Validating Zebrafish Chaf1b Antibody

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ABSTRACT

Zebrafish embryos that are homozygous for *gef* mutant alleles experience a small-eye phenotype, due to a coding change in the *chaf1b* gene that results in a prematurely truncated protein. It's thought that the Chaf1b protein is required for all dividing cells to survive the DNA replication phase of the cell cycle. Contradicting these findings, the proliferating cells of the *gef* mutant embryos do not start to prematurely die until they are two days old. One question of delayed death in zebrafish is whether the normal protein can be provided by the heterozygous mother. If so, this would account for how the dividing cells are surviving for days in the developing mutant embryo. This study will test for Chaf1b protein in cells dying during the proliferation step to detect Chaf1b protein. It is hypothesized that surviving, not dying, retinal cells may have the normal Chaf1b protein which could be detected by anti-Chaf1b antibodies. This research will verify this question by using antibodies against Chaf1b. Previous research obtained unclear Western Blot results that suggested 3 dpf *gef* mutant embryos lack detectable Chaf1b protein. Thus, validation of Chaf1b antibodies will be analyzed with a Dot Blot to confirm the efficiency of the antibody used in the Western Blot. If the antibody is valid, it is hypothesized that Dot Blot results will display wild-type zebrafish embryo extract glowing under chemiluminescence through anti-Chaf1b immunoprecipitation, while there will be reduced levels of Chaf1b detected in 3 dpf *gef* mutant embryos compared to wild-type.

INTRODUCTION

Retinal regeneration in zebrafish is often triggered by apoptosis of retinal neurons, which provokes rapid proliferation of the Müller glia and increases 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 occurred (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; 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).

METHODS

Immunoprecipitation was repeated on a membrane collected last year on a Western Blot in order to increase protein detection. Washing and blocking of the Western Blot membrane were first conducted with increased concentrations of anti-Chaf1b as the primary antibody and anti-rabbit IgG HRP Linked F(ab)2 as the secondary antibody. 0.1% PBST and blocking buffer were used during this experiment to wash and block-shake the membrane. This membrane was then visualized under a VisionWorks system using an ECL substrate. Additional tests were subsequentially performed that utilized different secondary antibodies that would fluoresce under the system to different wavelengths of light with chemiluminescence; G anti-rabbit TRITC and G anti-rabbit FITC. In order to validate the efficiency of the primary anti-Chaf1b antibody, a dot blot was performed to determine if there is Chaf1b present in *gef* mutants 3 dpf. This experiment is qualitative instead of quantitative and is thus strictly used for detection purposes. Zebrafish embryos were isolated at 3 dpf to be used in this experiment. Eight g5 x g6 gef mutant embryos were collected from breeding tanks at 3 dpf. Another eight embryos were collected from g5 x g6 at 3 dpf, except they were wild-type zebrafish embryos. The wild-type/normal embryos serve as a positive control because they are known to contain Chaf1b protein. These embryos were allowed to incubate at ~76 degrees for 3 dpf until they were ready to be extracted. Eight zebrafish heads were severed from the gut in the normal and *gef* g5 x g6 embryos and homogenized with 80 µl of RIPA buffer to lyse the cells. 10 µl of the original concentration of *gef* protein was blotted onto the membrane along with dilations of 10^-1 and 10^-2 and the area was circled with a pencil. This was repeated for the wt protein. 10 µl of 100 µg/ml of Chaf1b primary antibody was blotted onto the membrane. The membrane was incubated for an hour, immunoprecipitated, and then visualized under the VisionWorks system with an ECL substrate.

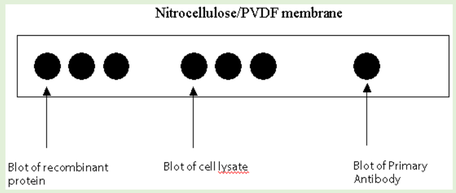

Figure 1: Example of Dot Blot procedure. How the nitrocellulose membrane is organized during the dot blot experiment.

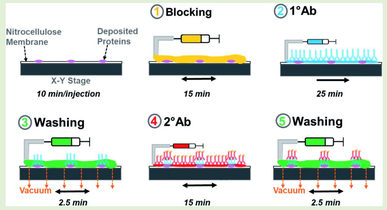

Figure 2: Diagram of Immunoprecipitation Procedure. Example procedure of blocking and washing.

Figure 3: 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.

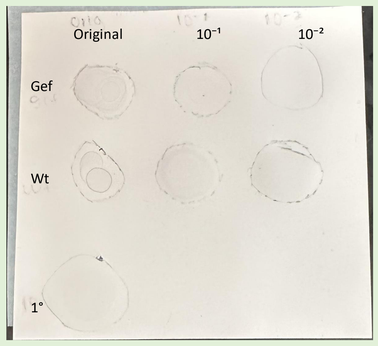

Figure 4: Nitrocellulose Membrane Setup. Results after blotting the membrane, before immunoprecipitation with washing and blocking occurs. Areas where the extracts were blotted was marked in a circle with a number two pencil.

RESULTS

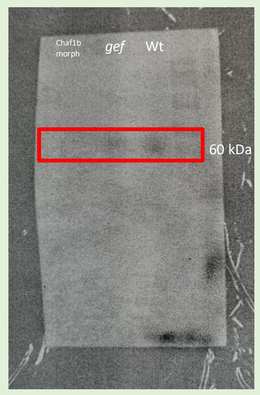

Figure 5: Results of Immunoblotting. Western blot of Chaf1b protein for Wt alb (+ control), gef mutants, and Chaf1b morpholino (- control). Results suggest the absence of Chaf1b protein in gef mutants, however, bands are very light, unclear, and hard to distinguish. This could be due to the fact that not enough protein sample was added to the blot. Further western blots are needed to validate the Chaf1b antibody as an effective detection tool, along with the anti-actin antibodies to confirm the western blot was performed correctly.

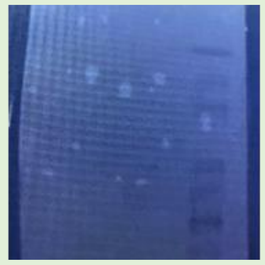

Figure 6: Image from VisionWorks system. There were no clear bands seen from either the G anti-rabbit TRITC and G anti-rabbit RITC secondary antibodies. TRITC was visualized using Filter 4 (green light) on BioLite External Source and Filter 5 in the BioSpectrum filter wheel that holds an Ethidium Bromide filter. FITC was visualized using Filter 5 (blue light) in the BioLite External Light Source and Filter 3 (SYBER Green) in the BioSpectrum filter wheel.

Figure 7: Chemiluminescence of Dot Blot under VisionWorks System. Two areas of the Dot Blot nitrocellulose membrane fluoresced under Chaf1b antibodies when placed in a VisionWorks System with an ECL substrate for ~20 minutes. When merged with a brightfield photo of the membrane, results indicated that the portions that are glowing are the wild-type original protein concentration and the 10^{-1} concentration. This could suggest that wild-type zebrafish have functional Chaf1b protein while *gef* mutants do not.

CONCLUSION

The use of several different secondary antibodies as a method of protein detection on a previous Western Blot membrane experiment proved to be ineffective in producing chemiluminescence in our protein of interest. Validation of the Chaf1b antibody using a dot blot proved to be more effective in terms of a qualitative assessment. Results indicated that there was a presence of Chaf1b protein in wild-type zebrafish embryos 3dpf but were not present in *gef* mutant zebrafish 3dpf. These results contradicted our hypothesis, as there might be some other potential reason as to why these mutants are able to survive for 2-3 dpf if they are able to survive without the presence of Chaf1b protein. The dot blot results could not be reliable, however, due to the fact that a very large concentration of primary antibody was blotted onto the membrane. As a result, the chemiluminescence could have been brighter in a shorter amount of exposure and thus the capture might have been stopped prematurely. A longer exposure time could have allowed for the *gef* mutant extract to glow, and thus in the future the dot blot procedure will be repeated with a smaller amount of primary antibody.

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