Chaf1b Protein in *gef* Mutant Zebrafish

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Abstract

Zebrafish embryos that are homozygous for *gef* mutant alleles experience a small-eye phenotype and are hypothesized to have decreased levels of the Chaf1b protein. This could be detected by anti-Chaf1b antibodies in a western blot. This western blot will be performed using Chaf1b morpholino 3 dpf embryos, wild type 3 dpf embryos, and *gef* mutant embryos 3 dpf. Embryos inoculated with Chaf1b morpholino serve as a negative control. This was then compared to the positive control of wild type zebrafish embryos, whose western blot was expected to have normal levels of Chaf1b. Proteins isolated from a *gef* mutant were then be analyzed and compared to the control proteins to determine the levels of the Chaf1b protein in the mutant. Future research can expand on the in vitro role of Chaf 1b in multicellular organisms and this protein's significance in regeneration.

Introduction:

Retinal regeneration in zebrafish is often triggered by apoptosis of retinal neurons, this provokes rapid proliferation of the Müller glia, increasing the number of progenitor cells. The newly created cells located within the inner nuclear layer will then migrate and differentiate, incorporating themselves within the system accounting for the damage that has occured (Bailey and Hyde, 2010). However, various mutations that may exist in the zebrafish genotype that can prevent normal development and regenerative abilities. The specific deviation of interest is the gef mutation (Fig. 2), fish with this genotype experience a small-eye phenotype and cannot make a Chaf1b protein detected by anti-Chaf1b antibodies. The Chaf1b protein is a vital subunit of the Chromatin Assembly Factor 1, as it is thought that this protein is necessary for the cell to be able to switch from cycling retinal progenitor cells to post-mitotic differentiating cells, allowing it to survive the synthesis (S) phase of the cell cycle (Bailey and Hyde, 2014). It has also been shown that Caf1b is required for differentiation of cells in the retina (Fischer, 2007).Without the presence of Chaf1b protein, zebrafish embryos are unable to add histones to DNA for replication, ultimately leading to cell death 2-3 days post fertilization (dpf).

Works Cited:

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Materials and Methods

Protein Extraction: 15 eggs were collected from the breeding ALB 7ax7a zebrafish, these wild type embryos served as the positive control. Additionally 38 Eggs were collected from breeding ALB 7ax7a zebrafish and a select amount were inoculated at the 1-2 cell stage with 1nl of 0.5M Chaf1b morpholino using a picospritzer apparatus, creating the negative control. Eggs were also collected from gef/tp53 3xg51 zebrafish, the samples from all conditions were then incubated for three days at 80℉. At 3dpf the heads of ten fish were lysed from the gut of wild type and morpholino injected ALB 7ax7a zebrafish. The isolated tissue was homogenized with 100µlof previously prepared RIPA buffer (Table. 1) in a 1.5ml microcentrifuge tube and kept frozen till ready for use. The same protocol was followed for isolating gef/tp53 3xg51 zebrafish retinas, except 9 heads were used in 90µl

Gel Electrophoresis, Protein Transfer and Immunoblotting: Protein samples were allowed to thaw at room temperature and were vortexed to ensure DNA was homogenized with the lysis buffer. 90µl of each protein sample, wild type ALB 7ax7a, Chaf1b morpholino ALB 7ax7a and gef/tp53 3xg51were then transferred to three separate .2ml white PCR strip tubes. 2µl of *Phosphatase Inhibitor Cocktail 2* and 2µl *Phosphatase Inhibitor Cocktail 3* were added to each sample, to inhibit protein denaturing. As well is 10µl of 10% 2-mercaptoethanol and 30µl of Laemmli 2X loading buffer (Table. 2), for a total volume of 132µl in each condition. These solutions were then incubated at 90℃ for 5 minutes. A Bio-Rad pre-filled cassette was used to run the SDS-PAGE, after removing the comb and assembling the stand the upper chamber was filled with 150mL of 1X tris-glycine buffer and the lower chamber was filled with 550mL of 1X tris-glycine buffer. This was made by diluting 100ml 1X tris-glycine buffer in 900ml of distilled water. Subsequently, 20µl of PageRuler Plus Prestained Protein Ladder was injected into the first lane. 20µl of the protein solutions, wild type ALB 7ax7a, Chaf1b morpholino ALB 7ax7a and gef/tp53 3xg51, were injected into the following lanes respectively. The SDS-PAGE was then run at 120v for 1.5 hours. A wet transfer to a PVDF membrane was then completed at 35℃ at a constant 200mA for one hour (Fig.3). Afterwards, the specific Chaf1b antibody Rabbit IgG HRP Linked F(ab′)2 will be introduced and will bind only to to the Chaf1b protein. Horseradish peroxidase will also be added to bind to and aid in the visualization of the antibodies, using 10X Tris and TBST wash buffer to remove excess reagents, ensuring Chaf1b is the only protein visible.

Results:

Lysis Buffer	Amount of reagents added for a 50 ml solution:
RIPA	
1%Triton X-100	5.00 x 10^3 ul
150 mM NaCl	0.438 g
0.5% sodium deoxycholate	0.104 g
0.1% SDS	5.00 x 10^-2 g
50 mM Tris, pH 8.0	0.304 g

Table 1: Needed concentrations of reagents for lysis buffer. 1%Triton X-100 was added first to purify proteins in the cell culture. 150mM NaCl is an ionic salt that was used as a neutralizing agent to regulate the osmolarity and pH of the solution. 0.5% sodium deoxycholate is an ionic detergent used to lyses cells and disrupt protein interactions. 0.1% SDS is a negatively charged anionic detergent that aids in aligning the proteins. Lastly, the 50 mM Tris was used to further stabilize the pH of the solution. This lysate buffer is helpful in extracting nuclear proteins and is used to expose the protein of interest.

Loading Buffer	Amount of reagents added for a 10 ml solution:
Laemmli 2X Buffer for SDS	
4% SDS	2000 ul of 20% SDS
10% 2-mercaptoethanol	1000 ul
20% glycerol	146 ul
0.004% bromophenol blue	100 ul

Table 2: Needed concentrations of reagents for loading buffer. The 4% SDS is used to denature proteins and assign them a negative charge. 20% glycerol is used to increase the density of the sample which allows it to settle at the bottom of the wells in the gel. Then the 0.004% bromophenol blue is added as a tracking dye. Lastly the 10% 2-mercaptoethanol is not added to the solution is not added until ready for use because it may denature the sample. However, it is a critical component of the buffer as it breaks the disulfide bonds in the proteins.

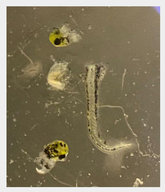

Figure 1. Wild type zebrafish embryos 6-7 dpf. Example of proper fish head severing from the gut. This is crucial to ensure that the gut contents of the fish do not contaminate the western blot and alter the results.

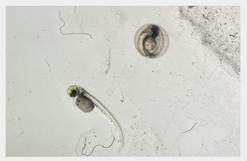

Figure 2: Example of gef mutant zebrafish 3dpf. Good effort is a mutation in the early stages of retinal development. It is characterized by normal cell growth for 2dpf, but failure to mature beyond this. This leads to standard morphological development of optic cells, but an abnormally small eye size compared to wild-type zebrafish around 2-3dpf.

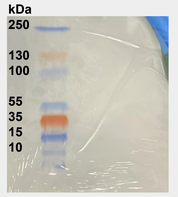

Figure 3: PVDF membrane post wet transfer. PageRuler Plus Prestained Protein Ladder was used as size standard in the protein electrophoresis. This ruler is also useful in monitoring protein transfer onto nitrocellulose paper. The presence of the bands from the ladder indicate the transfer was successful.

Discussion

Zebrafish have become a leading model organism in developmental biology research because of their unique ability to regenerate neuronal tissues in addition to rapid functional regeneration (Bailey and Hyde, 2010). Although mammals do not have all of these capabilities, studying retinal regeneration in zebrafish provides an excellent example for the molecular mechanisms and specific signals required for regeneration. This has proved to be exceptionally useful in researching blindness and other congenital diseases in humans (Wan and Goldman, 2016). The complexity of the zebrafish regenerative processes have been substantiated by recent research, as it has been shown that regeneration uses the same molecular elements as development but in a distinct manner (Bailey and Hyde, 2010). There were difficulties encountered when injecting the zebrafish embryos with Chaf1b morpholino. AB 1x1 embryos had been collected and injected with morpholino, the inoculations appeared to be successful when visualized 9 hpf under blue and green light. However, after incubation in 23℃ for 3 dpf these cells failed to developed correctly, with most experiencing necrosis. This could be due to the fact that the antisense oligonucleotide morpholino disrupted RNA function in such a way that necessary subunits were knocked down which inhibited cell growth. It is important to inoculate 1–8-cell-stage embryos; early embryonic cytoplasmic tissue allows for rapid diffusion of the hydrophilic morpholino (Bill, 2009). However, the failure of the initial set of AB 1x1 embryos could have been also due to experimental error possibly including that the inaccurate injection of morpholino, with a lack of precision, as anywhere from 1-3 nl was used. It is also possible that the incubator was not the right temperature for zebrafish development.

Once the blocking and immunoblotting procedures are complete, we expect to see that the *gef* mutant lane will have reduced bands if any on the western blot. This can be compared to its positive control of wild type zebrafish embryos that would contain normal levels of Chaf1b, therefore, wild type fish embryos would have a clear, distinct band ~ 60 kDa. Zebrafish inoculated with Chaf1b morpholino would be expected to have no Chaf1b protein and therefore would have no bands present on the western blot. *gef* mutants dying around 3 dpf should be similar to Chaf1b morpholino at 3 dpf, proving that cell death in these mutants could be due to the lack of Chaf1b protein. By expanding on this experiment with further research, understanding how zebrafish retinal regeneration functions can help scientists to apply this concept to human retinas.

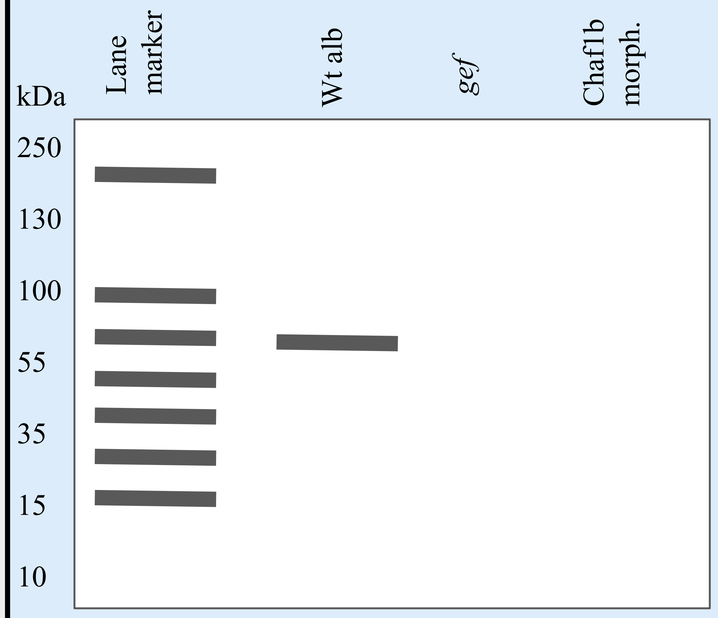

Figure 4. Expected results of western blot assuming no experimental error and that *gef* and Chaf1b mo will have no protein present (could have low levels present instead). Clearly shows presence of Chaf1b in wild type zebrafish at around 60 kDa.