish embryos, but it does cross the BBB and affects the fish' behavior, so it was used as a negative control. (see **Figure *18***).

The effects of the PTZ derivatives 079 and 085 have not yet been identified fully, but both are controls for the eight to be tested derivatives 083, 084, 086, 087-2, 087-3, 088-2, 088-3 and 089. In contrast to 079, which was clearly able to rescue hemoglobin levels in *rps29* null zebrafish embryos, was 085 more or less uneffective,

since no significant increase in hemoglobin was observed upon treatment of homozygous *rps29* mutant zebrafish embryos with this PTZ derivatives. In the behavior assay, there was no difference between DMSO and 085 treated zebrafish larvae either, but larvae, which were treated with 079 showed a significant deviation in their activity compared to the DMSO control from minute 50 to 100 (see **Figure 19**).

At low concentrations, such as 0.01µM and 0.1µM drug, no significant deviations between the drug 089 and DMSO could be noticed. But with an increased dose, the fish that were treated with the drug clearly moved less than the fish that were treated with DMSO. At a concentration of 1µM the larvae already showed a significant reduction in their swimming activity, except for a short time point after approximately 50 minutes, where they had a significant increase in number of bouts per minute compared to the control. Also, the fish which were treated with drug 089, show a more erratic movement than the control fish. At a concentration of 5µM, the drug treated fish did not move at all unlike the control fish, which indicates that this dose was already toxic for the animals (see **Figure 20**). Similar results were obtained from drug 088-3 and 083, although these were less toxic (see **Figure 21** and **Figure 23**)

The results from drug 088-2 were very similar to those of the positive control Chk2 inhibitor and there was no significant difference between the activity of the drug treated and the DMSO control larvae (see **Figure 22**). Although 088-2 was not the most effective drug in the drug screen (see **Figure 16**), these results were very promising.

So, the PTZ 088-2 was tested in a protein assay in human $CD34^+$ control cells and $CD34^+$ that were infected with lentivirus to produce an shRPS19 $CD34^+$ disease model for DBA (and other ribosomopathies), in order to check, if the drug would be able to rescue RP levels and if it would influence p53, the main regulator of the disease. There was a slight increase in P-RPS6 levels upon treatment with 088-2 at 1µM and 5µM, but not as high as in the positive control TFP or the wild type cells (see **Figure 24**). Unfortunately, we were not able to identify the effect of the drug on p53 yet. So further experiments are needed to confirm the results we obtained so far.

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