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Role of DeltaC and DeltaD in Zebrafish Retinal Development

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For the degree of  Doctor of Philosophy

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Head of the Departmental Graduate Program  Date
ROLE OF DELTAC AND DELTAD IN ZEBRAFISH RETINAL DEVELOPMENT

A Dissertation
Submitted to the Faculty
of
Purdue University
by
Sylvia Imelda Bonilla

In Partial Fulfillment of the
Requirements for the Degree
of
Doctor of Philosophy

May 2016
Purdue University
West Lafayette, Indiana
Find a path, or make one!

-John C. Fremont High School
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>CHAPTER 1. AN INTRODUCTION TO RETINAL DEVELOPMENT AND DELTA-NOTCH PATHWAY</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Retinal structure and development</td>
<td>1</td>
</tr>
<tr>
<td>1.2 The Notch pathway</td>
<td>2</td>
</tr>
<tr>
<td>1.3 The focus of the project</td>
<td>4</td>
</tr>
<tr>
<td>1.4 References</td>
<td>7</td>
</tr>
<tr>
<td>CHAPTER 2. THE EFFECTS OF DELTAC AND DELTAD ON ZEBRAFISH RETINAL DEVELOPMENT</td>
<td>11</td>
</tr>
<tr>
<td>2.1. Introduction</td>
<td>11</td>
</tr>
<tr>
<td>2.1.1 The Notch and Delta proteins</td>
<td>11</td>
</tr>
<tr>
<td>2.1.2 Notch activity promotes gliogenesis and inhibits ganglion and photoreceptor development</td>
<td>15</td>
</tr>
<tr>
<td>2.1.3 Delta signaling promotes and inhibits ganglion and photoreceptor development</td>
<td>15</td>
</tr>
<tr>
<td>2.1.4 Late inhibition of Delta-Notch signaling favors rod development</td>
<td>16</td>
</tr>
<tr>
<td>2.1.5 Does the function of Delta ligands differ during zebrafish retinal development?</td>
<td>16</td>
</tr>
<tr>
<td>2.2. Materials and Methods</td>
<td>17</td>
</tr>
<tr>
<td>2.2.1 Fish maintenance and embryo collection</td>
<td>17</td>
</tr>
<tr>
<td>2.2.2 Whole-mount in situ hybridization</td>
<td>18</td>
</tr>
<tr>
<td>2.2.3 Sequencing of dlc and dld in their corresponding mutant fish</td>
<td>19</td>
</tr>
<tr>
<td>2.2.4 Morphological analysis</td>
<td>20</td>
</tr>
<tr>
<td>2.2.5 Immunohistochemistry</td>
<td>20</td>
</tr>
</tbody>
</table>
2.2.6 Characterization of blue opsin staining pattern ........................................21
2.2.7 Quantitative Reverse Transcription PCR (qRT-PCR) ...............................21
2.2.8 Image acquisition ....................................................................................22

2.3. Results ........................................................................................................22

2.3.1 Differential expression patterns of dlc and dld during zebrafish retinogenesis .................................................................22
2.3.2 Phenotype and genotype of dlc\textsuperscript{b663} and dld\textsuperscript{g249} mutant zebrafish..............................................25
2.3.3 The dld\textsuperscript{g249} embryos have no change in eye size while dlc\textsuperscript{b663} embryos have thicker eyes ..........................................................28
2.3.4 No effect on retinal lamination in dlc\textsuperscript{b663} and dld\textsuperscript{g249} .................................................................29
2.3.5 Dlc and Dld affect photoreceptor development ........................................32
2.3.6 Expression of rhodopsin in dlc\textsuperscript{b663} and dld\textsuperscript{g249} eyes ..................................................................34
2.3.7 Expression of cone opsins in dlc\textsuperscript{b663} and dld\textsuperscript{g249} eyes ................................................................36
2.3.7.1 Blue opsin expression in 5-dpf dlc\textsuperscript{b663} and dld\textsuperscript{g249} embryos ..................................................37
2.3.7.2 Blue opsin expression in 36- and 55-hpf dlc\textsuperscript{b663} and dld\textsuperscript{g249} embryos ........................................40
2.3.7.3 Increased red opsin expression in dld\textsuperscript{g249} and not in dlc\textsuperscript{b663} retinas ............................................42
2.3.8.4 Green opsin expression was increased in dld\textsuperscript{g249} and not in dlc\textsuperscript{b663} retinas ............43
2.3.8 Decreased expression of nr2e3 in dlc\textsuperscript{b663} and dld\textsuperscript{g249} embryos .............................................44

2.4. Discussion ....................................................................................................47

2.4.1 Differential expression of dlc and dld during zebrafish retinogenesis might be the key to distinguish subpopulation of retinal proliferating cells ........................................50
2.4.2 Eye defects in dlc\textsuperscript{b663} and dld\textsuperscript{g249} .................................................................50
2.4.3 Dlc and Dld promote initial ventral differentiation of rod cells at 36-hpf ..........51
2.4.4 Dlc- and Dld- Notch signaling determine rod differentiation at 55-hpf ........51
2.4.5 The role of Dlc and Dld in rod patterning at 5-dpf ....................................51
2.4.6 Dld inhibits initial ventral differentiation of blue cones at 36-hpf ..................52
2.4.7 Dlc hinders the timing of blue cone development while Dld hinders the timing of blue and red cone development at 55-hpf ...........................................52
2.4.8 At 5-dpf, Dlc is required for proper blue cone patterning while Dld is required for constraining red and green cone differentiation ........................................53
2.4.9 Dlc and Dld signaling is upstream of \textit{nr2e3} ..................................................53
2.4.10 Conclusion .................................................................................................53
2.5. References ..................................................................................................55

CHAPTER 3. IMMEDIATE DOWNSTREAM TARGETS BY WHICH DELTAC AND DELTAD SIGNAL DURING RETINAL DEVELOPMENT ......................60
3.1 Introduction ................................................................................................60
3.1.1 Basic helix-loop-helix (bHLH).................................................................60
3.1.2 The contribution of hairy/Enhancer-of-slit-related family in retinal development ..61
3.1.3 Through which immediate downstream targets do Dlc and Dld ligands signal? .....62
3.2. Material and Methods .................................................................................63
3.2.1 Fish maintenance and embryo collection ................................................63
3.2.2 Whole-mount \textit{in situ} hybridization .......................................................63
3.2.3 Image acquisition and analysis ...............................................................64
3.3. Results .........................................................................................................64
3.3.1 Expression of \textit{her2}, \textit{hey1} and \textit{her13.2} at 1-day post fertilization ..........64
3.3.2 Expression of \textit{her4.2}, \textit{hey1} and \textit{her13.2} at 2-day post fertilization ..........65
3.3.3 Expression of \textit{her4.2}, \textit{hey1} and \textit{her13.2} at 3-day post fertilization ..........67
3.3.4 Expression of \textit{her6} at 36-hours post fertilization ....................................69
3.4. Discussion ...................................................................................................70
3.4.1 \textit{her6} is downregulated by Dlc and upregulated by Dld in 36-hpf embryos ....72
3.4.2 The expression dynamics of \textit{hey1}, \textit{her4.2} and \textit{her13.2} in 2-dpf WT, \textit{dle}^{b663} and \textit{dld}^{g249} eyes .................................................................73
3.4.3 The expression dynamics of \textit{hey1}, \textit{her4.2} and \textit{her13.2} in 3-dpf WT, \textit{dle}^{b663} and \textit{dld}^{g249} eyes .................................................................74
3.5. References ..................................................................................................75
CHAPTER 4. THE ALTERED VISUAL BEHAVIOR OF $DLC^{663}$ AND $DLD^{TG249}$ EMBRYOS

4.1 Introduction ........................................................................................................... 78
4.1.1 Photoreceptor cells and visual behavior ......................................................... 78
4.1.2 Optokinetic response (OKR) ............................................................................. 79
4.1.3 Visual-motor response (VMR) .......................................................................... 80
4.1.4 What are the visual behavior defects in the Delta mutant embryos? .............. 80
4.2. Materials and Methods ....................................................................................... 81
4.2.1. Fish maintenance and fish lines ................................................................. 81
4.2.2. Optokinetic response assay .......................................................................... 81
4.2.3. Visual-motor response assay ........................................................................ 82
4.3. Results .................................................................................................................. 83
4.3.1 The OKR of $dlc^{663}$ and $dld^{TG249}$ are decreased ......................................... 83
4.3.2. Bright light VMR: fewer $dld^{TG249}$ embryos responded during the onset of light, and their locomotor activity were decreased ........................................................................................................... 84
4.3.3. Dim light VMR: fewer $dld^{TG249}$ embryos responded to the onset of light, while $dlc^{663}$ embryos had higher activity during the offset of light ............................................................... 90
4.4. Discussion ............................................................................................................. 95
4.4.1 $dlc^{663}$ and $dld^{TG249}$ embryos can respond to visual signals .................... 96
4.4.2 The visual behavior of $dlc^{663}$ and $dld^{TG249}$ embryos is affected ................ 96
4.4.3 During the VMR assay at the onset of bright light, $dld^{TG249}$ embryos are less responsive and are less active ........................................................... 96
4.4.4 The dim light VMR assays demonstrated a differential effect in the Delta mutants: $dld^{TG249}$ embryos have altered activity during the offset of light while $dlc^{663}$ embryos have altered activity during the onset of light ............................................................... 98
4.4.5 Conclusion ....................................................................................................... 99
4.5. References ......................................................................................................... 100

CHAPTER 5: CONCLUSION ..................................................................................... 102
5.1 References ........................................................................................................106
APPENDIX ..............................................................................................................116
VITA .........................................................................................................................120
ABSTRACT

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Classical studies have shown that Notch-Delta signaling is essential for the maintenance of retinal progenitor cells. However, the specific roles of different Delta ligands on retinal development are not well characterized. The focus of this study was to investigate the specific functions of the Delta ligands, deltaC (Dlc) and deltaD (Dld), in zebrafish retinal development by using dlc<sup>b663</sup> and dld<sup>g8249</sup> mutant embryos. The retinal expression pattern of dlc and dld, determined by whole-mount in situ hybridization, demonstrated that both ligands have similar and distinct expression patterns in WT retinas throughout retinal development. In mutant retinas where dlc and dld are absent, whole-mount in situ hybridization and immunohistochemistry analyses indicated a disruption in the spatial patterning of rods and cones, as well as a stunted photoreceptor differentiation. Blue cone differentiation was affected in the dlc<sup>b663</sup> embryos, whereas red and green cone differentiation was affected in dld<sup>g8249</sup> embryos. In order to demonstrate the effects of a disrupted retinal development on the zebrafish visual behavior, the visual motor response (VMR) and optokinetic reflex (OKR) of mutant embryos were analyzed. The OKR showed a reduced ability of mutant embryos to detect moving objects on their visual field, whereas each mutant presented a characteristic VMR. This study demonstrated that different Delta ligands contribute to specific aspects of retinal functionality.
CHAPTER 1. AN INTRODUCTION TO RETINAL DEVELOPMENT AND DELTA-NOTCH PATHWAY

1.1 Retinal structure and development

During early development, the central nervous system (CNS) is composed of progenitor cells that give rise to diverse cell types. The retina is an ideal structure to study questions of cell fate and differentiation processes since it is an extension of the brain, the cellular architecture of the retina is well characterized, and it is easy to manipulate experimentally. In the vertebrate retina, there are six neuronal cell types (ganglion, amacrine, bipolar, horizontal, cone and rod cells), and one glial cell type (Müller glia) (Dowling and Boycott, 1966). The architecture of the retina is divided into three cellular layers: the ganglion cell layer (GCL) that contains ganglion and displaced amacrine cells, the inner nuclear layer (INL) that contains bipolar, horizontal, amacrine interneurons and Müller glia cells, and the outer nuclear layer (ONL) that contains cone and rod cells (Figure 1) (Dowling, 1987).

During retinal development, all retinal cell types develop from a pool of progenitor cells that give rise to the highly organized and laminated retina (Holt et al., 1988; Turner and Cepko, 1987). Neural progenitor cells initially undergo symmetric cell divisions to generate the growing pool of proliferating cells. Some neural progenitor cells undergo asymmetric cell division to produce one neural progenitor cell and one immature neuronal cell (Chenn and McConnell, 1995; Morrison and Kimble, 2006). The immature neuronal cell exits the cell cycle and migrates to the appropriate position in the neuroepithelium. The immature neural cell undergoes three developmental steps: specification, differentiation and functional maturation. In zebrafish, retinal cells that make up the GCL exit first from the cell cycle, followed by cells that make up the INL, and then cells that make up the ONL (Hu and Easter, 1999). Retinal differentiation occurs in the following order: ganglion, amacrine, photoreceptors, horizontal, bipolar and lastly
Müller glia cells (Hu and Easter, 1999; Schmitt and Dowling, 1996; Schmitt and Dowling, 1999).

The zebrafish (*Danio rerio*) is a useful research model to address questions of neural development. Its genome is fully sequenced facilitating genetic studies. An adult female fish can lay hundreds of eggs per clutch. Their development occurs rapidly, for example, the precursors of major organs are present as early as 36-hours post fertilization (hpf). In addition, the eyes of zebrafish are relatively large and easily accessible, which facilitates experimental procedures, and the retinal architecture and development is well characterized. The zebrafish retinal cells can be identified by their position in the retina, morphology, and by specific molecular markers (Fadool and Dowling, 2008; Schmitt and Dowling, 1994).

1.2 The Notch pathway

Diversity amongst cell types in the nervous system depends on the cells’ ability to communicate with one another. A molecular pathway that allows for neighboring cells to communicate to each other is the Delta-Notch pathway. The Delta-Notch pathway is an evolutionary conserved mechanism shown to have vast effects on many aspects of metazoans development. For instance, the Delta-Notch pathway functions to inhibit neural differentiation and to maintain neural cells proliferating (Kageyama et al., 2008).

The main components of this pathway are the transmembrane proteins Notch, Delta and Serrate (or Jagged in mammals and zebrafish). The signal-sending cell has the membrane-bound ligand Delta/Serrate/Lag-2 (DSL), and the neighboring signal-receiving cell has the membrane-bound receptor, Notch. Upon ligand-receptor interaction, two proteolytic cleavage events occur on the Notch receptor (Greenwald, 1998; Gridley, 1997; Gu et al., 1995). The first cleavage is catalyzed by an ADAM-family metalloprotease and the second cleavage is mediated by γ-secretase (Fortini, 2002; Mumm and Kopan, 2000; Selkoe and Kopan, 2003). These proteolytic events release the Notch intracellular domain (NICD), which travels to the nucleus to form a complex with the DNA-binding protein RBPJ (recombination signal sequence-binding protein Jκ). The NICD-RBPJ complex activates the transcription of various basic helix-loop-helix
(bHLH) transcriptional repressors, such as *hairy/Enhancer of split-related (her/hes)* (Fortini and Artavanis-Tsakonas, 1994). The her/hes proteins inhibit proneural genes, consequently impeding neural differentiation and maintaining cell proliferation (Kageyama and Ohtsuka, 1999). Retinal cells that do not activate the Notch pathway express proneural genes, and eventually differentiate into a neural or glial cell type.

The Delta-Notch pathway is involved in vertebrate retinal development. The activation of the Notch receptor (1) prevents neuronal differentiation, (2) promotes glial differentiation, (3) affects retinal cell patterning, and (4) maintains retinal progenitor cells proliferation (Ahmad et al., 2004; Ahmad et al., 1997; Austin et al., 1995; Bao and Cepko, 1997; Bernardos et al., 2005; Dorsky et al., 1997; Dorsky et al., 1995; Furukawa et al., 2000; Henrique et al., 1997; Hojo et al., 2000; Jadhav et al., 2006a; Livesey and Cepko, 2001; Mu and Klein, 2004; Perron and Harris, 2000; Pujic and Malicki, 2004; Rapaport and Dorsky, 1998; Scheer et al., 2001; Schneider et al., 2001). In contrast, the lack of Notch activity causes (1) premature exiting of retinal cells from the cell cycle, (2) an increase in number of ganglion and photoreceptor cells, and (3) a decrease in number of glial cells (Austin et al., 1995; Bernardos et al., 2005; Henrique et al., 1997; Jadhav et al., 2006b; Mizeracka et al., 2013a; Mizeracka et al., 2013b; Nelson et al., 2007; Silva et al., 2003; Yaron et al., 2006). The various effects of the Delta-Notch pathway are due to the existence of multiple proteins involved in the pathway. In vertebrate retinas, there are multiple ligands that activate the Notch receptor and multiple immediate downstream targets that can mediate the pathway’s outputs.

It was observed in a microarray study that not all Delta ligands were affected in the retinas of the zebrafish mutant *smarca4* (Leung et al., 2008). This mutant has a null mutation in the *brahma-related (Brg1)* gene, which encodes an ATPase involved in the SWI/SNF chromatin remodeling complex (Roberts and Orkin, 2004). In the *smarca4* eyes, retinal specification occurs but retinal differentiation does not (Link et al., 2000). Leung and collaborators (2008) observed that the transcription level of the Notch receptor did not change in *smarca4* retinas. However, the expression of two ligands, *deltaC (dlc)* and *deltaD (dld)*, was increased. The increase of *dlc* and *dld* in the *smarca4* retinas and the fact that *smarca4* retinal cells do not differentiate, suggests that *dlc* and *dld* have a
role on retinal cell differentiation. Yet it is unknown which ligand contributes to the differentiation of a specific retinal cell type and how they mediated their effect. Thus, it is important to elucidate these uncertainties to further understand the function of the signaling molecules involved in retinal cell differentiation. Understanding the aspects of the eye has been a focus in the field of developmental biology. The cellular complexity of the retina and the underlying genetic controls of its development are immediate challenges for designing better therapies for various retinal degenerative diseases.

1.3 The focus of the project

The purpose of this study was to elucidate the functions of Dlc and Dld in zebrafish retinal development, investigate the mechanism by which Dlc and Dld regulate retinal development and analyze the visual behavior when Dlc or Dld are absent. The completion of this study will define the roles of Dlc and Dld on retinal differentiation, identify the immediate downstream targets Dlc and Dld regulate, and determine the contribution of Dlc and Dld on vision. The project’s findings can potentially contribute to the development of novel therapies for various retinal degenerative diseases.
Figure 1.1. Retinal architecture. Schematic diagram of the neural retina, adopted by Goldman, 2004 (Goldman, 2014). The retina is divided into three layers: the ganglion cell layer (GCL), the inner nuclear layer (INL) and outer nuclear layer (ONL). The retinal pigment epithelium (RPE) is posterior to the retina. This diagram also shows the nerve fiber layer (NFL), inner plexiform layer (IPL), outer plexiform layer (OPL), inner limiting membrane (ILM) and outer limiting membrane (OLM).
Figure 1.2. The Delta-Notch pathway. Upon activation, the Notch intracellular domain transfers to the nucleus, forming a complex with RBPJ to induce transcription of *her/hes*. Her/hes proteins repress proneural genes expression needed for differentiation.
1.4 References
Austin, C. P., et al., 1995. Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. Development. 121, 3637-50.


CHAPTER 2. THE EFFECTS OF DELTAC AND DELTAD ON ZEBRAFISH RETINAL DEVELOPMENT

2.1. Introduction

The Delta-Notch pathway is a paracrine signaling system involved in cell identity, proliferation and differentiation during neuron development. Classical studies have shown that Delta-Notch signaling is essential for the maintenance of retinal progenitor cells during neural development in metazoans (Artavanis-Tsakonas et al., 1999). However, the specific function(s) of the Delta ligands on retinal development are not well understood. In this chapter, the retinal defects in dlc<sup>b663</sup> and dld<sup>tg249</sup> mutant embryos were evaluated in order to elucidate the function of deltaC (Dlc) and deltaD (Dld) in zebrafish retinal development.

2.1.1 The Notch and Delta proteins

The presence of multiple Notch orthologs in vertebrates contributes to the complexity of the Delta-Notch signaling pathway. Four Notch receptors have been identified in mammals, three in the frog (Xenopus tropicalis), two in the chicken (Gallus gallus) and four in the zebrafish (Danio rerio) (Figure 2.1) (Gazave et al., 2009; Kopan and Ilagan, 2009; Kortschak et al., 2001). Expression analysis experiments demonstrated the transcription of notch1 (including notch1a and notch1b from zebrafish) and notch3 are expressed in the developing retina, whereas notch2 was present in the retinal pigment epithelium (Bao and Cepko, 1997; Lindsell et al., 1996; Raymond et al., 2006). These data suggest that notch1 and notch3 contribute to retinal development. The Notch ligand Delta also has multiple orthologs in different vertebrate species. Three Delta ligands have been characterized in mammals, two in the frog and the chicken, and five in the zebrafish (Figure 2.2) (Eckalbar et al., 2012; Gazave et al., 2009; Kopan and Ilagan, 2009; Lindsell et al., 1996; Myat et al., 1996; Nelson and Reh, 2008). All Delta ligands are expressed
during retinal development (Dorsky et al., 1997; Nelson et al., 2009; Nelson and Reh, 2008; Raymond et al., 2006; Smithers et al., 2000).
Figure 2.1. Notch Bayesian phylogram adopted by Gazave and colleagues, 2009 (Gazave et al., 2009). The posterior probabilities (greater than 0.50) are marked next to the node. The Notch families 1, 2 and 3 are in yellow, blue and green boxes, respectively.
Figure 2.2. DSL Bayesian phylogram adopted by Gazave and colleagues, 2009 (Gazave et al., 2009). The posterior probabilities (greater than 0.50) are marked next to the node. The Deltas split in three clades shown in blue boxes. The Jagged proteins are in the red box.
2.1.2 Notch activity promotes gliogenesis and inhibits ganglion and photoreceptor development

The inhibition of Notch activity during retinal development allows progenitor cells to prematurely exit the cell cycle and adopt early cell fates, such as ganglion and cone cells (Austin et al., 1995; Jadhav et al., 2006b; Mizeracka et al., 2013a; Nelson et al., 2007; Silva et al., 2003; Yaron et al., 2006). The downregulation of Notch1 by antisense oligonucleotides in the chicken retinas caused an overproduction of ganglion cells (Austin et al., 1995; Silva et al., 2003). However, the knockout of Notch1 during mice retinal development caused a decrease in the eye size due to a reduced number of progenitor cells. The reduction of progenitor cells was produced by premature neurogenesis that led to an increase in the production of cone cells (Jadhav et al., 2006b; Nelson et al., 2007; Yaron et al., 2006).

Studies that increased Notch activity, during the retinal development of mice and zebrafish, maintained the proliferation of undifferentiated retinal cells that will eventually differentiate into Müller glia cells (Bao and Cepko, 1997; Furukawa et al., 2000; Hojo et al., 2000; Jadhav et al., 2006a; Rapaport and Dorsky, 1998; Scheer et al., 2001; Schneider et al., 2001). However, in mice, the increased activity of Notch signaling lowered the expression of short-wave-opsin, medium-wave-opsin and the neural retina leucine zipper (nrl) (Jadhav et al., 2006a). These observations suggest that Notch activity promotes gliogenesis and inhibits neurogenesis, specifically in ganglion and photoreceptor development.

2.1.3 Delta signaling promotes and inhibits ganglion and photoreceptor development

The Delta ligands’ roles differ among vertebrate species. In zebrafish, inhibition of the Delta ligands caused delayed photoreceptor differentiation, disrupted the arrangement of retinal neurons (including photoreceptors), and decreased the number of Müller glia cells (Bernardos et al., 2005). In the chicken, the reduction of delta-like-1 (c-dll1) increased ganglion cell differentiation, whereas the increase of c-dll1 reduced ganglion cell differentiation (Ahmad et al., 1997; Henrique et al., 1997). During early stages of the frog retinal development, cells that misexpressed x-delta-like-1 (x-dll1) became ganglion or cone cells, causing a reduction in the population of rod and Müller
glia cells. However, during the later stages of retinal development, the progenitor cells that misexpressed X-dll1 became rod and cone cells (Dorsky et al., 1997). This study suggests that X-dll1 can influence cell type differentiation, depending on the developmental time in which the ligand is expressed.

The function of Delta ligands also differs amongst paralogs. For example, in the mouse retina, the removal of delta-like-1 (m-dll1) accelerated neurogenesis and caused newly formed progenitor cells to arrange into a rosette pattern (Rocha et al., 2009). However, removal of delta-like-4 (m-dll4) caused an overproduction of photoreceptor cells and an abnormal organization of the photoreceptor layer (Luo et al., 2012). Overall, these studies demonstrated that Delta signaling is needed for ganglion, photoreceptor and Müller glia development.

2.1.4 Late inhibition of Delta-Notch signaling favors rod development

As previously mentioned, inhibition of the Delta-Notch pathway caused an increase in ganglion and cone cells and decreased the Müller glia cell population. Nevertheless, inhibiting the Delta-Notch signaling in cells at a later stage of retinal development may have a different outcome in retinogenesis. For example, the removal of Notch1 in early progenitor cells caused the cone cells population to increase. However, removal of Notch1 in postmitotic cells or during late retinal development, promoted the rod cell population to increase (Jadhav et al., 2006b; Luo et al., 2012; Mizeracka et al., 2013a; Nelson et al., 2007) and the Müller glia cells to decrease (Mizeracka et al., 2013a). These findings suggest that Delta-Notch signaling, in later stages of retinal development, inhibits rod development.

2.1.5 Does the function of Delta ligands differ during zebrafish retinal development?

The delta ligands may have similar or distinct expression patterns. For example, c-dll1 was expressed in retinal progenitor cells, whereas the expression of c-dll4 was found mostly in recently born neurons and detected in few retinal progenitor cells (Nelson and Reh, 2008). In zebrafish, deltaC (dlc) expression is first detected at 22-hours post fertilization (hpf) in the retinal neuroepithelium, but by 24-hpf dlc is broadly expressed in the retina. On the contrary, deltaD (dlld) expression is restricted to a subset population of cells in the retinal neuroepithelium (Smithers et al., 2000). These
observations suggest that different *deltas* can allow the development of distinct retinal cell types (Ahmad et al., 1997; Dorsky et al., 1997; Henrique et al., 1997; Luo et al., 2012; Nelson et al., 2009; Nelson and Reh, 2008; Rocha et al., 2009).

The objective of this chapter is to define the roles of *dlc* and *dld* in zebrafish retinal development, using mutants of these ligands. A previous microarray study identified molecular pathways that contribute to zebrafish retinal differentiation (Leung et al., 2008). One of these pathways was the Delta-Notch pathway. Specifically the transcription of *dlc* and *dld* increased. This result suggests that *dlc* and *dld* have a role in the inhibition of retinal cell differentiation. Furthermore, based on the expression differences of *dlc* and *dld* reported by Smithers and colleagues (2000), I hypothesize that *dlc* and *dld* affect differentiation of different retinal cell types. To test this hypothesis, I characterized the retinal defects in *dlc<sup>b663</sup>* and *dld<sup>g249</sup>* mutant embryos during retinal development.

### 2.2. Materials and Methods

#### 2.2.1 Fish maintenance and embryo collection

Zebrafish were maintained according to standard procedures (Westerfield, 2000). Homozygous mutant fish *dlc<sup>b663</sup>* and *dld<sup>g249</sup>* , as well as wild-type (WT) AB fish, were used for this study. Parental fish were bred for 15 minutes to ensure all embryos were at a similar developmental stage during collection. Embryos were maintained in medium for zebrafish embryos (E3 medium) (Westerfield, 2000) at 28˚C and their developmental stage was determined based on previous studies (Kimmel et al., 1995). To prevent melanization for *in situ* hybridization experiments, the embryos were treated with 0.003% phenylthiourea (PTU) (Sigma-Aldrich, St. Louis, MO) in E3 medium, between 12- and 23-hpf (Li et al., 2012; Nusslein-Volhard and Dahm, 2002). Embryos were fixed overnight, in 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO) in 1x phosphate-buffered saline (PBS) (0.137M NaCl, 0.0027M KCl, 0.010M Na<sub>2</sub>HPO<sub>4</sub>, 0.0018M KH<sub>2</sub>PO<sub>4</sub>) (IBI Scientific, Peosta, IA and Avantor Performance Materials, Phillipsburg, NJ), at 4˚C. After fixation, the samples for *in situ* hybridization were dehydrated and stored in 100% methanol (VWR International, West Chester, PA) at -20˚C. All protocols were approved by the Purdue Animal Care and Use Committee.
2.2.2 Whole-mount \textit{in situ} hybridization

The preparation of the riboprobes, as well as the whole-mount \textit{in situ} hybridization of the collected samples, were performed as described previously (Hensley et al., 2011). The primers that were used to make the riboprobes of \textit{dlc} and \textit{dld} are presented in Table 2.1. The other riboprobes used in this study were \textit{blue opsin}, \textit{uv opsin}, \textit{red opsin}, \textit{green opsin}, \textit{rhodopsin}, \textit{cone-rod homeobox (crx)}, \textit{neural retina leucine zipper (nrl)} and \textit{nuclear receptor subfamily 2 group E member 3 (nr2e3)} (Leung et al., 2008; Takechi and Kawamura, 2005; Zhang et al., 2012). A minimum of ten embryos was analyzed for the three genotypes, at each developmental stage throughout the study. Stringency washes after probe hybridization and before signal detection, were performed with the semi-automated \textit{in situ} hybridization machine Biolane (INTAVIS Bioanalytical Instruments, Koeln, Germany). The samples used for the characterization of the same gene were processed and stained for the same period of time to maximize comparability between conditions. Samples were destained by a 2:1 mixture of benzyl benzoate (Alfa Aesar, Lancashire, United Kingdom)-benzyl alcohol (Alfa Aesar, Ward Hill, MA), and stored in 70% glycerol (Sigma-Aldrich, St. Louis, MO)/ PBS at 4°C. For whole-mount images, the embryos were mounted in 3% methylcellulose (MP Biomedical LLC, Illkirch, France) on a depression slide for observation and imaging. To obtain cryosections from embryos after \textit{in situ} hybridization, each embryo was washed in 1x PBS and infiltrated with 5%, 10%, 15%, and 20% sucrose (AMRESCO, Solon, OH)/ PBS for 30 minutes at room temperature (RT). The final infiltration step used 30% sucrose/ PBS, overnight, at RT. Samples were incubated in an equal volume of 30% sucrose/ PBS to tissue freezing media (TFM) (Triangle Biomedical Sciences, Durham, NC), for 30 minutes, at RT. Five embryos were transferred in a cryomold, mounted with TFM, and frozen with liquid nitrogen. Twenty-micrometer-thick transverse or lateral cryosections were collected. Slides were warmed on a hot plate for 5 minutes at 50°C to fix the sections on the slide. Slides were aired dried for at least 1 h at RT and coverslipped in VectorMount (Vector Laboratories, Burlingame, CA).
Table 2.1. Primers used for riboprobes synthesis of *dlc* and *dld* riboprobes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primers: 5’-3’</th>
<th>Reverse Primers: 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>dlc</em></td>
<td>TGCACCTTGCAAAAGAAGGTTG</td>
<td>TGCAGGTGTACCCGTTGATA</td>
</tr>
<tr>
<td><em>dld</em></td>
<td>TGGGAGGACAGAGCTGAAGT</td>
<td>CCCTTAGAAACCAGGAGGACA</td>
</tr>
</tbody>
</table>

2.2.3 Sequencing of *dlc* and *dld* in their corresponding mutant fish

Adult WT, *dlc*<sup>b663</sup> and *dld*<sup>a8249</sup> fish were fin clipped to extract their genomic DNA following the protocol of Nusslein-Volhard and Dahm (2002) (Nusslein-Volhard and Dahm, 2002). Each DNA extraction was diluted 1:10 with EB buffer (Qiagen Science, Germantown, MD). PCR reactions were composed by 1.6 µl of diluted DNA, 0.2 µM of reverse and forward primers, 1 x PCR Buffer, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTP, and one unit of *Taq* polymerase (New England Biolabs, Ipswich, MA). The primers in Table 2.2 were designed using Primer3 v 0.4.0 software (Rozen and Skaletsky, 2000). The mutation in *dld*<sup>a8249</sup> fish is located in the second exon (Holley et al., 2000). The *dld* pair of primers (Table 2.2) amplified a 357 base pair fragment that included the mutation previously described by Holley and colleagues (2000). To identify the mutation in *dlc*<sup>b663</sup> fish, nine primer pairs were designed to cover the *dlc* gene (Table 2.2) (Appendix 1). The first set of primers (*dlc-1F* and *dlc-1R*) amplified a 531 base pair fragment that included the mutation at the start codon of *dlc*. PCR cycling conditions were as follows: 95°C for 10 min; 35 cycles of 94°C (25 sec), 65°C (-0.3°C/cycle) (35 sec), 72°C (60 sec); 10 cycles of 94°C (25 sec), 54°C (35 sec), 72°C (60 sec); and 72°C for 5 min. PCR products were sequenced at the Purdue Genomic Core Facility.
Table 2.2. PCR primers for dlc and dld.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primers: 5’-3’</th>
<th>Reverse Primers: 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>dld</td>
<td>GAAAGGAGGTGACGGAACCG</td>
<td>CGGGAAACTTGGAAGGAGTTT</td>
</tr>
<tr>
<td>dlc-1</td>
<td>TCGTGCTGCTTGTAAGAGACA</td>
<td>GCTTGCTTGTTGTAAGAGACA</td>
</tr>
<tr>
<td>dlc-2</td>
<td>AAAAAACGGTTATCATGTTT</td>
<td>CAAAACGGTTATCATGTTT</td>
</tr>
<tr>
<td>dlc-3</td>
<td>CTGACGGCTACGAGTTCG</td>
<td>CTTCTCAGGAAACGACGAG</td>
</tr>
<tr>
<td>dlc-4</td>
<td>GCAAGTTCAACGGCTTCTTC</td>
<td>ACCTCGCAGTTCTTGACATA</td>
</tr>
<tr>
<td>dlc-5</td>
<td>AGCAGCAGTTATTCGACCTT</td>
<td>GTTGCCCAAATCGAGACACT</td>
</tr>
<tr>
<td>dlc-6</td>
<td>GTGCTTGCCCTTGTTGACT</td>
<td>TGAAACAGAACATTGTGAACAGAA</td>
</tr>
<tr>
<td>dlc-7</td>
<td>AGCAGAAGATGGTGGACTACA</td>
<td>GCTCTCCAGAGATTTCTTGAC</td>
</tr>
<tr>
<td>dlc-8</td>
<td>CGTGGAACAACTTGCACTA</td>
<td>ACTCTGATTCAGAGACATTACA</td>
</tr>
<tr>
<td>dlc-9</td>
<td>GCCATGCTCTGCAACATTCACT</td>
<td>CGAGTTACAGGCTCTCTCTCGG</td>
</tr>
</tbody>
</table>

2.2.4 Morphological analysis

For the three genotypes, ten embryos were embedded in 3% methylcellulose (MP Biomedical LLC, Illkirch, France). For each embryo, the lateral and dorsal view images were acquired. Length measurements were conducted in i-Solution software (IMT i-Solution Inc., Vancouver, BC, Canada). Body lengths of the embryos were measured from the anterior tip of the snout to the posterior end of the caudal peduncle. Eye lengths were measured (1) anterior to posterior from a lateral view image, (2) dorsal to ventral from a lateral view image, and (3) distal to proximal from a dorsal view image. Differences between genotypes were determined by one-way ANOVA, using Tukey Honest Significant Difference (HSD) test to separate means. The statistical analyses were performed in R package version 2.15.3 (R.DCT, 2006). An alpha level of 0.05 was considered statistically significant.

2.2.5 Immunohistochemistry

Immunohistochemistry was performed on ten-micrometer-thick cryosections (Leung et al., 2008). The antibodies used and their corresponding dilutions were as follows: mouse anti-zpr1 (1:200, ZIRC, Eugene, OR), mouse anti-zpr3 (1:200, ZIRC, Eugene, OR), rabbit anti-phospho-Histone H3 (pH3) (1:500, Millipore, Billerica, MA), and Alexa Fluor 488/555 goat anti-rabbit/mouse IgG (1:1000, Invitrogen, Carlsbad, CA). The nuclei were counterstained with DAPI (100ng/mL) (Molecular Probes, Eugene, OR). Mitotic cells were counted based on the pH3 positive cells found in each genotype. Mitotic cell analyses were conducted on cryosections that contained the optic nerve.
number of mitotic cells was compared between genotypes, using an one-way ANOVA. In addition, the pH3 positive cells were categorized based on their location (ciliary marginal zone or neuroepithelium), and analyzed with a logistic regression. The statistical analyses were performed in R package version 2.15.3 (R.DCT, 2006). An alpha level of 0.05 was considered statistically significant.

2.2.6 Characterization of blue opsin staining pattern

In situ hybridization data from blue opsin showed three distinct staining patterns in the eyes of WT and mutant fish (Pattern 1 [P1], Pattern 2 [P2] and Pattern 3 [P3]), at 5-dpf. Therefore, five independent in situ hybridization experiments were conducted (as described before in this section). A total of 98 WT, 82 dlc<sup>b663</sup> and 109 dld<sup>g249</sup> embryos were analyzed based on staining patterns. The percent distribution of the three staining patterns was analyzed by a Chi-squared test, using the R package version 2.15.3 (R.DCT, 2006). In addition, five eyes were dissected for each staining pattern in each genotype. The number of blue opsin cells was counted in four different regions of the eye: anterior-medial, posterior-medial, ventral and dorsal. A linear mixed-effect model was performed using the SAS software (SAS Institute Inc., Cary NC) using PROC MIXED and REML (restricted maximum likelihood) as an estimate method. Post-hoc comparisons of the DLSM (Differences of Least Squares Means), were conducted with the Tukey-Kramer adjustment method. The department of Statistics at Purdue University provided consultation services for this analysis.

**Fixed effects:** Region + Staining Pattern + Genotype + (genotype*staining pattern*region) + (genotype*staining pattern) + (genotype*region) + (staining pattern *region)

**Random effect:** Experimental Group

2.2.7 Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was extracted from 30 eyes microdissected from 5-dpf embryos and reverse-transcribed into cDNA, as previously described (Leung and Dowling, 2005). qRT-PCR was performed using FastStart Essential DNA Green Master Mix on a LightCycler 96 System following the manufacturer protocol (Roche Diagnostics Corporation, Indianapolis, IN). Primers used for qRT-PCR (Table 2.3) were purchased...
from Integrated DNA Technologies (IDT, Coralville, IA). For each genotype, two biological replicates that included three technical replicates were analyzed. Data was analyzed by the ΔΔCt method (Livak and Schmittgen, 2001) and the standard error propagation was used to combine errors. The data was normalized to the reference gene β-actin. The results were reported in ratio of mRNA in the mutant group to that of WT as $2^{\Delta \Delta \text{Ct}}$ and the range of deviation as $2^{\Delta \Delta \text{Ct}} \pm \Delta \Delta \text{CtErr}$. Welch Student $t$-test was used to compare the ΔCt values between mutant and WT. The statistical analyses were performed in R package version 2.15.3 (R.DCT, 2006). An alpha level of 0.05 was considered statistically significant.

### Table 2.3. Primers sequences for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’–3’ Forward Primer</th>
<th>5’-3’ Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>TGCTGTTTTCCCTCCAATTG</td>
<td>GTCCCATGCAAACCATCCTAC</td>
</tr>
<tr>
<td>rhodopsin</td>
<td>AGTCTGGCCAGACATCTAG</td>
<td>GTACTGTGGTATTCGTATGGG</td>
</tr>
<tr>
<td>opn1sw1 (uv opsin)</td>
<td>TCAATTTCCTACTCACCAGCTC</td>
<td>CACAAAAGAGCACAACCACATCAC</td>
</tr>
<tr>
<td>opn1sw2 (blue opsin)</td>
<td>GGTTCCTTTCCAGCACCATTG</td>
<td>AGAAGCCGAACACCACATTACC</td>
</tr>
<tr>
<td>opn1mw1 (green opsin)</td>
<td>GGCTGTGTAATGGAGGGATTTC</td>
<td>ATGGTTTGCGGAGAATTTGAAG</td>
</tr>
<tr>
<td>opn1lw2 (red opsin)</td>
<td>CCAACAGCAATAACACAAGGG</td>
<td>GCGACAACCACAAAGAACATC</td>
</tr>
</tbody>
</table>

#### 2.2.8 Image acquisition

All images were acquired by a SPOT-RT3™ color slider camera (Diagnostic Instruments, Sterling Heights, MA) mounted on an Olympus SZX16 stereomicroscope or Olympus BX51 compound microscope.

#### 2.3. Results

2.3.1 Differential expression patterns of dlc and dld during zebrafish retinogenesis

In order to determine the specific roles of dlc and dld on retinal development, their respective time-series gene expression profiles were determined, in WT embryos, by whole-mount in situ hybridization. At 24-hpf, the expression of dlc was observed throughout the nasal and temporal regions of the retinal neuroepithelium (Figure 2.3A,
black arrows), whereas the expression of \textit{dld} was detected throughout the ventral and apical sides of the dorsal-temporal regions, of the retinal neuroepithelium (Figure 2.3B, black arrows). At 30-hpf, the expression of \textit{dlc} was not detected throughout the basal side of the retinal neuroepithelium, but its expression was strongest in the apical side of the nasal and temporal regions (Figure 2.3C, black arrows). The expression of \textit{dld}, at 30-hpf, was observed throughout the retinal neuroepithelium (Figure 2.3D, black arrows), except at the ventral-nasal region (Figure 2.3D, red asterisk).

At 36-hpf, the expression of \textit{dlc} was strongest in the ventral region of the retinal neuroepithelium (Figure 2.3E, black arrow) and absent in the basal side of the nasal, dorsal and temporal regions (Figure 2.3E, red asterisks). However, the expression of \textit{dld} was found in the basal and apical sides of the retinal neuroepithelium (Figure 2.3F, yellow & black arrows). At 42-hpf, \textit{dlc} expression was detected in the middle and central regions of the ciliary marginal zone (CMZ) (Figure 2.3G, red arrow), as well as in the ventral region of the retina (Figure 2.3G, black arrow), whereas \textit{dld} signal was weakly detected in the CMZ (Figure 2.3H). By 48-hpf, \textit{dlc} expression was restricted to the peripheral region of the CMZ (Figure 2.3I, green arrow). The same expression pattern was observed at 52- and 72-hpf (Figure 2.4A & C, green arrows). In contrast, the expression of \textit{dld} was not detected at 48-, 52-, or 72-hpf (Figures 2.3J, 2.4B & D). Overall, the expression patterns of \textit{dlc} and \textit{dld} at 24-, 30-, 36-, 42-, 48-, 52- and 72-hpf, in WT retinas, differ spatially and temporally. These data suggest that \textit{dlc} and \textit{dld} could have different functions during zebrafish retinogenesis.
Figure 2.3. Differential expression of *dlc* and *dld* during retinogenesis. *In situ* hybridization of *dlc* (A, C, E, G, & I) and *dld* (B, D, F, H, & J) on 24-, 30-, 36-, 42- and 48-hpf WT embryos. Images are 20µm-thick lateral (A-F) or transverse (G-J) cryosections. In the lateral cryosections, anterior (a) is to the left and dorsal (d) is up (lenses are outlined by black dashed lines). In the transverse cryosections, lateral is to the left and dorsal is up. Black arrows indicate expression in the retinal neuroepithelium. Yellow arrow indicates expression on the basal region of the eye. Red asterisks indicate the lack of expression. Red arrow indicates expression in the middle-central region of the ciliary marginal zone (CMZ). Green arrow indicates expression in the peripheral region of the CMZ. Scale bar = 50 µm.
2.3.2 Phenotype and genotype of \textit{dlc}^{b663} and \textit{dld}^{tg249} mutant zebrafish

To determine the function of Dlc and Dld, the retinal development of the mutants \textit{dlc}^{b663} (previously named: \textit{beamter}) and \textit{dld}^{tg249} (previously named: \textit{after eight}) were analyzed (Henry et al., 2005; Henry et al., 2002; Holley et al., 2000; van Eeden et al., 1996). Homozygous mutant fish \textit{dlc}^{b663} and \textit{dld}^{tg249} are viable and fertile. These mutant fish have been used extensively to study the function of Dlc and Dld on somite development (Holley et al., 2000; Holley and Takeda, 2002; Jiang et al., 2000). It has...

**Figure 2.4. Differential expression of \textit{dlc} and \textit{dld} in the eye at 52- and 72-hpf.** \textit{In situ} hybridization of \textit{dlc} (A & C) and \textit{dld} (B & D) on 52- and 72-hpf WT embryos. Transverse cryosections are shown for 52-hpf embryos (A & B). Ventral whole-mount images are shown for 72-hpf embryos (C & D). In cryosections images, the lateral side is to the left and dorsal side is up. In whole-mount images, the lateral side are to the left and nasal is up. Green arrows indicate expression in the peripheral region of the CMZ. Scale bars = 50 µm.
been determined that Dlc and Dld are needed for proper somite segmentation by coordinating the oscillation of gene expression in the presomitic mesoderm (PSM) (Holley et al., 2000; Julich et al., 2005; Mara et al., 2007; Wright et al., 2011). Their embryos can be differentiated by their specific defects in somite formation (van Eeden et al., 1996). For example, at 14-hpf, \( dlc^{b663} \) embryos had six abnormal somites with obscured boundaries (Figure 2.5B, red asterisks), which contrast with the ten somites with clear boundaries observed in WT embryos (Figure 2.5A, black asterisks). The \( dld^{g249} \) mutants have defects on their posterior somites and only the first eight somites develop normally (Figure 2.5C, black and red asterisks indicate normal or defected somites, respectively).

The somite defects in the homozygous embryos allows for the phenotypic identification of the mutants as early as 14-hpf, well before the first retinal cells are born. Yet, it is not clear how their mutations differ genetically. The \( dld^{g249} \) zebrafish has a premature stop codon at the 63rd amino acid from the amino terminus (Holley et al., 2000). This premature stop codon is located before the Delta:Serrate:Lag-2 (DSL) domain that is needed to mediate ligand-receptor interaction; thus, the \( dld^{g249} \) fish have no functional Dld. However, the mutation in \( dlc^{b663} \) has not been identified. Therefore, in order to determine the mutation in \( dlc^{b663} \), as well as confirming the mutation in \( dld^{g249} \), genomic DNA from WT and homozygous mutant (\( dlc^{b663} \) and \( dld^{g249} \)) adult fish were extracted and sequenced. As expected, the \( dld^{g249} \) mutant had an A to T substitution (Figure 2.6B & D), whereas the sequence of \( dlc^{b663} \) revealed an A to T substitution located at the start codon (Figure 2.6A & C).
Figure 2.5. Phenotype of WT, \textit{dle}^{b663} and \textit{dll}^{g249} embryos at 14-hpf and 5-dpf. Lateral views (A-I) of 14-hpf (A-C) and 5-dpf (D-I) WT (A, D & G), \textit{dle}^{b663} (B, E & H) and \textit{dll}^{g249} (C, F & I) embryos are shown. Dorsal views are shown (J-M) of 5-dpf embryos. At 14-hpf, WT embryos had 10 somites, while \textit{dle}^{b663} had five somites with abnormal boundaries, and \textit{dll}^{g249} had an abnormal and eight normal somites. Black and red asterisks indicate normal and defected somites, respectively. The lateral images of 5-dpf embryos were utilized to measure body length (D-F) and eye size from three anatomical locations, anterior to posterior (G-I) and dorsal to ventral (G-I). In lateral images, nasal is left and dorsal is up. Dorsal images of 5-dpf embryos were utilized to measure eye thickness by measuring the distal to proximal ends of the eyes (J-M). In dorsal images, nasal is up. Scale bar =100 µm.
Figure 2.6. Sequence analysis of $dlc^{b663}$ and $dld^{g249}$ mutations. Sequence trace profiles of the regions altered in the mutants and the corresponding WT sequences. Genomic DNA was extracted from WT (A & B), $dlc^{b663}$ (C) and $dld^{g249}$ (D) adult fish. Sequencing of $dld^{g249}$ verified the substitution of A to T which translates into a premature stop codon (Holley et al., 2000). The sequencing of $dlc^{b663}$ revealed an A to T substitution in its start codon.

2.3.3 The $dld^{g249}$ embryos have no change in eye size while $dlc^{b663}$ embryos have thicker eyes.

Since $dlc^{b663}$ and $dld^{g249}$ have not been previously studied for retinal defects, the eye size of 5-dpf WT, $dlc^{b663}$ and $dld^{g249}$ embryos was measured and compared. In order to determine if an embryo’s body length could be used to normalize its eye size measurement, the body length of ten 5-dpf embryos, per each genotype, was measured. Mean body length of $dlc^{b663}$ (mean ± SD) (3597 ± 145 µm) and $dld^{g249}$ (3762 ± 82 µm) were significantly shorter than WT embryos (3934 ± 83 µm) ($F(2, 27) = 24.6, p < 0.001$) (Figure 2.5D-F). Tukey’s Honest Significant Difference (HSD) test, determined significant differences between $dlc^{b663}$ and WT ($p < 0.004$), and between $dld^{g249}$ and WT.
Therefore, normalizing the eye size data to body length was not feasible due to the change in mutants’ body length.

The mean eye size for WT, \( dlc^{b663} \), and \( dld^{g249} \) embryos was measured in three different anatomical locations: (1) anterior to posterior (A-P) from a lateral view, (2) dorsal to ventral (D-V) from a lateral view, and (3) distal to proximal (Di-Pr) from a dorsal view (Figure 2.5G-M). The means and standard deviations are presented in Table 2.4. There were no significant differences between genotypes on A-P and D-V eye measurements (Table 2.4). Yet, there were significant differences between genotypes on Di-Pr eye measurements (Table 2.4). Tukey’s HSD determined that \( dlc^{b663} \) embryos had significant larger Di-Pr lengths compared to WT \((p = 0.0165)\) (Tables 2.4 & 2.5).

### Table 2.4. Mean values and ANOVA results of eye measurements on 5-dpf WT, \( dlc^{b663} \) and \( dld^{g249} \) embryos.

<table>
<thead>
<tr>
<th>Genotype (mean ± SD ( \mu m ))</th>
<th>One-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>( dlc^{b663} )</td>
</tr>
<tr>
<td>A-P</td>
<td>324.8 ± 10.8</td>
</tr>
<tr>
<td>D-V</td>
<td>270.7 ± 9.00</td>
</tr>
<tr>
<td>Di-Pr</td>
<td>184.1 ± 10.6</td>
</tr>
</tbody>
</table>

### Table 2.5. The \( p \)-values of the post hoc multiple comparison tests of eye measurements on 5-dpf WT, \( dlc^{b663} \) and \( dld^{g249} \) embryos.

The Tukeys’ HSD \( p \)-values are shown for each comparison.

<table>
<thead>
<tr>
<th>Tukey’s HSD ( p )-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - ( dlc^{b663} )</td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>A-P</td>
</tr>
<tr>
<td>D-V</td>
</tr>
<tr>
<td>Di-Pr</td>
</tr>
</tbody>
</table>

### 2.3.4 No effect on retinal lamination in \( dlc^{b663} \) and \( dld^{g249} \)

During retinal development, Delta-Notch signaling contributes to the maintenance, specification and differentiation of progenitor cells (Austin et al., 1995;
Bernardos et al., 2005; Dorsky et al., 1997; Dorsky et al., 1995; Furukawa et al., 2000; Henrique et al., 1997; Jadhav et al., 2006b; Mizeracka et al., 2013a; Rapaport and Dorsky, 1998; Scheer et al., 2001; Tomita et al., 1996; Yaron et al., 2006). Previous studies have demonstrated that inhibition of Delta-Notch signaling will allow progenitor cells to prematurely exit the cell cycle and disrupt retinal lamination (Ahmad et al., 1997; Austin et al., 1995; Bernardos et al., 2005). In the case of the dlc$^{b663}$ and dld$^{tg249}$ mutants, Delta-Notch signaling is decreased. Therefore, this study hypothesized that both mutants have disrupted retinal lamination and decreased mitotic cell population. To test this hypothesis, retinal lamination and mitotic cells were analyzed in 3-dpf WT, dlc$^{b663}$ and dld$^{tg249}$ embryos by using immunohistochemistry.

The retinas of WT embryos at 3-dpf have recognizable retinal cell layers, including the ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL) (Figure 2.7A). In mutant embryos, the GCL, INL and ONL are also identifiable (Figure 2.7B & C). Overall, lamination in the mutant retinas seems largely unaffected.

In order to compare the mitotic cell population in WT, dlc$^{b663}$ and dld$^{tg249}$ retinas, mitotic cells in G2/M phase were labeled with anti-phospho-histone H3 (pH3) cells, in 3-dpf eyes. There were no significant differences in number of mitotic cells between genotypes ($F (2, 19) = 1.36, p = 0.2805$) (Figure 2.5D-F) (Table 2.6). These results indicate that the pool of mitotically active progenitor cells is not depleted by the lack of Dlc or Dld signaling. However, the position of pH3 cells in WT and mutant eyes was different. In WT eyes, most pH3 cells were located in the proliferating zone, known as the ciliary marginal zone (CMZ) (Figure 2.7D), whereas mutant retinas had pH3 cells in the retinal neuroepithelium or the border of the CMZ (Figure 2.7E & F, yellow arrows). A logistic regression analysis of pH3 cell count (base on location) demonstrated that the WT proportion of pH3 cells in the CMZ was 85.3% compared to 90.0% and 51.9% of dlc$^{b663}$ and dld$^{tg249}$ embryos, respectively. This indicates that a significant amount of pH3 cells are not in the CMZ of dld$^{tg249}$ retinas ($p = 0.024$).
Table 2.6. Mitotic cell counts in 3-dpf WT, \textit{dlc}^{b663} and \textit{dld}^{g249} embryos. N is the number of embryos analyzed. SD is the standard deviation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Mean # of total pH3 cells (SD)</th>
<th>Mean # of pH3 cells in the CMZ (SD)</th>
<th>Mean # of pH3 cells not in the CMZ (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6</td>
<td>5.67 (2.66)</td>
<td>4.83 (2.40)</td>
<td>0.833 (0.753)</td>
</tr>
<tr>
<td>\textit{dlc}^{b663}</td>
<td>6</td>
<td>8.17 (5.00)</td>
<td>7.33 (5.32)</td>
<td>0.833 (1.17)</td>
</tr>
<tr>
<td>\textit{dld}^{g249}</td>
<td>10</td>
<td>5.40 (2.55)</td>
<td>2.80 (2.04)</td>
<td>2.6 (1.43)</td>
</tr>
</tbody>
</table>

Figure 2.7. Retinal lamination and pH3 cells in 3-dpf WT, \textit{dlc}^{b663} and \textit{dld}^{g249} embryos. Transverse cryosections labeled with DAPI (A-C) and phosphorylated histone H3 (pH3) (D-F). Yellow arrows indicate ectopic pH3 positive cells in the retina. Lateral is to the left and dorsal is up. Eyes are outlined by red dots. GCL (ganglion cell layer), INL (inner nuclear layer) and ONL (outer nuclear layer) are labeled. Scale bar= 50 µm.
2.3.5 Dlc and Dld affect photoreceptor development

In addition to contributing to the maintenance of retinal progenitor cells and lamination, Delta-Notch signaling is essential for proper development of neurogenesis and gliogenesis (Austin et al., 1995; Bernardos et al., 2005; Dorsky et al., 1997; Furukawa et al., 2000; Jadhav et al., 2006a; Scheer et al., 2001). To identify the cell types that are affected in \( dlc^{b663} \) and \( dld^{g249} \) retinas, several retinal-specific markers were used to screen retinal glial and neural cells in 3-dpf embryos. Transverse sections were stained with islet-1 (ganglion, amacrine, bipolar, and horizontal cell maker), anti-GS (Müller glia cell marker), zpr-1 (green/red double cone cell marker) or zpr-3 (rod cell marker).

Ganglion, amacrine, bipolar, and horizontal cells were detected in WT and mutant retinas (Figure 2.8A-C). In addition, Müller glia cells were detected in both WT and mutant retinas (Figure 2.8D-F). This data showed no drastic differences on islet-1 and anti-GS staining in mutant retinas compared to WT. Nonetheless, photoreceptor staining in mutant retinas was abnormal (Figure 2.9). In WT eyes, zpr-1 signal was present throughout the ventral to dorsal regions of the ONL, while no zpr-1 signal was detected in the dorsal region of mutant eyes (Figure 2.9A-C). Moreover, a stronger zpr-1 signal was detected in the ventral region of both mutant retinas when compared to the WT. Nevertheless, \( dld^{g249} \) retinas had zpr-1 staining in the central region of the ONL whereas \( dlc^{b663} \) did not (Figure 2.9A-C). A smaller range of zpr-3 signal was observed in \( dlc^{b663} \) and \( dld^{g249} \) retinas when compared to WT (Figure 2.9D-F). WT retinas had zpr-3 staining throughout the ventral to dorsal regions of the ONL (Figure 2.9D), whereas \( dlc^{b663} \) and \( dld^{g249} \) retinas had staining only in the ventral to central regions of the ONL (\( dld^{g249} \) showed slightly more signal towards the dorsal side, without covering the entire ONL) (Figure 2.9E & F). These results indicate that the lack of Dlc or Dld affects photoreceptor development.
Figure 2.8. Ganglion, amacrine, bipolar, horizontal and Müller glia cell staining in 3-dpf WT, $dlc^{b665}$ and $dlld^{g249}$ eyes. Transverse cryosections of 3-dpf WT and mutant eyes labeled with islet-1 (ganglion, amacrine, bipolar and horizontal cells) (A-C) or anti-GS (Müller glia cells) (D-E). Lateral is to the left and dorsal is up. Eyes are outlined by red dots. Scale bar= 50 µm.
2.3.6 Expression of rhodopsin in \textit{dlc}^{b663} and \textit{dld}^{g249} eyes

To further examine the role of Dlc and Dld on rod development, the expression of rhodopsin (\textit{rho}) was analyzed by whole-mount \textit{in situ} hybridization and quantitative reverse-transcription PCR (qRT-PCR), in mutant and WT embryos. At 36-hpf, WT embryos had few cells with \textit{rho} expression in the ventral side of the retina, while no signal was detected in the mutant retinas (Figure 2.10A-C). By 55-hpf, \textit{rho} signal was observed in cells located in the ventral side of WT and mutant eyes (Figure 2.10D-F), but mutant retinas had few scattered cells that expressed \textit{rho} in the central region of the ONL (Figure 2.10D’-F’). At 5-dpf, \textit{dlc}^{b663} and \textit{dld}^{g249} embryos had different staining patterns of \textit{rho} in the ventral side when compared to WT (Figure 2.10G-H). In addition, mutant retinas had fewer cells expressing \textit{rho} in the central region of the ONL (Figure 2.10G’-I’). At 5-dpf, \textit{rho} staining did not extend to the medial side of the mutant eyes, probably
due to a reduction of the transcript or due to a disruption of \textit{rho} distribution. To address this uncertainty, \textit{rho} expression level was compared, between 5-dpf WT and mutant eyes, by using qRT-PCR. These experiments showed no significant differences between mutant and WT retinas (Tables 2.7 & 2.8). The results suggest that Dlc and Dld (1) allows for early rod development, and (2) contributes to the spatial distribution of \textit{rho} expression.

![Figure 2.10. Expression of rhodopsin (rho) at 36-hpf, 55-hpf and 5-dpf in WT and mutant retinas. In situ hybridization of rho (rod cell differentiation maker) on 36-hpf (A-C), 55-hpf (D-F), and 5-dpf (G-I) WT (A, D, & G), \textit{dlc}^{b663} (B, E, & H), and \textit{dld}^{tg249} (C, F, & I) embryos. Images of whole embryos from the ventral view (A-C), and from dissected eyes from ventral (D-F & G-I) and medial views (D’-F’ & G’-I’) are shown. (A-C) Nasal is to the top. (D-F & G-I) Nasal is to the left and medial is up. (D’-F’ & G’-I’) Nasal is to the left and dorsal is to the top. Red arrows indicate cells with \textit{rho} expression in the medial region of the eye (E’ & F’). A minimum of ten embryos was analyzed for each genotype.}
### Table 2.7. qRT-PCR and statistical analyses of opsins between dlc<sup>b663</sup> and WT.

30 eyes of 5-dpf WT and dlc<sup>b663</sup> embryos were dissected for analyses (two biological replicates each with three technical replicates). The mean fold-changes and ranges are listed. None of the opsins was significantly different between dlc<sup>b663</sup> and WT.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Log Fold Change</th>
<th>Range</th>
<th>Welch Student t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodopsin</td>
<td>1.15</td>
<td>0.934 - 1.41</td>
<td>0.525 8.78 0.613</td>
</tr>
<tr>
<td>Uv opsin</td>
<td>1.12</td>
<td>0.898 - 1.41</td>
<td>0.563 7.03 0.591</td>
</tr>
<tr>
<td>Blue opsin</td>
<td>1.28</td>
<td>1.13 - 1.44</td>
<td>0.987 5.52 0.365</td>
</tr>
<tr>
<td>Green opsin</td>
<td>0.887</td>
<td>0.746 - 1.05</td>
<td>0.0906 6.69 0.930</td>
</tr>
<tr>
<td>Red opsin</td>
<td>0.924</td>
<td>0.764 - 1.12</td>
<td>-0.211 9.02 0.838</td>
</tr>
</tbody>
</table>

### Table 2.8. qRT-PCR and statistical analyses of opsins between dld<sup>tg249</sup> and WT.

30 eyes of 5-dpf WT and dld<sup>tg249</sup> embryos were dissected for analyses (two biological replicates each with three technical replicates). Green and red opsins were significantly overexpressed in dld<sup>tg249</sup> eyes compared to WT, Welch Student t-test, p < 0.01.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Log Fold Change</th>
<th>Range</th>
<th>Welch Student t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodopsin</td>
<td>1.20</td>
<td>1.09 - 1.31</td>
<td>1.57 9.96 0.147</td>
</tr>
<tr>
<td>Uv opsin</td>
<td>1.22</td>
<td>1.06 - 1.41</td>
<td>1.85 5.46 0.119</td>
</tr>
<tr>
<td>Blue opsin</td>
<td>1.27</td>
<td>1.15 - 1.41</td>
<td>1.98 8.92 0.0799</td>
</tr>
<tr>
<td>Green opsin</td>
<td><strong>1.98</strong></td>
<td>1.78 - 2.21</td>
<td>5.39 7.73 <strong>0.000732</strong></td>
</tr>
<tr>
<td>Red opsin</td>
<td><strong>2.71</strong></td>
<td>2.45 - 3.00</td>
<td>6.80 6.11 <strong>0.000458</strong></td>
</tr>
</tbody>
</table>

#### 2.3.7 Expression of cone opsins in dlc<sup>b663</sup> and dld<sup>tg249</sup> eyes

To further analyze the defects in cone development observed in Figure 2.9, cone opsins expressions were analyzed, by whole-mount in situ hybridization and qRT-PCR, in mutant and WT embryos. There was no change detected, by in situ hybridization or qRT-PCR, in the expression of uv opsin in dlc<sup>b663</sup> and dld<sup>tg249</sup> compared to 5-dpf WT embryos (Figure 2.11A-C) (Tables 2.7 & 2.8).
2.3.7.1 Blue opsin expression in 5-dpf dlc\textsuperscript{663} and dld\textsuperscript{8249} embryos

By in situ hybridization, an increased expression of blue opsin was observed in dlc\textsuperscript{663} embryos compared to WT and dld\textsuperscript{8249} embryos (Figure 2.11D-F). However, by qRT-PCR there were no significant changes in expression of blue opsin between dlc\textsuperscript{663} and WT eyes (Table 2.7). Suggesting that the expression pattern of blue opsin was disrupted in dlc\textsuperscript{663} embryos. To examine this possibility, the blue opsin staining patterns of each genotype were analyzed. At 5-dpf, blue opsin expression had three staining patterns (P1-P3; Figure 2.12), being P1 the pattern with the lowest expression and P3 the one with the highest. In WT, dlc\textsuperscript{663} and dld\textsuperscript{8249} embryos, P3 was the most prominent staining pattern (Figure 2.12). Nevertheless, dlc\textsuperscript{663} embryos had significantly more embryos with the P3 pattern than WT or dld\textsuperscript{8249} embryos, $X^2$ (4, N = 289), $p < 0.0001$.

To analyze the blue opsin expression pattern in the three staining groups, five eyes per genotype per staining pattern were dissected. Cells with blue opsin expression were counted in four regions of the eye: anterior-medial (A-M), posterior-medial (P-M), ventral and dorsal regions. Five different in situ experiments were conducted. A linear mixed-effect model was used to fit the data with the number of blue opsin cells as the response variable. The fixed effects were staining pattern, genotype and region. In addition, experimental group was entered to the model as a random effect. The number of blue opsin cells per area was significantly affected by genotype, $F(1, 91) = 33.55$, $p < 0.0001$). The individual group comparisons confirmed that, in the P3 staining pattern, the number of blue opsin cells per area were significantly higher ($p < 0.0001$, Tukey-Kramer adjusted) in dlc\textsuperscript{663} (0.0783, ± 0.00929 $\mu$m$^2$) than in WT (0.0712, ± 0.0130 $\mu$m$^2$). Specifically, blue opsin cells per area in the dorsal and posterior-medial region in the P3 staining pattern were different (Figure 2.13, Table 2.9).
Figure 2.11. Expression of cone opsins at 5-dpf in WT, \( dlc^{b663} \) and \( dld^{g249} \) embryos. Whole-mount \textit{in situ} hybridization of \textit{uv}, \textit{blue}, \textit{green} and \textit{red opsins} of WT, \( dlc^{b663} \) and \( dld^{g249} \) embryos. Ventral views of the embryos are shown, nasal is up. A minimum of ten embryos was analyzed for each genotype.
Figure 2.12. Staining pattern distribution for blue opsin of 5-dpf WT, dlc<sup>b663</sup> and dld<sup>g8249</sup> embryos. There were three staining patterns, P1, P2 and P3 for blue opsin. There were significant differences among the genotypes’ staining pattern distributions (Genotype: N = P3, P2, P1; WT: N = 39, 22, 37; dlc<sup>b663</sup>: N = 74, 7, 1; dld<sup>g8249</sup>: N = 52, 30, 27), $X^2$ (4, N = 289), p < 0.0001).

Table 2.9. Mean count of blue opsin cells per area (µm<sup>2</sup>) of 5-dpf WT and dlc<sup>b663</sup> eyes. The percent distribution for P3 was calculated (based on data from Table 2.10), and reported in the second column. All cell counts per area (µm<sup>2</sup>) were reported as means with their standard deviations (±SD). A linear mixed-effect model was used to examine the effects of genotype, region and staining pattern on blue opsin cell count. The pos hoc comparison (with Tukey-Kramer multiple comparison adjustment) revealed mutant embryos with P3 staining pattern were significantly different to WT embryos from P3 (p < 0.0001). Additionally, the P3 dorsal and posterior-medial regions were significantly different (p < 0.0001 and p = 0.0170, respectively).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Dist. of embryos</th>
<th>Total (± SD)</th>
<th>Dorsal (± SD)</th>
<th>Ventral (± SD)</th>
<th>A-M (± SD)</th>
<th>P-M (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dlc&lt;sup&gt;b663&lt;/sup&gt;</td>
<td>90.3%</td>
<td>0.0783 (± 0.00929)</td>
<td>0.0201 (± 0.00265)</td>
<td>0.00956 (± 0.00677)</td>
<td>0.0244 (± 0.00424)</td>
<td>0.0242 (± 0.00436)</td>
</tr>
<tr>
<td>WT</td>
<td>39.8%</td>
<td>0.0712 (± 0.0130)</td>
<td>0.0214 (± 0.00285)</td>
<td>0.00578 (± 0.00466)</td>
<td>0.0219 (± 0.00664)</td>
<td>0.0221 (± 0.00522)</td>
</tr>
</tbody>
</table>
Figure 2.13. *Blue opsin expression in 5-dpf WT and dlc<sup>b663</sup> embryos.* *In situ* hybridization of blue opsin on 5-dpf WT (A) and dlc<sup>b663</sup> (B) embryos. Dissected eyes are shown. In dorsal (A & B) and ventral views (A’ & B’), nasal is to the left and medial is up. In the medial views (A” & B”), nasal is to the left and dorsal is to the top.

2.3.7.2 *Blue opsin expression in 36- and 55- hpf dlc<sup>b663</sup> and dld<sup>g249</sup> embryos*

Since there were spatial defects of blue opsin at 5-dpf in dlc<sup>b663</sup> retinas, blue opsin expression was analyzed at 36- and 55- hpf (Figure 2.14 & 2.15). At 36-hpf, the signal of blue opsin was observed in the posterior side in WT and dlc<sup>b663</sup> eyes (Figure 2.14A & B). Precisely, the blue opsin expression was observed on the periphery of the eye (Figure 2.15A & B). At 36-hpf, blue opsin expression in dld<sup>g249</sup> eyes was detected in the retinal neuroepithelium (Figure 2.15C). At 55-hpf, there were two groups with different staining patterns in WT, dlc<sup>b663</sup> and dld<sup>g249</sup> eyes (Figure 2.14D-I). Group one had blue opsin expression in the ventral side and small amounts of positive signal in the medial side of the eyes (Figure 2.14D-F). Group two had blue opsin expression in the ventral side and larger amounts of positive signal in the medial side (Figure 2.14G-I). Both mutants had increased blue opsin expression in the medial region of the eye compared to WT at 55-hpf. These data suggest that Dlc and Dld hinder blue cone development.
**Figure 2.14. Expression of blue opsin in 36- and 55-hpf WT and mutant embryos.** *In situ* hybridization of blue opsin on 36-hpf (A-C) and 55-hpf (D-I) on WT (A, D, & G), *dlc*<sup>b663</sup> (B, E & H), and *dlb*<sup>tg249</sup> (C, F & I) embryos. Images of whole embryos from the ventral view (A-C), and dissected eyes from the ventral view (D’-F’ & G’-I’), dorsal view (G”-I”) and medial view (D-I). In the ventral view of A-C, nasal is to the top. In the medial view of D-I, nasal is to the left and dorsal is to the top. In the ventral and dorsal views of D’-F’, G’-I’ and G”-I”, nasal is to the left and medial is up. Red arrows indicate cells with blue opsin expression in the medial or ventral regions of the eye.
2.3.7.3 Increased red opsin expression in dld\(^{tg249}\) and not in dlc\(^{b663}\) retinas

At 5-dpf, there was an increase expression of red opsin in dld\(^{tg249}\) embryos compared to WT and dlc\(^{b663}\) embryos (Figure 2.11J-L). Moreover, qRT-PCR experiments confirmed there was a significant increase in the expression of red opsin between dld\(^{tg249}\) and WT eyes (dld\(^{tg249}\) vs. WT = 2.71, 2.45 - 3.00; Welch Student t-test, \(p = 0.000458\)) (Tables 2.8). As a result, it is possible that red cone development could be increased at an earlier time point of development in dld\(^{tg249}\) embryos. To exam this possibility, in situ hybridization of red opsin was conducted at 55-hpf (Figure 2.16). At 55-hpf, there were two groups with different staining patterns in WT, dlc\(^{b663}\) and dld\(^{tg249}\) eyes (Figure 2.16A-F). Group one had red opsin expression in the ventral side and small amounts of signal in the medial side of the eyes (Figure 2.16A-C). Group two had red opsin expression in the ventral side and larger amounts of signal in the medial side (Figure 2.16D-F). In the group one, dld\(^{tg249}\) had more red opsin expression in the medial side than WT and dlc\(^{b663}\) (Figure 2.16A-C). These data suggest that Dld hinders early and late red cone development.
Figure 2.16. Expression of red opsin in 55-hpf WT, dlc<sup>b663</sup> and dld<sup>g249</sup> eyes. In situ hybridization of red opsin on 55-hpf WT (A & D), dlc<sup>b663</sup> (B & E) and dld<sup>g249</sup> (C & F) eyes. In the ventral and dorsal views of A’-F’ and D”-F”, nasal is to the left and medial is up. In the medial views of A-F, nasal is to the left and dorsal is to the top. Red arrows indicate cells with red opsin expression in the medial or ventral regions of the eye.

2.3.7.4 Green opsin expression was increased in dld<sup>g249</sup> and not in dlc<sup>b663</sup> retinas

At 5-dpf, there were no differences in green opsin expression between mutant and WT eyes (Figure 2.11G-I). However, the qRT-PCR analysis showed a significant increase in green opsin expression in dld<sup>g249</sup> compared to WT eyes (dld<sup>g249</sup> vs. WT = 1.98, 1.78 - 2.21; Welch Student t-test, p = 0.000732) (Table 2.8). Thus, these results suggest that green cone development could be increased at an earlier time point in dld<sup>g249</sup> embryos. To test this possibility, in situ hybridization of green opsin was conducted at 52-hpf (Figure 2.17). At 52-hpf, green opsin expression was present in few cells in the ventral region of WT, dlc<sup>b663</sup> and dld<sup>g249</sup> eyes. There were no changes in green opsin expression between mutant and WT eyes at 52-hpf. These data suggest that Dld hinders late green cone development.
2.3.8 Decreased expression of \textit{nr2e3} in \textit{dlc}^{b663} and \textit{dlld}^{g249} embryos

Since \textit{dlc}^{b663} and \textit{dlld}^{g249} embryos had defects in photoreceptor differentiation, photoreceptor precursor cells were analyzed at 55-hpf and 5-dpf. Gene expression of \textit{cone-rod homeobox (crx)} was analyzed by \textit{in situ} hybridization in WT, \textit{dlc}^{b663} and \textit{dlld}^{g249} embryos. No differences were detected in \textit{crx} expression in 55-hpf (Figure 2.18A-C) and 5-dpf (Figure 2.19A-C) \textit{dlc}^{b663} and \textit{dlld}^{g249} embryos, when compared to WT. These
results indicate that the effect of Dlc and Dld on photoreceptor development is downstream of crx; thus, Dlc and Dld do not affect or delay photoreceptor precursor cells.

During rod development, photoreceptor precursor cells express crx and specific transcription factors needed for rod differentiation, like nrl (neural retina leucine zipper) and nr2e3 (nuclear receptor subfamily 2, group E, member3) (Chen et al., 2005; Mears et al., 2001; Peng et al., 2005). To further investigate the rod differentiation defects in dlc^{b663} and dld^{g8249} embryos, in situ hybridization of nrl and nr2e3 was conducted in WT and mutant embryos. At 55-hpf, nrl is expressed scarcely in the ventral region of the retina (Figure 2.18D). There were no changes of nrl in the mutant retinas, with the exception of a higher nrl expression in the brain of dlc^{b663} embryos (Figure 2.18D-F). At 5-dpf, nrl was not expressed in WT or mutant retinas (data not shown). Nevertheless, there was a decreased expression of nr2e3 in both mutant retinas at 55-hpf and 5-dpf when compared to WT (Figure 2.18G-L) (Figure 2.19D-I). At 55-hpf, decreased expression of nr2e3 was observed in the medial region of the ONL in mutant eyes (Figure 2.18J-L). In addition, at 5-dpf, decreased expression of nr2e3 was observed in the CMZ and INL in mutant eyes (Figure 2.19D-I). These results, suggest that both Dlc and Dld promote expression of nr2e3, which is needed for rod differentiation.
Figure 2.18. \textit{dlc}^{b663} and \textit{dld}^{tg249} have altered expression of transcription factors for rod cells. Ventral views of whole-mount \textit{in situ} hybridization of \textit{crx}, \textit{nrl} and \textit{nr2e3} on 55-hpf WT (A, D, & G), \textit{dlc}^{b663} (B, E & H) and \textit{dld}^{tg249} (C, F & I) embryos. In the ventral views (A-I), nasal is to the top. Transverse cryosections are shown for \textit{nr2e3} (J-L), lateral side is to the left and dorsal side is up. (A-C) Gene expression of \textit{crx} was not affected in mutant retinas. (D-F) Gene expression of \textit{nrl} was not affected in mutant retinas (\textit{dlc}^{b663} retinas had high signal in the brain). (G-L) Gene expression of \textit{nr2e3} was decreased in \textit{dlc}^{b663} and \textit{dld}^{tg249} retinas. Red arrows indicate the lack of expression in the ONL.
Figure 2.19. Expression of *crx* and *nr2e3* in WT, *dlec*<sup>b663</sup>, and *dld*<sup>g249</sup> eyes at 5-dpf. *In situ* hybridization of *crx* and *nr2e3* on 5-dpf WT (A & D), *dlec*<sup>b663</sup> (B & E) and *dld*<sup>g249</sup> (C & F) embryos. Whole-mount for *crx* are shown in the ventral view (A-C), nasal is up. Dissected eyes and transverse cryosections are shown for *nr2e3* (D-F & G-I). In the dissected eyes, the lateral view is shown, nasal to the left and dorsal side is up. In the transverse sections, lateral is to the left and dorsal is up. (A-C) Gene expression of *crx* was not affected in mutant retinas. (D & G) Gene expression in WT eyes of *nr2e3* was observed in the CMZ and in the boundaries of the INL. Red arrowheads indicate expression in the boundaries of the INL. (H & I) Mutant eyes had expression of *nr2e3* in the CMZ.

2.4. Discussion

In this chapter, we showed that the expression profiles of *dlc* and *dld* differ in WT retinas during retinal development (Figures 2.3 & 2.4). Moreover, we showed that Dlc and Dld have similar and different functions on photoreceptor development (summarized
in Figure 2.20). We found that the loss of *dd* function at 36-hpf delayed rod development (Figure 2.10C). Similarly, the loss of *dlc* function had the same effect (Figure 2.10B), but in addition it accelerated blue cone development (Figure 2.15C). The progressive lost of *dlc* and *dd* function affected the distribution of rod cells (Figure 2.10E, F, H & I). Ultimately, the continued loss of *dlc* function hindered the distribution of blue cones while loss of *dd* function increased red and green cones.
Figure 2.20. Schematic diagram of Dlc and Dld effects on photoreceptor development during retinal development.
2.4.1 Differential expression of dlc and dld during zebrafish retinogenesis might be the key to distinguish subpopulation of retinal proliferating cells

An initial report from Smithers and colleagues (2000) demonstrated that only dlc is expressed in the retinal neuroepithelium at 22-hpf (Smithers et al., 2000). In this chapter, dlc was detected throughout the retinal neuroepithelium while dld expression was detected in a narrower domain at 24-hpf (Figure 2.3A & B). At 24-hpf, the neuroepithelium consists mainly of proliferating cells (Schmitt and Dowling, 1994), suggesting that the expression of dlc and dld resides in proliferating retinal cells. These observations were consistent with previous reports in other taxonomic groups (Nelson et al., 2009; Nelson and Reh, 2008). For example, expression analysis in the mouse and chicken, reported that delta genes are expressed in retinal progenitor cells during early retinal development (Nelson et al., 2009; Nelson and Reh, 2008). Nelson and colleagues (2009) reported that m-dll1 (an ortholog of zebrafish dla and dld) and m-dll3 (an ortholog of zebrafish dlb and dlc) were found in proliferating retinal cells throughout retinal development (Dornseifer et al., 1997; Eckalbar et al., 2012; Nelson et al., 2009).

The different expression patterns of dlc and dld were observed throughout 30- to 72-hpf (Figure 2.3 & 2.4). The different levels of dlc and dld in retinal proliferating cells may influence these cells to acquire different retinal identities.

2.4.2 Eye defects in dlc<sup>b663</sup> and dld<sup>g249</sup>

No major defects were found in retinal lamination (Figure 2.7A-C) or on eye size (Figure 2.5D-M), except that dlc<sup>b663</sup> mutants had significantly thicker eyes compared to WT (Table 2.4). This observation might be explained by the slight increase of mitotic cells in dlc<sup>b663</sup> eyes that was observed (Table 2.6). However, the mean count of mitotic cells was not statistically significant. Nevertheless, the location of mitotic cells did differ in mutant eyes compared to WT (Figure 2.7D-F). At 3-dpf, mitotic cells were located in the CMZ, but in dld<sup>g249</sup> eyes mitotic cells were outside the CMZ and in the neuroepithelium. This suggests that Dld prevents mitotic cells to continue into the neuroepithelium at 3-dpf. Furthermore, neural and glial differentiation in mutant eyes was analyzed. The decreased of photoreceptor development was observed in mutants’ eyes compared to WT (Figures 2.8 & 2.9). The involvement of the Delta-Notch pathway
on photoreceptor development has been reported in other studies in the mouse, chicken and frog (Bernardos et al., 2005; Dorsky et al., 1997; Jadhav et al., 2006b; Luo et al., 2012; Mizeracka et al., 2013b; Nelson et al., 2007; Tomita et al., 1996; Yaron et al., 2006).

2.4.3 Dlc and Dld promote initial ventral differentiation of rod cells at 36-hpf

In zebrafish, the initial expression of *rhodopsin* (*rho*) is detected in the ventral region of the retina (Raymond et al., 1995; Stenkamp et al., 1996). In this study, the ventral expression of *rho* was observed at 36-hpf, in WT embryos (Figure 2.10A). In contrast, the loss of *dlc* or *dld* function inhibited or delayed the initial expression of *rho* at 36-hpf (Figure 2.10B & C). This suggests that Dlc and Dld allow for the initial differentiation of rod cells. Previous studies on the frog retinal development observed that the misexpression of *x-dll1* in progenitor cells promoted rod differentiation (Dorsky et al., 1997; Dorsky et al., 1995). The observation seen on this zebrafish study further supports the model in which rod fate is promoted by the presence of Delta in progenitor cells.

2.4.4 Dlc- and Dld- Notch signaling deter rod differentiation at 55-hpf

In this study, *rho* expression at 55-hpf in WT was detected in the ventral region of the retina (Figure 2.10D). In *dlc* \(^{b663}\) and *dld* \(^{g249}\) retinas, *rho* expression was also detected in the ventral region but additionally in the central region of the retina (Figure 2.10E & F). Moreover, the expression of *crx* (a transcription factor required for rod and cone specification) and *nrl* (a transcription factor required for rod development) were not decreased at 55-hpf in mutant retinas compared to WT (Figure 2.18A-F). These data suggest that Dlc and Dld at 55-hpf deters rod differentiation in the central region of the retina. This statement is supported by previous studies in the mouse retinal development, where the removal of Notch1 in progenitor or postmitotic cells caused the overproduction of rod cells (Jadhav et al., 2006b; Mizeracka et al., 2013a). Considering that Delta activates Notch signaling, the results obtained in this study may indicate that Dlc and Dld deters rod development via Notch signaling in progenitor and/or postmitotic cells.

2.4.5 The role of Dlc and Dld in rod patterning at 5-dpf

At 5-dpf, *rho* expression was observed in the ventral, central and dorsal regions of the retina in WT embryos (Figure 2.10G). However, in *dlc* \(^{b633}\) and *dld* \(^{g249}\) embryos, *rho*
expression was reduced in the medial region of the retinas (Figure 2.10H & I). Transcript expression levels of *rho* in mutant eyes were not significantly different compared to WT (Tables 2.7 & 2.8). Moreover, the expression of *crx* was not decreased at 5-dpf in mutant retinas compared to WT (Figure 2.19A-C). These data suggest that Dlc and Dld affect the patterning of rod cells. A similar result was observed in the mouse, the removal of Dll1 (ortholog of zebrafish Dla and Dld) disrupted photoreceptor patterning (Rocha et al., 2009). However, the removal of Dll3 in the mouse (ortholog of zebrafish Dlb and Dlc) did not affect photoreceptor development (Nelson et al., 2009). To confirm the role of Dlc and Dld on rod patterning, further studies would be required (including the immunolocalization of rhodopsin).

### 2.4.6 Dld inhibits initial ventral differentiation of blue cones at 36-hpf

There was no expression difference on *red* or *green opsin* at 36-hpf (data not shown) or 52-hpf (Figure 2.17) between WT and mutant retinas, respectively. At 36-hpf, *blue opsin* expression is absent in the retinal neuroepithelium in WT retinas; however, in *dld*<sup>tg249</sup> retinas, *blue opsin* was localized in the retinal neuroepithelium (Figure 2.15). These results suggest that Dld functions to inhibit blue cone development at early stages of retinal development.

### 2.4.7 Dlc hinders the timing of blue cone development while Dld hinders the timing of blue and red cone development at 55-hpf

At 55-hpf, *red opsin* expression in WT retinas had two distinct patterns: (1) *red opsin* in the ventral region of the retina, and (2) *red opsin* in the ventral and central regions of the retina (Figure 2.16A & D). In *dlc*<sup>bg249</sup> retinas, pattern one for *red opsin* was different from WT. The expression of *red opsin* was expanded to the medial region of the retina in *dlc*<sup>bg249</sup> embryos (Figure 2.16C). These data showed that *dlc*<sup>bg249</sup> retinas had accelerated red cone differentiation; thus, suggesting that Dld partially prevents red cone differentiation in the medial region of the retina at 55-hpf. This was also the case for the expression of *blue opsin* at 55-hpf. The *blue opsin* expression was increased in the medial region of the retinas of *dlc*<sup>bg249</sup>, but also in *dlc*<sup>b663</sup> retinas (Figure 2.14D & F). This overall suggests that Dld delays the timing of blue and red cone differentiation whereas Dlc delays the timing of red cone differentiation. In the mouse, the removal of Dll1 (ortholog
of zebrafish Dla and Dld) accelerated neurogenesis, suggesting that DLL inhibits neurogenesis (Rocha et al., 2009). In addition, the inhibition of cone differentiation was observed in other studies, where the inhibition of Notch activity caused the overproduction of cone differentiation (Jadhav et al., 2006b; Luo et al., 2012; Nelson et al., 2007; Yaron et al., 2006).

2.4.8 At 5-dpf, Dlc is required for proper blue cone patterning while Dld is required for constraining red and green cone differentiation

At 5-dpf, the expression of red and green opsin was significantly overexpressed in dld<sup>b249</sup> retinas compared to WT (Table 2.8). This indicates that Dld constrains red and green cone differentiation at 5-dpf. In the case of dlc<sup>b663</sup> retinas, blue opsin patterning was disrupted at 5-dpf (Figure 2.13). Transcript levels of blue opsin in dlc<sup>b663</sup> eyes were not different compared to WT (Table 2.7). The total mean number of blue opsin cells in dlc<sup>b663</sup> (0.0783 ± 0.00929 μm<sup>2</sup>) was significantly higher than WT (0.0712 ± 0.0130 μm<sup>2</sup>) (p < 0.0001) (Table 2.9). These data indicate that the loss of dlc misexpressed blue opsin at 5-dpf, suggesting that Dlc inhibits blue cone fate and/or is required for proper patterning of blue cones.

2.4.9 Dlc and Dld signaling is upstream of nr2e3

Mutant retinas had downregulation of nr2e3 compared to WT at 55-hpf (Figure 2.18G-L) and 5-dpf (Figure 2.19D-I). Nr2e3 is a transcription factor required to suppress cone opsin and allow rod differentiation to occur (Chen et al., 2005). As a result, the overexpression of cone opsin in the mutant retinas could be due to the decreased expression of nr2e3. Thus, Dlc- and Dld- signaling promotes the expression of nr2e3 which inhibit cone opsin. However, how Dlc and Dld mediate differential effects on nr2e3 and photoreceptor differentiation is not understood, but a possible explanation can lie within their downstream effectors of the Delta-Notch pathway.

2.4.10 Conclusion

In zebrafish, there are five photoreceptor cell types. However, the mechanisms that permit the diverse photoreceptor population are not well understood. This study showed that Dlc and Dld have similar functions on rod development, but have different functions on cone development. In addition, this study showed that Dlc and Dld function
upstream of *nr2e3* to inhibit photoreceptor differentiation. Dlc and Dld are thus part of the mechanism that regulates and allows for the diversity of photoreceptor development.
2.5. References
Austin, C. P., et al., 1995. Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. Development. 121, 3637-50.


Peng, G. H., et al., 2005. The photoreceptor-specific nuclear receptor Nr2e3 interacts with Crx and exerts opposing effects on the transcription of rod versus cone genes. Human molecular genetics. 14, 747-64.


CHAPTER 3. IMMEDIATE DOWNSTREAM TARGETS BY WHICH DELTAC AND DELTAD SIGNAL DURING RETINAL DEVELOPMENT

3.1. Introduction

DeltaC and DeltaD have similar and different effects on retinal development (CHAPTER TWO). The methods by which DeltaC and DeltaD exerts their effects on retinal development can be mediated through Her/Hes proteins, immediate downstream targets of the Delta-Notch pathway. In this chapter, the transcription of the downstream effectors of the Delta-Notch pathway in \( dlc^{b663} \) and \( dld^{tg249} \) mutants was analyzed.

3.1.1 Basic helix-loop-helix (bHLH)

During retinal development, there are three successive steps that occur: (1) the proliferation of progenitor cells, (2) neurogenesis, and (3) gliogenesis. The progression of the steps is under the control of extrinsic and intrinsic regulators. Multiple basic helix-loop-helix (bHLH) proteins are intrinsic regulators shown to control retinal development (Harris, 1997; Livesey and Cepko, 2001; Marquardt and Gruss, 2002). There are two functional distinct groups of bHLH genes, activators and repressors. bHLH activators like \( mash1 \) (homolog of Drosophila proneural gene \( acheate-scute \)) promote neuronal and glial differentiation, while bHLH repressors like \( hes1 \) (homolog of Drosophila \( hairy/Enhancer-of-slit-related \)) inhibit bHLH activators to negatively regulate neuronal differentiation (Hatakeyama and Kageyama, 2004).

Upon Delta ligand stimulation, the intracellular domain of Notch (NICD) is cleaved off from the transmembrane region and translocates into the nucleus. Once in the nucleus, the NICD will form a complex with the DNA-binding protein RBPJ and upregulate the transcription of \( her/hes \) (Artavanis-Tsakonas et al., 1999; Honjo, 1996). As a result, the Delta-Notch signaling, via the immediate downstream targets (Her/Hes), inhibits neural differentiation and promotes maintenance of proliferating progenitor cells.
3.1.2 The contribution of hairy/Enhancer-of-slit-related family in retinal development

There are several Her/Hes proteins; in this chapter, the focus is on a set of zebrafish Her/Hes proteins in which their known orthologs in the mice affect retinal development (Tables 3.1 & 3.2). In the mice, Hes5 is initially detected in retinal progenitor cells and is progressively restricted to differentiated Müller glia cells during retinal development (Hojo et al., 2000; Nelson et al., 2006). The removal of Hes5 in the mice decreased the population of Müller glia cells; as a result, Hes5 contributes to Müller glia cell differentiation (Hojo et al., 2000).

In the mice, Hes1 also promotes Müller glia cell differentiation and maintains retinal cells proliferating; additionally, Hes1 inhibits neuronal differentiation (Furukawa et al., 2000; Kageyama et al., 1997; Takatsuka et al., 2004; Tomita et al., 1996). Hes1 is present in retinal progenitor cells. The inactivation of Hes1 increases ganglion, rod and horizontal cell populations, while it decreases the Müller glia cell population (Furukawa et al., 2000; Nelson et al., 2006; Takatsuka et al., 2004; Tomita et al., 1996).

Hes-related bHLH gene, hey1, is expressed in retinal progenitor cells (Furukawa et al., 2000; Satow et al., 2001). In mice, the constitutive activation of Notch in mitotic cells increased hey1 and hes1. Consequently, it increased the Müller glia cell population and decreased the photoreceptor cell population (Jadhav et al., 2006a). However, the removal of Notch reduced hey1 and not hes1, causing an increase in the photoreceptor cell population (Jadhav et al., 2006b). These studies concluded that Hey1 inhibits photoreceptor development.

Another member of the Her/Hes family is Hes6; however, this protein promotes neural differentiation by suppressing Hes1 activity (Bae et al., 2000; Koyano-Nakagawa et al., 2000). The misexpression of hes6 promotes neural differentiation, specifically rod differentiation.
Table 3.1. Zebrafish Her/Hes orthologs. (Bae et al., 2000; Bernardos et al., 2005; Gajewski et al., 2006; Jouve et al., 2000; Koyano-Nakagawa et al., 2000; Sieger et al., 2004)

<table>
<thead>
<tr>
<th>Hairy/Enhancer-of-split-related proteins</th>
<th>Zebrafish</th>
<th>Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hairy-related 2</td>
<td>Her2</td>
<td>Hes5</td>
</tr>
<tr>
<td>Hairy-related 4, tandem duplicate 2</td>
<td>Her4.2</td>
<td>Hes5</td>
</tr>
<tr>
<td>Hairy-related 6</td>
<td>Her6</td>
<td>Hes1</td>
</tr>
<tr>
<td>Hes family bHLH transcription factor 6</td>
<td>Her13.2</td>
<td>Hes6</td>
</tr>
<tr>
<td>Hes-related family bHLH transcription factor with YRPW motif 1</td>
<td>Hey1</td>
<td>Hey1</td>
</tr>
</tbody>
</table>

Table 3.2. Percent identity of zebrafish (z) and mice (m) Her/Hes orthologs. Identity percentages were based on BLASTP analysis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>z-Her2</th>
<th>z-Her4.2</th>
<th>z-Her6</th>
<th>z-Her13.2</th>
<th>z-Hey1</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-Hes1</td>
<td>46%</td>
<td>42%</td>
<td><strong>72%</strong></td>
<td>38%</td>
<td>43%</td>
</tr>
<tr>
<td>m-Hes5</td>
<td><strong>55%</strong></td>
<td><strong>51%</strong></td>
<td>39%</td>
<td>34%</td>
<td>33%</td>
</tr>
<tr>
<td>m-Hes6</td>
<td>35%</td>
<td>37%</td>
<td>37%</td>
<td><strong>48%</strong></td>
<td>35%</td>
</tr>
<tr>
<td>m-Hey1</td>
<td>40%</td>
<td>32%</td>
<td>37%</td>
<td>34%</td>
<td><strong>68%</strong></td>
</tr>
</tbody>
</table>

3.1.3 Through which immediate downstream targets do Dlc and Dld ligands signal?

The objective of this chapter is to define the immediate downstream genetic circuit, which DeltaC and DeltaD signal. Based on the similar and distinct effects of *dlc* and *dld* on retinal development (*CHAPTER TWO*), I hypothesized that *dlc* and *dld* would signal through similar and distinct immediate downstream factors. The effect on *her/hes* in *dlc* and *dld* embryos during retinal development was evaluated by whole-mount *in situ* hybridization.
3.2. Material and Methods

3.2.1 Fish maintenance and embryo collection

Zebrafish were maintained according to standard procedure (Westerfield, 2000). Homozygous mutant fish \(dlc^{663}\) and \(dlf^{9249}\), as well as wild-type (WT) AB fish, were used. Parental fishes were bred for 15 minutes to ensure all embryos were at a similar developmental stage during collection. Embryos were maintained in E3 medium (Westerfield, 2000) at 28°C. The developmental stage of the zebrafish were determined by previous studies (Kimmel et al., 1995). The embryos were treated with 0.003% phenylthiourea (PTU) (Sigma-Aldrich, St. Louis, MO) in E3 medium, between 12- and 23-hpf to prevent melanization for \textit{in situ} hybridization experiments (Li et al., 2012; Nusslein-Volhard and Dahm, 2002). Embryos were fixed overnight, in 4% PFA (Sigma-Aldrich, St. Louis, MO) in 1x PBS (0.137M NaCl, 0.0027M KCl, 0.010M \(\text{Na}_2\text{HPO}_4\), 0.0018M \(\text{KH}_2\text{PO}_4\)) (IBI Scientific, Peosta, IA and Avantor Performance Materials, Phillipsburg, NJ), at 4°C. After fixation, the samples for \textit{in situ} hybridization were dehydrated and stored in 100% methanol (VWR International, West Chester, PA) at -20°C. The protocols were approved by the Purdue Animal Care and Use Committee.

3.2.2 Whole-mount \textit{in situ} hybridization

The preparation of the riboprobes as well as the whole-mount \textit{in situ} hybridization were performed as described (Hensley et al., 2011). The riboprobes used in this study were \(\text{her}2\), \(\text{her}4.2\), \(\text{her}6\), \(\text{her}13.2\) and \(\text{hey}1\). The primers used to make the riboprobes are listed in Table 3.3. A minimum of ten embryos was analyzed for the three genotypes, at each developmental stage throughout the study. The samples used for the characterization of the same gene were processed and stained for the same period of time to maximize comparability between conditions. Samples were destained by a 2:1 mixture of benzyl benzoate (Alfa Aesar, Lancashire, United Kingdom)-benzyl alcohol (Alfa Aesar, Ward Hill, MA), and stored in 70% glycerol (Sigma-Aldrich, St. Louis, MO)/ PBS at 4°C.
Table 3.3. Primers used to riboprobes synthesis of her riboprobes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primers: 5’-3’</th>
<th>Reverse Primers: 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>her2</td>
<td>CCTTCTCTTTCCAGCTGAGG</td>
<td>GCGCGTGAAGTAAAGCAATA</td>
</tr>
<tr>
<td>her4.2</td>
<td>TGGATCAATCAGCAGCAGAG</td>
<td>TTCAGTCCATGCCAATCTCA</td>
</tr>
<tr>
<td>her6</td>
<td>AGAGAAGATGCCTGCGGATA</td>
<td>TTGAACCATGGGTGACTGA</td>
</tr>
<tr>
<td>hey1</td>
<td>GAATTCTCCACTCGGGGTCAA</td>
<td>TTTGAGGATGGAGGACTGCT</td>
</tr>
<tr>
<td>her13.2</td>
<td>TCACGACGAGGATAATTACGG</td>
<td>CTGTGTCGTCAGGTCAAGAA</td>
</tr>
</tbody>
</table>

3.2.3 Image acquisition and analysis

The embryos were mounted in 3% methylcellulose (MP Biomedical LLC, Illkirch, France) on a depression slide, for observation and imaging. All images were acquired by a SPOT-RT3 color slider camera (Diagnostic Instruments, Sterling Heights, MA) mounted on an Olympus SZX16 stereomicroscope.

3.3. Results

3.3.1 Expression of her2, hey1 and her13.2 at 1-day post fertilization

By 1-day post fertilization (dpf), the optic cups are formed and the newly formed lenses are detached from the ectoderm (Fadool and Dowling, 2008; Schmitt and Dowling, 1994). At this stage of development, her2 and hey1 were in the lens (Figure 3.1A & G). The expression of her4.2 was not found in the eye (Figure 3.1D), while her13.2 was found in the presumptive neural basal retina (Figure 3.1J). Expression locations of these genes were not changed in dlc^{663} and dld^{8249} eyes (Figure 3.1).
Figure 3.1. The transcript expression of her2, her4.2, hey1 and her13.2 in 1-dpf WT, dlc\textsuperscript{b663} and dld\textsuperscript{g249} embryos. \textit{In situ} hybridization of her2 (A-C), her4.2 (D-F), hey1 (G-I) and her13.2 (J-L) on 1-dpf WT, dlc\textsuperscript{b663} and dld\textsuperscript{g249} embryos. Ventral views of whole-mount embryos are shown; dorsal is to the top. Black arrows indicate expression of a gene in the retinal neuroepithelium. Red arrows indicate expression of a gene in the lens.

3.3.2 Expression of her4.2, hey1 and her13.2 at 2-day post fertilization

At 2-dpf, the progenitor cells in the neuroepithelium have exited the cell cycle; these cells will form the ganglion cell layer (GCL), inner nuclear layer (INL) and the outer nuclear layer (ONL) (Hu and Easter, 1999). At this stage of development her2 is not expressed in the WT, dlc\textsuperscript{b663} and dld\textsuperscript{g249} eyes (Figure 3.2A-C). In WT eyes, her4.2 is in the ciliary marginal zone (CMZ) and in the temporal-ventral region of the retina (Figure 3.2D, H & H’). In dlc\textsuperscript{b663} embryos, there were two expression pattern groups for her4.2. In 11 of the 18 embryos, the expression of her4.2 did not change compared to WT embryos (Figure 3.2E, I & I’). In seven of the 18 dlc\textsuperscript{b663} embryos, there was an upregulation of her4.2. The expression of her4.2 was present in the apical side of the
retina (Figure 3.2F, J & J’). There was no drastic change of her4.2 in dld^g249 eyes (Figure 3.2G, K & K’). hey1 was found in the retinas of WT embryos (Figure 3.2L); however, in dlc^b663 and dld^g249 eyes, hey1 was reduced in the retina (Figure 3.2M-P). In dlc^b663 and dld^g249 eyes, hey1 was in the CMZ, which was not the case in WT eyes (Figure 3.2M-P).

The expression of her13.2 in WT eyes was detected throughout the retina and CMZ (Figure 3.2Q). However, her13.2 in the retina was drastically decreased in dlc^b663 and dld^g249 retinas (Figure 3.2R-T). These data suggest that the loss of function of (1) dlc inhibits the expression of her4.2 in the retina, (2) dlc and dld inhibits hey1 in the CMZ, (3) dlc and dld upregulates hey1 in the retina, and (4) dlc and dld upregulates her13.2 in the retina (summarized in Figures 3.5 & 3.6).
Figure 3.2. The expression patterns of her2, her4.2, hey1 and her13.2 in 2-dpf WT, dlc^b663 and dld^b249 embryos. *In situ* hybridization of her2 (A-C), her4.2 (D-K), hey1 (L-P) and her13.2 (Q-T) on 2-dpf WT, dlc^b663 and dld^b249 embryos. The ventral view of whole-mount embryos are shown; nasal is to the top. For her4.2, eyes were dissected and images of the lateral (H-K) and ventral (H'-K') views of the eyes are shown. In the lateral view of dissected eyes, nasal (n) is to the left and dorsal (d) is up. In ventral views of dissected eyes, nasal (n) is to the left. Black and yellow arrows indicate expression of a gene in the retinal neuroepithelium. Red arrows indicate expression of the gene in the ciliary marginal zone (CMZ).

3.3.3 Expression of her4.2, hey1 and her13.2 at 3-day post fertilization

At 3-dpf, the neural retina and the Müller glial cells are differentiating (Hu and Easter, 1999; Schmitt and Dowling, 1999). At this developmental stage, her4.2 was observed in the CMZ (Figure 3A). In dlc^b663 eyes, her4.2 was downregulated in the CMZ.
(Figure 3.3B) but five of the 38 dlc\textsuperscript{b663} embryos had her4.2 expression in the retina (Figure 3.3C). In dld\textsuperscript{g8249} eyes, her4.2 was not changed compared to WT (Figure 3.3D). hey1 was in the CMZ in WT and dlc\textsuperscript{b663} eyes (Figure 3.3E & F). Eyes from dld\textsuperscript{g8249} embryos had hey1 in the CMZ and also in the retina (Figure 3.3G). In WT, dlc\textsuperscript{b663} and dld\textsuperscript{g8249} eyes, her13.2 was present in the CMZ (Figure 3.3H, I & K). However, there were 12 of 35 dlc\textsuperscript{b663} embryos that had her13.2 in the CMZ and retina (Figure 3.3J). These data suggest that the loss of function of (1) dlc inhibits her4.2 in the retina and upregulates it in the CMZ, (2) dld inhibits hey1 in the retina, and (3) dlc inhibits her13.2 in the retina (summarized in Figures 3.5 & 3.6).

Figure 3.3. The expression patterns of her4.2, hey1 and her13.2 in 3-dpf WT, dlc\textsuperscript{b663} and dld\textsuperscript{g8249} embryos. In situ hybridization of her4.2 (A-D), hey1 (E-G) and her13.2 (H-K) on 3-dpf WT, dlc\textsuperscript{b663} and dld\textsuperscript{g8249} embryos. Ventral view of whole-mount embryos are shown; nasal is to the top. Blue arrows indicate expression of a gene in the retinal neuroepithelium. Red arrows indicate expression of a gene in the ciliary marginal zone (CMZ).
3.3.4 Expression of her6 at 36-hours post fertilization

Signal of her6 was not detected at 24-, 30-, 42-, 45-, 48- or 50- hpf in WT or mutant eyes (data not shown). However, her6 was detected at 36-hpf in the CMZ and in the optic nerve (Figure 3.4D). At this developmental stage, the closure of the choroid fissure and the development of ganglion and photoreceptor cells have commenced (Schmitt and Dowling, 1994; Schmitt and Dowling, 1996). In dlc<sup>b663</sup> eyes, her6 is in the CMZ, optic nerve, and retina (Figure 3.4E). In dld<sup>g249</sup> eyes, her6 is restricted to the optic nerve (Figure 3.4F). Thus, these data suggest that the presence of dlc inhibit her6 in the retina while dld upregulates her6 in the CMZ (Figure 3.6).

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**Figure 3.4. The expression of her6 in 36-hpf WT, dlc<sup>b663</sup> and dld<sup>g249</sup> embryos.** *In situ* hybridization of her6 on 36-hpf WT, dlc<sup>b663</sup> and dld<sup>g249</sup> embryos. The lateral view of whole-mount embryos are shown; nasal is to the left and dorsal is to the top (A-C). Transverse cryosections are shown (D-F); lateral is to the left and dorsal is up.
3.4. Discussion

In this chapter, the expression patterns of *her2, her4.2, her6, her13.2* and *hey1* in WT, *dlc*<sup>b663</sup> and *dld*<sup>tg249</sup> embryos were shown during retinal development. At 1-dpf, *her2* and *hey1* was detected in the developing lens, while *her13.2* was detected in the retina. The expression of these genes in 1-dpf mutant embryos was not altered in the mutants’ eyes (Figure 3.1). The expression of *her6* was detected in the eye at 36-hpf; loss of *dlc* function increased *her6* in the retina, whereas the loss of *dld* function decreased *her6* in the CMZ (Figure 3.4). The summary of the expression patterns of *her2, her4.2, her13.2* and *hey1* are shown in Figure 3.5. Overall, in *dlc*<sup>b663</sup> eyes, the expression of *her4.2, her6, her13.2* and *hey1* were changed compared to WT. In the *dld*<sup>tg249</sup> eyes, the expression of *her6, her13.2* and *hey1* was altered compared to WT. The interpretations of these results are summarized in Figure 3.6.
Figure 3.5. Expression pattern summary of *her2*, *her4.2*, *her13.2* and *hey1* of WT, *dlc*<sup>b663</sup> and *dll*<sup>b249</sup> eyes. The downstream targets were found in three regions of the eye; lens, ciliary marginal zone (CMZ) and retina. White boxes represent the positive signal of a gene with no change in level compared to WT. Red boxes represent upregulation of a gene and green boxes represent downregulation of a gene, compared to WT.
Figure 3.6. Possible regulatory mechanisms on her4.2, her6, her13.2 and hey1 from dlc and dld signaling during zebrafish retinal development.

3.4.1 her6 is downregulated by Dlc and upregulated by Dld in 36-hpf embryos

At 36-hpf, her6/hes1 was detected in cells that were located in the optic nerve and CMZ in WT embryos (Figure 3.4D). The signal of her6/hes1 in zebrafish is different compared to mouse and chicken, where her6/hes1 is found in the retina and CMZ (Nelson et al., 2006; Nelson et al., 2009; Nelson and Reh, 2008).
In \textit{dlc}\textsuperscript{b663} and \textit{dld}\textsuperscript{tg249} embryos, there were no changes in the signal of \textit{her6/hes1} in the optic nerve; suggesting that \textit{dlc} and \textit{dld} do not regulate \textit{her6/hes1} in the optic nerve. However, the loss of \textit{dlc} function increased \textit{her6/hes1} in the retina (Figure 3.4E), suggesting that \textit{Dlc} inhibits transcription of \textit{her6/hes1} (Figure 3.6). The loss of \textit{dld} function decreased \textit{her6/hes1} in the CMZ (Figure 3.4F), suggesting that \textit{Dld} promotes transcription of \textit{her6/hes1} (Figure 3.6). These observations showed that \textit{Dlc} and \textit{Dld} pathways have different effects on \textit{her6/hes1}.

In the mouse, the inactivation of m-Notch1 causes downregulation of \textit{her6/nes1} in the peripheral retina (Mizeracka et al., 2013b; Yaron et al., 2006). This suggests that Notch signaling upregulated \textit{her6/nes1}. In this chapter it was shown that \textit{Dld} (not \textit{Dlc}) promoted the transcription of \textit{her6/nes1}. Thus, we can conclude that \textit{Dld} could signal via Notch1 to upregulate the transcription of \textit{her6/nes1}. A possible reason why \textit{Dlc} did not promote the transcription of \textit{her6/nes1} might be due to the inability of \textit{Dlc} to directly activate Notch signaling. This observation was reported in m-Dll3 (an ortholog of zebrafish Dlc) (Ladi et al., 2005). It was observed that m-Dll3/Dlc did not directly interact with Notch but instead affected other delta ligands’ localization and activity within the same cell (Ladi et al., 2005; Wright et al., 2011). Thus, m-Dll3/Dlc indirectly affects the Notch pathway.

### 3.4.2 The expression dynamics of \textit{hey1}, \textit{her4.2} and \textit{her13.2} in 2-dpf WT, \textit{dlc}\textsuperscript{b663} and \textit{dld}\textsuperscript{tg249} eyes

At 2-dpf, the expression of \textit{hey1} was detected in the retina of WT embryos whereas it was decreased in \textit{dlc}\textsuperscript{b663} and \textit{dld}\textsuperscript{tg249} retinas, suggesting that \textit{Dlc} and \textit{Dld} in the retina promotes transcription of \textit{hey1} (Figure 3.2L-P) (Figure 3.5). In the \textit{dlc}\textsuperscript{b663} and \textit{dld}\textsuperscript{tg249} embryos the \textit{hey1} signals were present in the CMZ, while WT was not. These results suggest that both \textit{Dlc} and \textit{Dld} are needed to inhibit \textit{hey1} in the CMZ (Figure 3.6). Previous studies on the mouse showed that inactivation of Notch1 decreased \textit{hey1} in the eye (Jadhav et al., 2006b); thus, these studies suggest that \textit{hey1} is regulated upstream of Notch via \textit{Dlc} and \textit{Dld} within the retina and CMZ.

At 2-dpf, the expression of \textit{her4.2/nes5} was detected in the retina and CMZ of WT, \textit{dlc}\textsuperscript{b663} and \textit{dld}\textsuperscript{tg249} embryos (Figure 3.2H’-K’). In the mouse and the chicken,
her4.2/ges5 was exclusively present in the retina, which was not the case in the zebrafish (Nelson et al., 2006; Nelson et al., 2009; Nelson and Reh, 2008). her4.2/ges5 in the retina was increased in dlc<sup>b663</sup> embryos compared to WT. These results suggest that Dlc inhibits her4.2/ges5 in the retina, and her4.2/ges5 in the CMZ is independent of Dlc or Dld. The ability for Dlc to inhibit her4.2/ges5 was also observed during mouse somitogenesis. The deletion of <i>m-dll3</i> (an ortholog of zebrafish Dlc) decreased her4.2/ges5 and her6/ges1 in the presomitic mesoderm, which was also the case in zebrafish retinal development (Sewell et al., 2009).

At 2-dpf, her13.2/ges6 was detected in the retina and CMZ of WT, dlc<sup>b663</sup> and dld<sup>tg249</sup> embryos (Figure 3.2Q-T). However, her13.2/ges6 in mutant retinas was decreased. Thus, these observations suggest that Dlc and Dld promote the transcription of her13.2/ges6 in the retina. In other previous studies done on mice, <i>m-dll1</i> (an ortholog of zebrafish dld), <i>m-dll3</i> (an ortholog of zebrafish dlc) and her13.2/ges6 were decreased in various Delta-Notch mutants (Nelson et al., 2009; Rocha et al., 2009). Our observations link Dlc- and Dld-mediated Notch signaling to the transcription of her13.2/ges6.

3.4.3 The expression dynamics of <i>hey1</i>, her4.2 and her13.2 in 3-dpf WT, dlc<sup>b663</sup> and dld<sup>tg249</sup> eyes

At 3-dpf, hey1, her4.2/ges5 and her13.2/ges6 in WT eyes were detected in the CMZ (Figure 3.3). The expression of hey1 and her13.2/ges6 in the CMZ of mutant eyes were not altered; however, hey1 in dld<sup>tg249</sup> (Figure 3.3G) and her13.2/ges6 in dlc<sup>b663</sup> retinas were upregulated (Figure 3.3J). In addition, her4.2/ges5 in dlc<sup>b663</sup> embryos was altered in the CMZ and retina. In 33 embryos (from a total of 38) there was a decrease of her4.2/ges5 in the CMZ, whereas eight embryos had an increase of her4.2/ges5 in the retinas. Overall, these results suggest that Dlc inhibits transcription of her13.2/ges6 in the retina and promotes the transcription of her4.2/ges5 in the CMZ. Moreover, these results suggest that Dld inhibits the transcription of hey1 in the retina.

In conclusion, this chapter demonstrated that Dlc- and Dld-signaling differently regulate spatial and temporal aspects of her/ges during zebrafish retinal development. These findings can lead to further identification of roles and mechanisms that Dlc and Dld have on retinal development.
3.5. References


CHAPTER 4. THE ALTERED VISUAL BEHAVIOR OF DLC\textsuperscript{B663} AND DLD\textsuperscript{TG249} EMBRYOS

4.1. Introduction

Vision depends on retinal cells to transform light stimuli into electrochemical nerve impulses. Zebrafish embryos exhibit several visual-mediated behaviors that can be easily analyzed, making the zebrafish a good research model to assess vision and screen for visual system defects (Brockerhoff et al., 1995). In this chapter, the visual behavior of \textit{dlc}\textsuperscript{B663} and \textit{dld}\textsuperscript{TG249} embryos was evaluated by two visual behavior assays: the optokinetic response (OKR) and visual-motor response (VMR).

4.1.1 Photoreceptor cells and visual behavior

Visual defects can be evaluated in young zebrafish since their visual behaviors develop rapidly. For instance, the first visual behavior response is detectable in 3-day post fertilization (dpf) embryos (Easter and Nicola, 1996). In addition, the zebrafish visual system is fully functional by 5-dpf (Biehlmaier et al., 2003). As a result, as young as 3-dpf zebrafish can be use to evaluate their visual behavior.

The visual system is initiated by light stimulation in the outer retina via photoreceptor cells. Furthermore, proper formation of vision depends on accurate photoreceptor development. During photoreceptor development, photoreceptor precursor cells develop into rod or cone cells (Hu and Easter, 1999). Rod and cone cells differ by their physical shape and their ability to respond to different light conditions. For example, cone cells function under bright light and are important for color vision, while rod cells function under dim light (Dowling, 1987). In zebrafish rod and cone cells have different wavelength of maximum absorbance (Table 4.1). Moreover, cone cells themselves differ in the type of photopigment they exhibit. There are four types of cone cells that respond to different wavelengths of light (Table 4.1) (Allison et al., 2004; Robinson et al., 1993). Photoreceptor cells can respond to different wavelength of light because each type
contains a distinct photopigment. The rod cells contain a single photopigment, rhodopsin. The different types of photopigments for cone cells are: (1) long-wavelength-sensitive pigment (red opsin), (2) middle-wavelength-sensitive pigment (green opsin), (3) short-wavelength-sensitive pigment (blue opsin), and (4) ultraviolet-sensitive pigment (uv opsin) (Shen and Raymond, 2004).

Table 4.1. Mean wavelength of maximum absorbance for zebrafish photoreceptors.
The mean maximum absorbance values ($\lambda_{\text{max}}$) and standard deviations (SD) in nanometers (nm) are listed for each photoreceptor type found in zebrafish (Allison et al., 2004; Robinson et al., 1993).

<table>
<thead>
<tr>
<th>Photoreceptor type</th>
<th>Mean $\lambda_{\text{max}}$ in nm ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rods</td>
<td>503 ± 5</td>
</tr>
<tr>
<td>Red cones</td>
<td>566 ± 10</td>
</tr>
<tr>
<td>Green cones</td>
<td>482 ± 6</td>
</tr>
<tr>
<td>Blue cones</td>
<td>411 ± 5</td>
</tr>
<tr>
<td>Uv cones</td>
<td>361 ± 3</td>
</tr>
</tbody>
</table>

The specific wavelength absorbance values for each type of photoreceptor can be used as a parameter during visual behavior assays to elicit a specific photoreceptor response (Krauss and Neumeyer, 2003). Another parameter that can be used to specifically stimulate rod or cone cells is light intensity. Exclusively, zebrafish rod cells are active in dim light, they respond to light intensity range of $5.3 \times 10^{-2}$ - 0.053 $\mu$W/cm$^2$, whereas at higher levels of light rod cells are saturated and inactivated (Moyano et al., 2013). In contrast, cone cells are activated in bright light; they have a higher light intensity threshold than rod cells because they are less sensitive to light (Chen et al., 2007). These two parameters, wavelength absorbance and light intensity, can be manipulated to induce specific photoreceptor responses during OKR and VMR assays.

4.1.2 Optokinetic response (OKR)

The optokinetic response (OKR) is a behavior exhibited by most vertebrates in which the eyes move in response to movement in their external environment (Brockerhoff et al., 1995; Easter and Nicola, 1996). The OKR is elicited by moving
vertical stripes through the embryos visual field. An OKR is indicative that the retina can transduce visual information to the brain in order to elicit eye movements. This assay is robust and has been utilized in several studies for mutational screening of visual system genes (Brockerhoff et al., 1998; Brockerhoff et al., 1995; Brockerhoff et al., 1997; Neuhauss et al., 1999). In zebrafish, OKR is detectable after 3-dpf and continues throughout adulthood (Easter and Nicola, 1996), which makes it a feasible assay for this animal model.

4.1.3 Visual-motor response (VMR)

The visual startle response is an escape mechanism that can be triggered by potential predators (Easter and Nicola, 1996; Kimmel et al., 1974). In zebrafish, the startle response can be invoked by light changes in the environment causing the embryos to have brief spikes of motor activity. Thus, the locomotor activity indicates the ability of zebrafish to perceive light changes in their environment. Zebrafish are receptive to light as early as 3-dpf, allowing them to produce a startle response (Emran et al., 2008). The visual motor response (VMR) assay allows the quantification of the locomotor activity from zebrafish embryos (Emran et al., 2007; Emran et al., 2008). The VMR assay has been used recently to demonstrate effects of drugs on the visual system (Deeti et al., 2014; Rihel et al., 2010).

4.1.4 What are the visual behavior defects in the Delta mutant embryos?

The visual system depends on proper retina formation. In CHAPTER TWO, I identified photoreceptor development defects in dlc<sup>b663</sup> and dld<sup>g8249</sup> mutant embryos at 5-dpf. Consequently, the number of photoreceptor cells or the expression level of opsin may affect vision. For example, in zebrafish, behavioral visual sensitivity is associated with opsin expression. The behavior visual sensitivity of zebrafish is highest when the expression of opsin is the highest. For instance, opsin expression is highest in the afternoon (Li et al., 2005). Thus, it is essential to determine if the defects caused by the lack of deltaC (Dlc) or deltaD (Dld) can affect zebrafish vision. The objective of this chapter was to assess the visual behaviors of dlc<sup>b663</sup> and dld<sup>g8249</sup> mutant embryos at 5-dpf. I hypothesized that both dlc<sup>b663</sup> and dld<sup>g8249</sup> mutants have defects in their visual behavior, since both mutants have abnormal retinal development. Furthermore, I hypothesized that
each mutant would have different abnormal visual behaviors, given that their defects in retinal development are not the same. To test these hypotheses, I conducted the OKR and VMR assays at 5 dpf.

4.2. Materials and Methods

4.2.1. Fish maintenance and fish lines

Zebrafish were maintained according to standard procedures (Westerfield, 2000). Heterozygous fish, \textit{dlc}^{b663/+} and \textit{ddl}^{g249/+}, were utilized to collect and separate phenotypic homozygous mutant (\textit{dlc}^{b663} and \textit{ddl}^{g249}) and wild-type (WT) (\textit{dlc}^{+} and \textit{ddl}^{+}) embryos. All protocols were approved by the Purdue Animal Care and Use Committee.

4.2.2. Optokinetic response assay

The optokinetic response (OKR) of 5-dpf WT (\textit{dlc}^{+} and \textit{ddl}^{+}) and mutant (\textit{dlc}^{b663} and \textit{ddl}^{g249}) embryos was measured. Before the OKR assay, embryos were light-adapted in the apparatus for at least 1.5 hours. Embryos were partially immobilized during the test by immersing them in a 10x35 mm Petri dish containing 3% methylcellulose (MP Biomedical LLC, Illkirch, France). The Petri dish was placed in the center of a circular drum that rotates mechanically. The OKR apparatus was assembled as described in previous studies (Brockerhoff et al., 1995). A Fiber Lite M1-150 illuminator was used for the apparatus. The illuminance at the level of the Petri dish was 20,000 Lux measured by a LX1010B light meter (Mastech, Taipei, Taiwan). The internal walls of the drum contained black-and-white vertical stripes each 18 degrees wide. The drum rotation was set to 6 rounds per minute. For each embryo, the tested conditions were: 1) 15 seconds of clockwise drum rotation followed by 15 seconds of counterclockwise rotation, 2) rest phase of 30 seconds of no drum rotation, and 3) 15 seconds of clockwise drum rotation followed by 15 seconds of counterclockwise rotation. The embryo’s eye movement in response to the rotation of the drum was manually recorded every 15 seconds during the tested conditions (excluding the rest phase). A total of 90 embryos were tested for each genotype. The average eye movements per 15 seconds were calculated from the four rotations cycles for each embryo. The summation of individual eye tracking movements was divided by the total number of embryos and reported as the \textit{eye tracking movements}. 
Statistical significance of the OKR data was determined by Student’s $t$-tests. The statistical analyses were performed in R package version 2.15.3 (R.DCT, 2006).

4.2.3. Visual-motor response assay

The visual motor response (VMR) of 5-dpf WT ($dlc^+$ and $dld^+$) and mutant ($dlc^{b663}$ and $dld^{tg249}$) embryos was measured. The VMR experiments were based on a design previously reported (Emran et al., 2007; Emran et al., 2008). The VMR assays were conducted in a 96-well plate in a ZebraBox machine (ViewPoint Life Sciences, Lyon, France). Individual embryos were placed in each well with medium for zebrafish embryos (E3 medium) (Westerfield, 2000). A total of 48 embryos from each genotype ($dlc^+$, $dld^+$, $dlc^{b663}$ and $dld^{tg249}$) were analyzed in two separate experiments (24 embryos per experiment). The embryos were dark-adapted for at least two hours at room temperature in the ZebraBox machine. Following dark adaptation, the embryos were stimulated with three consecutive trials in which the light turned on and off every 30 minutes for a total of three hours. The light source of the ZebraBox was measured by an EPP2000 Spectrometer (StellarNet Inc, Tampa, Fl) at nine different locations across the surface of the light box were the 96-well plate were placed. To stimulate cones, a bright light was used (mean of total irradiance of $1.24 \times 10^4 \mu W/cm^2 \pm 0.203 \times 10^4 \mu W/cm^2$). To stimulate rods, a dim light was used (mean of total irradiance of $5.57 \times 10^2 \mu W/cm^2 \pm 0.705 \times 10^2 \mu W/cm^2$). The white light source wavelengths ranged from about 400 to 700 nm (excluding the infrared light utilized by the recording camera). The camera recorded locomotor activity by capturing videos in 30 frames per second. Activity is defined as the movement duration per second. The following parameters were utilized to detect movement per pixel: (1) detection sensitivity per pixel per image was set at six, (2) threshold was four pixels, and (3) bin size was one second. The data was processed in the Zebralab software (ViewPoint Life Sciences, Lyon, France). The activity is reported as an average of the pooled data of the three ON stimuli or OFF stimuli of the 48 embryos for each genotype ($N = 3 \times 48 = 144$). Activity was plotted against time. Each plotted graph displays 60 seconds before and 120 seconds after the light stimulus. The VMR activity, at time equal 0 seconds, was
compared between mutant and WT embryos. Also, the number of embryos that responded to the light stimuli was determined. Embryos that had zero activity were counted as non-responsive (-), while embryos that had activity were counted as responsive (+). The VMR activity of responsive (+) embryos was averaged and compared between mutant and WT embryos. Student’s $t$-tests or Welch $t$-tests were utilized to determined statistical significance of the VMR activity of overall (+/-) or responsive (+) embryos, respectively. Moreover, statistical significance of responsive (+) and non-responsive (-) between WT and mutant embryos were determined by Chi-square test with Yate’s continuity correction. Statistical analyses were performed in R package version 2.15.3 (R.DCT, 2006).

4.3. Results

The Delta-Notch mutants are known to have aberrant retinal development. Nevertheless, Delta-Notch mutants’ visual mediated behaviors have not been assessed. This study evaluated the visual behavior potential of $dlc^{b663}$ and $dld^{g249}$ embryos to establish the effect of Dlc and Dld on the visual system.

4.3.1 The OKR of $dlc^{b663}$ and $dld^{g249}$ are decreased

The OKR of 5-dpf WT ($dlc^{+}$ and $dld^{+}$) and mutant ($dlc^{b663}$ and $dld^{g249}$) embryos were quantified. All embryos were placed individually within the OKR apparatus and the average number of eye tracking movements per 15 seconds (ETMs/15s) was determined. The data distribution is visualized by a boxplot (Figure 4.1). Student’s $t$-tests were conducted to compare the average ETMs/15s for (1) $dlc^{b663}$ embryos to their WT siblings ($dlc^{+}$), and (2) $dld^{g249}$ embryos to their WT siblings ($dld^{+}$). There was a significant decreased in the average ETMs/15s of $dlc^{b663}$ (mean = 7.12, SD = 1.48) compared to $dlc^{+}$ embryos (mean = 7.76, SD = 1.64) condition; $t$ (176) = -2.71, $p = 0.007$. There was also a significant decreased in the average ETMs/15s of $dld^{g249}$ (mean = 6.32, SD = 1.35) compared to $dld^{+}$ embryos (mean = 8.69, SD= 1.57) conditions; $t$ (174) = -10.8, $p < 2.2e-16$. These results suggest that mutant embryos had abnormal OKR, specifically, when Dlc or Dld were not functional, the OKR was decreased.
Figure 4.1. Boxplot from the OKR of 5-dpf WT and mutant embryos. The data from the ETMs/15s from 5-dpf WT ($dlc^+$ and $dlld^+$) and mutant ($dlc^{b663}$ and $dlld^{g249}$) embryos is shown. For each genotype, 90 embryos were analyzed as describe in Materials and Methods. The dark horizontal lines represent the median, the boxes represent the 25th and 75th percentiles, the whiskers represent the 5th and 95th percentiles, and the outliers are represented by dots.

4.3.2. Bright light VMR: fewer $dlld^{g249}$ embryos responded during the onset of light, and their locomotor activity were decreased

The VMR of 5-dpf WT ($dlc^+$ and $dlld^+$) and mutant ($dlc^{b663}$ and $dlld^{g249}$) embryos were analyzed under bright light. At bright light ($1.24 \times 10^5 \mu W/cm^2$) rods do not activate while cones are activated (Moyano et al., 2013); thus, in this section we analyzed the VMR of cones’ output. The overall activity of WT and mutant embryos are shown in Figure 4.2. Student’s $t$-tests were conducted to compare the locomotor activity from (1)
$dlc^{b663}$ embryos to their WT siblings ($dlc^+$), and (2) $dlld^{g249}$ embryos to their WT siblings ($dlld^+$) (Tables 4.2 & 4.3). There was no significant difference between $dlc^{b663}$ and $dlc^+$ embryos (Tables 4.2 & 4.3). A significant decrease in the overall activity of the ON peak response between $dlld^{g249}$ and $dlld^+$ embryos was detected (Table 4.2). These results suggest that $dlld^{g249}$ embryos have a decreased VMR during the ON response that can be attributed to the abnormal function of cone cells.
Figure 4.2. Bright light VMR profiles of 5-dpf mutant and WT embryos. Graphs trace the overall locomotor activity of mutant (\textit{dlc}^{b633} and \textit{dd}^{g249}) (red traces) and WT (\textit{dlc}^{+} and \textit{dd}^{+}) (black traces) embryos during the VMR tests. Horizontal grey and black bars represent periods of lights ON and OFF, respectively. The ON response (A & B) and the OFF response (C & D) are shown. Arrows indicate the activity at t = 0 (time of light change). The \textit{dd}^{g249} embryos had a significantly reduced ON peak response activity upon light stimuli compared to \textit{dd}^{+} embryos (B).
Table 4.2. Student’s t-test analyses of the VMR ON peak activity of 5-dpf mutant and WT embryos. Data is from t = 0, the time at which the bright light is turned on. SD is the standard deviation.

<table>
<thead>
<tr>
<th>Overall VMR activity</th>
<th>Student’s t test</th>
<th>95% Confidence Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>N</td>
<td>Mean</td>
</tr>
<tr>
<td>dlc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>144</td>
<td>0.0292</td>
</tr>
<tr>
<td>dlc&lt;sup&gt;b663&lt;/sup&gt;</td>
<td>144</td>
<td>0.0375</td>
</tr>
<tr>
<td>dld&lt;sup&gt;+&lt;/sup&gt;</td>
<td>144</td>
<td>0.0278</td>
</tr>
<tr>
<td>dld&lt;sup&gt;g249&lt;/sup&gt;</td>
<td>144</td>
<td>0.0125</td>
</tr>
</tbody>
</table>

Table 4.3. Student’s t-test analyses of the VMR OFF peak activity of 5-dpf mutant and WT embryos. Data is from t = 0, the time at which the bright light is turned off. SD is the standard deviation.

<table>
<thead>
<tr>
<th>Overall VMR activity</th>
<th>Student’s t test</th>
<th>95% Confidence Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>N</td>
<td>Mean</td>
</tr>
<tr>
<td>dlc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>144</td>
<td>0.0708</td>
</tr>
<tr>
<td>dlc&lt;sup&gt;b663&lt;/sup&gt;</td>
<td>144</td>
<td>0.0653</td>
</tr>
<tr>
<td>dld&lt;sup&gt;+&lt;/sup&gt;</td>
<td>144</td>
<td>0.0771</td>
</tr>
<tr>
<td>dld&lt;sup&gt;g249&lt;/sup&gt;</td>
<td>144</td>
<td>0.0792</td>
</tr>
</tbody>
</table>

The overall VMR activity (reported above) is an average that includes responsive and unresponsive embryos during light changes. The overall VMR activity values can vary due to (1) a change in the ratio of responsive/unresponsive embryos, and/or (2) an increase or decrease activity of exclusively from responsive embryos.

To address the first point, we counted how many embryos had a locomotor activity and how many did not at t = 0. Person’s Chi-square tests of independence (with Yate’s continuity correction) were performed to determine differences in responsive (+) and unresponsive (-) embryos to ON- or OFF-light stimuli within each genotype (WT vs
There were no significant differences between \( dlc^{b663} \) and their WT siblings (Tables 4.4 & 4.5). The \( dld^{tg249} \) embryos compared to their WT siblings had no significant difference during the OFF stimuli (Table 4.5), but had fewer embryos that responded to the ON stimuli (Table 4.4).

**Table 4.4.** Chi-square tests with Yate’s continuity correction of the ON-response. VMR assay with bright light of 5-dpf mutant and WT embryos. (+) \( n \) is the number of embryos that responded to the light change at \( t = 0 \). (-) \( n \) is the number of the embryos that were unresponsive to the light change at \( t = 0 \).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>+ (n (%))</th>
<th>- (n (%))</th>
<th>N</th>
<th>Value</th>
<th>df</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( dlc^+ )</td>
<td>31 (21.5)</td>
<td>113 (78.5)</td>
<td>144</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( dlc^{b663} )</td>
<td>38 (26.4)</td>
<td>106 (73.6)</td>
<td>144</td>
<td>0.686</td>
<td>1</td>
<td>0.407</td>
</tr>
<tr>
<td>( dld^+ )</td>
<td>36 (25)</td>
<td>108 (75)</td>
<td>144</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( dld^{tg249} )</td>
<td>18 (12.5)</td>
<td>126 (87.5)</td>
<td>144</td>
<td>6.587</td>
<td>1</td>
<td>0.0103</td>
</tr>
</tbody>
</table>

**Table 4.5.** Chi-square tests with Yate’s continuity correction of the OFF-response. VMR assay with bright light of 5-dpf mutant and WT embryos. (+) \( n \) is the number of embryos that responded to the light change at \( t = 0 \). (-) \( n \) is the number of the embryos that were unresponsive to the light change at \( t = 0 \).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>+ (n (%))</th>
<th>- (n (%))</th>
<th>N</th>
<th>Value</th>
<th>df</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( dlc^+ )</td>
<td>75 (52.1)</td>
<td>69 (47.9)</td>
<td>144</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( dlc^{b663} )</td>
<td>68 (47.2)</td>
<td>76 (52.8)</td>
<td>144</td>
<td>0.500</td>
<td>1</td>
<td>0.479</td>
</tr>
<tr>
<td>( dld^+ )</td>
<td>80 (55.6)</td>
<td>64 (44.4)</td>
<td>144</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( dld^{tg249} )</td>
<td>75 (52.1)</td>
<td>69 (47.9)</td>
<td>144</td>
<td>0.223</td>
<td>1</td>
<td>0.636</td>
</tr>
</tbody>
</table>

The previous analyses showed that there were more unresponsive \( dld^{tg249} \) embryos during the onset of light (Table 4.4), which may have contributed to the decreased of the overall activity (Figure 4.2). Nevertheless, it is unclear if the responsive embryos have an aberrant VMR activity. To address this point, we compared the activity of responsive embryos for each of the light stimuli at \( t = 0 \). Welch \( t \)-tests were conducted to compare the locomotor activity from (1) \( dlc^{b663} \) embryos to their WT siblings (\( dlc^+ \)), and (2)
$dlfd^{249}$ embryos to their WT siblings ($dlfd^+$) (Tables 4.6 & 4.7). There were no differences in the responsive embryos activity between $dlc^{b663}$ and $dlc^{+}$ embryos. However, there was a decrease in the responsive embryos’ activity at the ON peak in $dlfd^{249}$ when compared to $dlfd^+$ embryos (Table 4.6). This suggest that responsive embryos without functional Dld, have decreased VMR in their ON peak activity.

Table 4.6. Welch $t$-test analyses of the VMR ON peak activity of responsive 5-dpf mutant and WT embryos. Data is from $t = 0$, the time at which the bright light is turned on. N is the number of responsive embryos. SD is the standard deviation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>$t$</th>
<th>df</th>
<th>$p$</th>
<th>95% Confidence Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>$dlc^{+}$</td>
<td>31</td>
<td>0.135</td>
<td>0.0661</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$dlc^{b663}$</td>
<td>38</td>
<td>0.142</td>
<td>0.0683</td>
<td>0.408</td>
<td>65.0</td>
<td>0.685</td>
<td>-0.0258, 0.0390</td>
</tr>
<tr>
<td>$dlfd^+$</td>
<td>36</td>
<td>0.111</td>
<td>0.0319</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$dlfd^{249}$</td>
<td>18</td>
<td>0.100</td>
<td>0.0000</td>
<td>-2.09</td>
<td>35</td>
<td>0.0438</td>
<td>-0.0219, -0.000327</td>
</tr>
</tbody>
</table>

Table 4.7. Welch $t$-test analyses of the VMR OFF peak activity of responsive 5-dpf mutant and WT embryos. Data is from $t = 0$, the time at which the bright light is turned off. N is the number of responsive embryos. SD is the standard deviation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>$t$</th>
<th>df</th>
<th>$p$</th>
<th>95% Confidence Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>$dlc^{+}$</td>
<td>75</td>
<td>0.136</td>
<td>0.0607</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$dlc^{b663}$</td>
<td>68</td>
<td>0.138</td>
<td>0.0734</td>
<td>0.197</td>
<td>130.5</td>
<td>0.844</td>
<td>-0.0202, 0.0246</td>
</tr>
<tr>
<td>$dlfd^+$</td>
<td>80</td>
<td>0.140</td>
<td>0.0670</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$dlfd^{249}$</td>
<td>75</td>
<td>0.152</td>
<td>0.0704</td>
<td>1.04</td>
<td>150.4</td>
<td>0.302</td>
<td>-0.0104, 0.0334</td>
</tr>
</tbody>
</table>
4.3.3. Dim light VMR: fewer $dll^{tg249}$ embryos responded to the onset of light, while $dlc^{b663}$ embryos had higher activity during the offset of light

The VMR of 5-dpf WT ($dlc^{+}$ and $dlld^{+}$) and mutant ($dlc^{b663}$ and $dll^{tg249}$) embryos were analyzed under dim light. At dim light ($5.57 \times 10^2$ $\mu$W/cm$^2$) cone cells are not active while rod cells are (Moyano et al., 2013); thus, we were able to analyze the VMR of rod cells’ output. The overall activity of WT and mutant embryos are shown in Figure 4.3. The major difference between the VMR profiles was during the ON-response, particularly during the onset of light change ($t = 0$). Student’s $t$ tests were conducted to compare the locomotor activity from (1) $dlc^{b663}$ embryos to their WT siblings ($dlc^{+}$), and (2) $dll^{tg249}$ embryos to their WT siblings ($dlld^{+}$) (Table 4.8 & 4.9). There was no significant difference between $dlc^{b663}$ and $dlc^{+}$ embryos. However, there was a significant decrease in the overall activity at the onset of light between $dll^{tg249}$ and $dlld^{+}$ embryos (Table 4.8). These results suggest that when Dld is not functional, the VMR during the onset of light is decreased, and this can be attributed to the abnormal function of rod cells.
Figure 4.3. Dim light VMR profiles of 5-dpf mutant and WT embryos. Graph tracing the overall locomotor activity of mutant (\(dlc^{b663}\) and \(dlc^{tg249}\)) (red traces) and WT (\(dlc^{+}\) and \(dld^{+}\)) (black traces) embryos during the VMR assays. Horizontal blue and black bars represent periods of lights ON and OFF, respectively. The ON response (A & B) and the OFF response (C & D) are shown. Arrows indicate the activity at \(t = 0\). The \(dld^{tg249}\) embryos had significantly reduced ON peak response activity upon light stimuli, compared to \(dld^{+}\) embryos (B).
Table 4.8. Student’s $t$-test analyses of the overall VMR ON peak activity of 5-dpf mutant and WT embryos. Data is from $t = 0$, the time at which the dim light is turned on. SD is the standard deviation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>t</th>
<th>df</th>
<th>p-value</th>
<th>95% Confidence Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>$dlc^+$</td>
<td>144</td>
<td>0.0174</td>
<td>0.0478</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$dlc^{b663}$</td>
<td>144</td>
<td>0.0187</td>
<td>0.0473</td>
<td>0.248</td>
<td>286.0</td>
<td>0.804</td>
<td>-0.00964 - 0.0124</td>
</tr>
<tr>
<td>$dlc^{b663}$ &amp; $dlc^{b663}$</td>
<td>144</td>
<td>0.0194</td>
<td>0.0447</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$dlc^{b663}$ &amp; $dlc^{b663}$</td>
<td>144</td>
<td>0.00833</td>
<td>0.0277</td>
<td>-2.54</td>
<td>238.9</td>
<td>0.0119</td>
<td>-0.0197 - 0.00248</td>
</tr>
</tbody>
</table>

Table 4.9. Student’s $t$-test analyses of the VMR OFF peak activity of 5-dpf mutant and WT embryos. Data is from $t = 0$, the time at which the dim light is turned off. SD is the standard deviation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>t</th>
<th>df</th>
<th>p-value</th>
<th>95% Confidence Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>$dlc^+$</td>
<td>144</td>
<td>0.113</td>
<td>0.0926</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$dlc^{b663}$ &amp; $dlc^{b663}$</td>
<td>144</td>
<td>0.121</td>
<td>0.108</td>
<td>0.643</td>
<td>279.2</td>
<td>0.520</td>
<td>-0.0157 - 0.0310</td>
</tr>
<tr>
<td>$dlc^{b663}$ &amp; $dlc^{b663}$</td>
<td>144</td>
<td>0.109</td>
<td>0.0938</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$dlc^{b663}$ &amp; $dlc^{b663}$</td>
<td>144</td>
<td>0.101</td>
<td>0.0982</td>
<td>-0.675</td>
<td>285.4</td>
<td>0.500</td>
<td>-0.0299 - 0.0146</td>
</tr>
</tbody>
</table>

The averaged overall activity does not segregate embryos that are responsive (+) and unresponsive (-) to the dim light stimuli. As a result, we counted how many embryos had a locomotor activity and how many did not, at $t = 0$. Person’s Chi-square tests of independence (with Yate’s continuity correction) were performed to determine differences in responsive (+) and unresponsive (-) embryos to ON- or OFF-light stimuli within each genotype (WT vs mutant) (Tables 4.10 & 4.11). There were no significant differences between $dlc^{b663}$ and their WT siblings (Tables 4.10 & 4.11). However, there...
were significantly fewer responsive \( dld^{tg249} \) embryos compared to their WT siblings during the ON-stimuli (Table 4.10).

**Table 4.10. Chi-square tests with Yate’s continuity correction during the ON-response.** VMR with dim light of 5-dpf mutant and WT embryos. (+) \( n \) is the number of embryos that respond to the light change at \( t = 0 \). (-) \( n \) is the number of the embryos that are unresponsive to the light change at \( t = 0 \).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>VMR (ON-response)</th>
<th>Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ ( n (%) )</td>
<td>- ( n (%) )</td>
</tr>
<tr>
<td>( dlc^+ )</td>
<td>20 (13.9)</td>
<td>124 (86.1)</td>
</tr>
<tr>
<td>( dlc^{b663} )</td>
<td>22 (15.3)</td>
<td>122 (84.7)</td>
</tr>
<tr>
<td>( dld^+ )</td>
<td>25 (17.4)</td>
<td>119 (82.6)</td>
</tr>
<tr>
<td>( dld^{tg249} )</td>
<td>12 (8.3)</td>
<td>132 (91.7)</td>
</tr>
</tbody>
</table>

**Table 4.11. Chi-square tests with Yate’s continuity correction during the OFF-response.** VMR with dim light of 5-dpf mutant and WT embryos. (+) \( n \) is the number of embryos that respond to the light change at \( t = 0 \). (-) \( n \) is the number of the embryos that are unresponsive to the light change at \( t = 0 \).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>VMR (OFF-response)</th>
<th>Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ ( n (%) )</td>
<td>- ( n (%) )</td>
</tr>
<tr>
<td>( dlc^+ )</td>
<td>106 (73.6)</td>
<td>38 (26.4)</td>
</tr>
<tr>
<td>( dlc^{b663} )</td>
<td>97 (67.4)</td>
<td>47 (32.6)</td>
</tr>
<tr>
<td>( dld^+ )</td>
<td>100 (69.4)</td>
<td>44 (30.6)</td>
</tr>
<tr>
<td>( dld^{tg249} )</td>
<td>92 (63.9)</td>
<td>52 (36.1)</td>
</tr>
</tbody>
</table>

The previous analyses showed that there are fewer responsive \( dlc^{b663} \) embryos during the ON-light stimuli (Table 4.10), which may cause a decrease in overall activity. Nevertheless, it is unclear if the responsive embryos have irregular VMR. To address this uncertainty, we compared the activity of responsive embryos for each of the light stimuli at \( t = 0 \). Welch \( t \)-tests were conducted to compare the locomotor activity from (1) \( dlc^{b663} \) embryos to their WT siblings (\( dlc^+ \)), and (2) \( dld^{tg249} \) embryos to their WT siblings (\( dld^+ \)) (Tables 4.12 & 4.13). There was significant increase in the overall activity at the offset of light in \( dlc^{b663} \) compared to \( dlc^+ \) embryos (Table 4.13). In addition, there were no
differences in the responsive embryos’ activity between \( dld^{8249} \) and \( dld^+ \) embryos during the ON or OFF stimuli. These results suggest that responsive embryos, without functional Dlc, have increase VMR activity at the offset of dim light. Moreover, the loss of function of Dld decreases the number of embryos that can respond to dim light changes but does not affect the activity of responsive embryos.

**Table 4.12. Welch \( t \)-test analyses of the VMR ON peak activity of responsive 5-dpf mutant and WT embryos.** Data is from \( t = 0 \), the time at which the dim light is turned on. SD is the standard deviation.

<table>
<thead>
<tr>
<th>VMR activity from responsive embryos</th>
<th>Welch ( t )-test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype</strong></td>
<td>( N )</td>
</tr>
<tr>
<td>( dlc^+ )</td>
<td>20</td>
</tr>
<tr>
<td>( dlc^{8663} )</td>
<td>22</td>
</tr>
<tr>
<td>( dld^+ )</td>
<td>25</td>
</tr>
<tr>
<td>( dld^{8249} )</td>
<td>12</td>
</tr>
</tbody>
</table>

**Table 4.13. Welch \( t \)-test analyses of the VMR OFF peak activity of responsive 5-dpf mutant and WT embryos.** Data is from \( t = 0 \), the time at which the dim light is turned off. SD is the standard deviation.

<table>
<thead>
<tr>
<th>VMR activity from responsive embryos</th>
<th>Welch ( t )-test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype</strong></td>
<td>( N )</td>
</tr>
<tr>
<td>( dlc^+ )</td>
<td>106</td>
</tr>
<tr>
<td>( dlc^{8663} )</td>
<td>97</td>
</tr>
<tr>
<td>( dld^+ )</td>
<td>100</td>
</tr>
<tr>
<td>( dld^{8249} )</td>
<td>92</td>
</tr>
</tbody>
</table>
4.4. Discussion

The lack of Dlc or Dld affects photoreceptor development (as described in Chapter Two) but it is unclear if this negatively affects the visual system. Since defects in the retina are not directly correlated to defects in vision, in this chapter we examined the visual behavior of Dlc and Dld mutant embryos to determine if the lack of these proteins affects vision. The mutant embryos’ visual behavior was evaluated by OKR and VMR. The results showed that \( dlc^{b663} \) and \( dld^{g8249} \) embryos had decreased OKR compared to WT embryos (Figure 4.1). Differences in the VMR between \( dlc^{b663} \) and \( dld^{g8249} \) embryos were observed (summarized in Figure 4.4). For instance, the \( dld^{g8249} \) embryos had altered VMR with bright and dim light stimuli (during the ON response), while \( dlc^{b663} \) embryos had altered VMR with dim light stimuli (during the OFF response) when compared to their WT embryos.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>VMR response</th>
<th># of (-)</th>
<th># of (+)</th>
<th>Activity from (+)</th>
<th>Activity from (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bright Light</td>
<td>( dlc^{b663} )</td>
<td>ON</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OFF</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>( dld^{g8249} )</td>
<td>ON</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OFF</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dim Light</td>
<td>( dlc^{b663} )</td>
<td>ON</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OFF</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>( dld^{g8249} )</td>
<td>ON</td>
<td>↑</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OFF</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 4.4. Summary table of the visual motor response (VMR) of \( dlc^{b663} \) and \( dld^{g8249} \) 5-dpf embryos. The results correspond to the ON- and OFF-VMR peak response (t = 0). In columns four and five, the number of responsive embryos (+) and non-responsive embryos (-) were compared between mutant and WT. In columns six and seven, the activity of only responsive (+) and the total number of embryos [(+)/(-)] were compared between mutant and WT. An up-arrow indicates an increase, down-arrow indicates a decrease and a hyphen indicates no change compare to WT.
4.4.1 *dlc*b663 and *dld*g249 embryos can respond to visual signals

Since both mutants’ embryos have aberrant visual behavior, we questioned the mutants’ ability to detect the visual stimuli and their ability to command motor outputs (eye tracking movements or locomotor activity during the OKR or VMR assays, respectively). The OKR assay demonstrated that both mutant embryos have eye tracking movements (Figure 4.1). This suggests that the mutant embryos can detect motion in their environment and have motor outputs. Moreover, another observation that demonstrates the mutant embryos’ ability to command motor function via visual stimulus is the unaffected OFF response with bright light stimuli during the VMR assay (Figure 4.2C & D). Mutant embryos’ OFF response does not differ compared to WT, suggesting that mutant embryos can process visual signals and have proper motor command outputs. The positive OKR response and the unaffected OFF response from the VMR assays are suggestive that the defects on visual behavior exhibited by the mutant embryos are most likely due to retina defects and not brain or muscle defects.

4.4.2 The visual behavior of *dlc*b663 and *dld*g249 embryos is affected

The fact that both WT and mutant embryos have an OKR (Figure 4.1), this indicates that they are able to track the movement in their external environment resulting in a muscle output. Nevertheless, the mutant embryos had a significantly (p < 0.01) reduced number of OKRs compared to WT, suggesting that the mutant embryos’ visual behavior is affected. Specifically, the ETMs/15s for *dlc*b663 embryos were reduced by 8.1%, and *dld*g249 embryos were reduced by 27.3% compared to their respective WT. This data indicates a deficit in the visual function for both mutant embryos. In addition, the difference in the percent of OKR of the mutant embryos, suggests that the lack of Dld causes a considerable more severe effect on the visual behavior than the lack of Dlc.

4.4.3 During the VMR assay at the onset of bright light, *dld*g249 embryos are less responsive and are less active

The VMR is able to test whether zebrafish embryos can response to light and dark transitions. The overall VMR profiles of WT and *dlc*b663 embryos were similar with bright light stimuli (Figure 4.1A & C). In addition, there was no significant difference at the onset or offset of light between WT and *dlc*b663 embryos (Tables 4.2 & 4.3).
Moreover, there was no change in the number of embryos that were responsive and unresponsive during the light changes between WT and $dlc^{b663}$ embryos (Tables 4.4 & 4.5). For the OFF-response, $dlld^{g249}$ embryos had no significant change compared to WT embryos. Specifically, the overall VMR profile (Figure 4.1D), the activity peak (Table 4.3), and the number of responsive and unresponsive (Table 4.5) $dlld^{g249}$ embryos did not differ to WT for the OFF response (Summarized in Figure 4.4).

However, during the ON response there were significant changes for $dlld^{g249}$ embryos. The VMR profile during the ON response (Figure 1B), showed a significant ($p < 0.003$) decreased activity for $dlld^{g249}$ embryos compared to WT embryos at the light onset ($t = 0$) (Table 4.2). Particularly, $dlld^{g249}$ embryos had 55% less activity than WT embryos. We speculate that the decrease in activity could be affected by a reduction on the number of embryos that responded to the light change. We observed there were 50% fewer responsive embryos compared to WT (Table 4.4). Furthermore, when we compared the locomotor activity during the onset of light, $dlld^{g249}$ responsive embryos had a 10% decrease in activity compared to responsive WT embryos. These data suggest that $dlld^{g249}$ embryos have a higher proportion of embryos that cannot detect the onset of light (50%) and the visual behavior of $dlld^{g249}$ embryos that do detect the light change have a decreased activity (by 10%). We can conclude that the lack of Dld affected the ability of embryos to detect the onset of light, yet does not affect the offset of light.

Since $dlld^{g249}$ embryos have decreased ON response during bright light stimuli, we can postulate that these defects in visual behavior are due to defects in the retinal pathways that produce the ON response. To elaborate on this idea, photoreceptor cell terminals synapse to horizontal and bipolar cells (Dowling, 1987). These photoreceptor terminals forms two types of synapses that produces the ON and OFF channels. The ON channel allows the detection for light increments via ON bipolar and horizontal cells, while the OFF channel allows for detecting light decrements via OFF bipolar cells (Dowling, 1987; Schiller, 1986; Vardi et al., 2002). Thus, we can suggest that the OFF channels from the retinal pathways are defective with the lack of functional Dld. Furthermore, this assay utilized a bright light, we can attribute the VMR defects to abnormalities from the OFF channel cone pathways.
4.4.4 The dim light VMR assays demonstrated a differential effect in the Delta mutants: *dld*\textsubscript{tg249} embryos have altered activity during the offset of light while *dlc*\textsubscript{b663} embryos have altered activity during the onset of light.

The overall VMR profiles of WT and *dlc*\textsubscript{b663} embryos were similar with dim light stimuli (Figure 4.3A & C). There was no significant difference in activity at the onset or offset of light between WT and *dlc*\textsubscript{b663} embryos (Tables 4.8 & 4.9). Moreover, there was no change in the number of embryos that were responsive and unresponsive during the light changes between WT and *dlc*\textsubscript{b663} embryos (Tables 4.10 & 4.11). However, when we analyzed the locomotor activity of responsive *dlc*\textsubscript{b663} embryos, these embryos had an increase in activity (p < 0.004) (Table 4.13). Specifically, responsive *dlc*\textsubscript{b663} embryos had a 16% increase in activity compared to responsive WT embryos during the offset of light (Summarized in Figure 4.4).

For the OFF response, *dld*\textsubscript{tg249} embryos had no significant change compared to WT embryos. Specifically, the overall VMR profile (Figure 4.3D), the activity peak (Table 4.9), and the number of responsive and unresponsive (Table 4.11) *dld*\textsubscript{tg249} embryos did not differ to WT for the OFF-response (Summarized in Figure 4.4). However, during the ON response there were significant changes for *dld*\textsubscript{tg249} embryos. At the onset of light, the activity of *dld*\textsubscript{tg249} embryos was decreased by 57% compared to WT (p < 0.02) (Table 4.8). Moreover, when we analyzed the number of responsive *dld*\textsubscript{tg249} embryos, there were 57% fewer responsive embryos compared to WT during the onset of lights (p < 0.035) (Table 4.10). Nevertheless, there was no significant difference in activity of responsive *dld*\textsubscript{tg249} embryos (p > 0.05) (Table 4.11). These data suggest that the lack of Dld inhibits the embryos’ ability to detect the onset of dim light but does not affect the degree of locomotor activity.

Dim light stimulation is perceived from the initial transduction of rod photoreceptor cells. Thus, aberrant visual behavior during dim light can be attributed to defects in the rod circuitry. In this study, we can propose that *dld*\textsubscript{tg249} embryos have defects in their ON rod circuitry, since they have fewer responsive embryos during the ON response. Moreover, we can speculate that *dlc*\textsubscript{b663} embryos have defects in their OFF rod circuitry, since they have increased activity during their OFF response. We can
conclude that the Dld is import for the ON rod circuitry, and if it is not present, the transduction of the rod pathway during the light stimulation is affected. However, Dlc is important for the OFF rod circuitry, and if not present, the rod pathway triggers a larger locomotor activity in response to the light stimulation.

4.4.5 Conclusion

To our knowledge, this study is the first of its kind to analyze the visual behavior of the Delta-Notch mutants. We demonstrated the adverse effects in $dlc^{b663}$ and $dld^{g249}$ on the visual behavior by OKR and VMR assays. For instance, we observed that the mutant embryos have attenuated abilities of detecting moving objects by OKR assay. Moreover, finite differences between Dlc and Dld were identified by the VMR assay. As a result, we were able to correlate Dlc and Dld to particular retinal pathways of the OFF- or ON-circuitry, respectively. Yet, further studies are needed to elucidate downstream cellular and molecular components that underlie the development of the visual circuitry. Overall, we conclude that Delta-Notch signaling contributes to vision; more notably, different Delta ligands contribute to specific aspects of retinal functionality that produce vision.
4.5. References


CHAPTER 5. CONCLUSION

The purpose of this study was to identify the functions of Dlc and Dld in zebrafish retinal development. Zebrafish mutants of these genes, dlc<sup>b663</sup> and dld<sup>g249</sup>, were used to identify retinal defects (CHAPTER TWO), determine gene expression changes of the immediate downstream targets (CHAPTER THREE), and evaluate their visual behavior (CHAPTER FOUR). The results of this investigation elucidated the contributions of Dlc and Dld. The overall outcomes of this study showed that Dlc and Dld (1) affect photoreceptor differentiation, (2) have dynamic effects on her/hes, and (3) contribute to the visual behavior.

At 36-hpf, it was observed that Dlc and Dld promote the differentiation of rod cells and Dld inhibits blue cone differentiation (summarized in Figure 2.20). Furthermore, it was demonstrated that Dlc downregulated the expression of her<sub>6</sub>/hes<sub>1</sub> in the retina, whereas Dld upregulated the expression of her<sub>6</sub>/hes<sub>1</sub> in the CMZ (summarized in Figure 3.6). These observations suggest that Dlc promotes neural differentiation in the retina, whereas Dld inhibits neural differentiation in the CMZ (summarized in Figure 5.1). The expression of dlc and dld supports these ideas. dlc was transcribed in cells located in the retina, specifically in the ventral patch (where initial retinal differentiation occurs), and dld was transcribed in cells located in the CMZ (Figure 2.3). Since the function of her<sub>6</sub>/hes<sub>1</sub> is to inhibit neural differentiation and maintain retinal progenitor cells proliferating (Furukawa et al., 2000; Kageyama et al., 1997; Takatsuka et al., 2004; Tomita et al., 1996), we can make the following conclusions. Dlc functions to inhibit the transcription of her<sub>6</sub>/hes<sub>1</sub> to allow rod cell differentiation. Dld functions to upregulate the transcription of her<sub>6</sub>/hes<sub>1</sub> in the CMZ to inhibit blue cone differentiation. However, it is not clear how Dld promotes rod cell differentiation. A possible method could be via the regulation of other Her/Hes proteins not analyzed in this study.
At 2-dpf, \textit{dlc} and \textit{dld} were expressed in cells located in the CMZ (Figure 2.3). During the second dpf, Dlc and Dld hinder the differentiation of rod, red cone and blue cone cells in the medial region of the retina (summarized in Figure 2.20). At this developmental stage, it was observed that (1) Dlc and Dld downregulated the transcription of \textit{hey1} in the CMZ, (2) Dlc and Dld upregulated the transcription of \textit{her13.2/ hes6} and \textit{hey1} in the retina, and (3) Dlc downregulated the transcription of \textit{her4.2/ hes5} in the retina. \textit{Hey1} is associated with the function of inhibiting photoreceptor differentiation (Jadhav et al., 2006b). \textit{Her13.2/Hes6} is known to promote neural differentiation (Bae et al., 2000; Koyano-Nakagawa et al., 2000), and \textit{Her4.2/Hes5} inhibits neural differentiation (Hojo et al., 2000; Nelson et al., 2006). Thus, we conclude that Dlc and Dld inhibit photoreceptor differentiation via the upregulation of \textit{hey1} in the retina. In addition, since Dlc and Dld upregulated \textit{her13.2/ hes6} and Dlc downregulated \textit{her4.2/ hes5}, we conclude that Dlc and Dld promotes retinal differentiation. However, it is unclear what are the contributions of these functions in retinal development.

In this study, it was demonstrated that at 5-dpf Dlc affected the distribution of rod and blue cone cells in the retina, while Dld inhibited the differentiation of green and red cone cells and affected the distribution of rod cells in the retina (Figure 2.20). Future experiments are needed to identify how Dlc and Dld mediate their effects on photoreceptor distribution and differentiation. However, based on Dlc and Dld transcriptional regulation on \textit{her/hes} at 3-dpf (Figure 5.1), we can suggest that (1) Dld inhibits cone differentiation via \textit{her4.2/ hes5} in the CMZ, while allowing rod differentiation via the inhibition of \textit{hey1} in the retina, and (2) Dlc allows for proper photoreceptor patterning via the inhibition of \textit{her13.2/ hes6}.

This study showed that \textit{dlc}\textsuperscript{b663} and \textit{dld}\textsuperscript{g8249} mutants have defects in retinal development, suggesting that Dlc and Dld contribute to the proper formation of the retina. Nevertheless, defects in retinal development do not always translate to functional defects on vision. To our knowledge, the effects of Delta-Notch mutants on the visual behavior have not been evaluated before. In this study, we demonstrated that defects in the Delta-Notch pathway affect the visual behavior of zebrafish. The OKR was decreased in \textit{dlc}\textsuperscript{b663} and \textit{dld}\textsuperscript{g8249} mutants (Figure 4.1). Furthermore, the mutants had different defects
in their VMR (summarized in Table 4.9). \textit{dld}^{g249} mutants had difficulties detecting the onset of light (in bright and dim light), while \textit{dlc}^{b663} mutants were more sensitive to changes in dim light. These studies showed that defects in Delta-Notch signaling cause defects on vision.

The overall conclusions of this dissertation were made by using the \textit{dlc}^{b663} and \textit{dld}^{g249} mutant zebrafish, which allowed the identification of specific retinal functions for Dlc and Dld, determined immediate downstream targets in which Dlc and Dld signal through, and concluded that Dlc and Dld contribute to the functionality of the eye.
Figure 5.1. Schematic diagram of Dlc and Dld signaling contribution to retinal development via immediate downstream targets (her/hes).
5.1 References
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APPENDIX

Appendix 1. Nucleotide sequence of deltaC. Primer pairs used to identify the mutation in dlC are shown.

.....AAGCG

\textcolor{red}{\textbf{dlC1F}} AATAAATTAATGGAAGAAAC
ATTTATGACTTTCAATTTGGCATTTGGCCACGGCCTCTACTACGGGCTGCTACAAAGGCC
AAGCGCAGCCTTCTCCTATTGAAAGACGTAATGGGTAAGGTCTAACGACTTACCTT
GGAGCTAGAATCTGTGTTTTATTTTATTTTATTTGCTTTTAATTTTATTTGCTTTTAAT
TTTACAATCTCATCTCCCTCTTAAAATCGTGGATATTACTACAAACATTCTG
TCGGACTACTCTCAGTCTGATCGTATCGGAGCA \textcolor{blue}{\textbf{dlC2F}} AAGAAGGCA

AAGATGGGCTCGT
GGTTTTATTAACGTGCTTTTTGATATTGTTTTGCTTTTAATTTTATTTGCTTTTAAT
TTTACAATCTCATCTCCCTCTTAAAATCGTGGATATTACTACAAACATTCTG
TCGGACTACTCTCAGTCTGATCGTATCGGAGCA \textcolor{blue}{\textbf{dlC2F}} AAGAAGGCA

\textcolor{red}{\textbf{dlC1R}} AAGATGGGCTCGT
GGTTTTATTAACGTGCTTTTTGATATTGTTTTGCTTTTAATTTTATTTGCTTTTAAT
TTTACAATCTCATCTCCCTCTTAAAATCGTGGATATTACTACAAACATTCTG
TCGGACTACTCTCAGTCTGATCGTATCGGAGCA \textcolor{blue}{\textbf{dlC2F}} AAGAAGGCA

\textcolor{red}{\textbf{dlC1R}} AAGATGGGCTCGT
GGTTTTATTAACGTGCTTTTTGATATTGTTTTGCTTTTAATTTTATTTGCTTTTAAT
TTTACAATCTCATCTCCCTCTTAAAATCGTGGATATTACTACAAACATTCTG
TCGGACTACTCTCAGTCTGATCGTATCGGAGCA \textcolor{blue}{\textbf{dlC2F}} AAGAAGGCA

\textcolor{red}{\textbf{dlC1R}} AAGATGGGCTCGT
GGTTTTATTAACGTGCTTTTTGATATTGTTTTGCTTTTAATTTTATTTGCTTTTAAT
TTTACAATCTCATCTCCCTCTTAAAATCGTGGATATTACTACAAACATTCTG
TCGGACTACTCTCAGTCTGATCGTATCGGAGCA \textcolor{blue}{\textbf{dlC2F}} AAGAAGGCA

\textcolor{red}{\textbf{dlC1R}} AAGATGGGCTCGT
GGTTTTATTAACGTGCTTTTTGATATTGTTTTGCTTTTAATTTTATTTGCTTTTAAT
TTTACAATCTCATCTCCCTCTTAAAATCGTGGATATTACTACAAACATTCTG
TCGGACTACTCTCAGTCTGATCGTATCGGAGCA \textcolor{blue}{\textbf{dlC2F}} AAGAAGGCA

\textcolor{red}{\textbf{dlC1R}} AAGATGGGCTCGT
GGTTTTATTAACGTGCTTTTTGATATTGTTTTGCTTTTAATTTTATTTGCTTTTAAT
TTTACAATCTCATCTCCCTCTTAAAATCGTGGATATTACTACAAACATTCTG
TCGGACTACTCTCAGTCTGATCGTATCGGAGCA \textcolor{blue}{\textbf{dlC2F}} AAGAAGGCA

\textcolor{red}{\textbf{dlC1R}} AAGATGGGCTCGT
GGTTTTATTAACGTGCTTTTTGATATTGTTTTGCTTTTAATTTTATTTGCTTTTAAT
TTTACAATCTCATCTCCCTCTTAAAATCGTGGATATTACTACAAACATTCTG
TCGGACTACTCTCAGTCTGATCGTATCGGAGCA \textcolor{blue}{\textbf{dlC2F}} AAGAAGGCA

\textcolor{red}{\textbf{dlC1R}} AAGATGGGCTCGT
GGTTTTATTAACGTGCTTTTTGATATTGTTTTGCTTTTAATTTTATTTGCTTTTAAT
TTTACAATCTCATCTCCCTCTTAAAATCGTGGATATTACTACAAACATTCTG
TCGGACTACTCTCAGTCTGATCGTATCGGAGCA \textcolor{blue}{\textbf{dlC2F}} AAGAAGGCA

\textcolor{red}{\textbf{dlC1R}} AAGATGGGCTCGT
GGTTTTATTAACGTGCTTTTTGATATTGTTTTGCTTTTAATTTTATTTGCTTTTAAT
TTTACAATCTCATCTCCCTCTTAAAATCGTGGATATTACTACAAACATTCTG
TCGGACTACTCTCAGTCTGATCGTATCGGAGCA \textcolor{blue}{\textbf{dlC2F}} AAGAAGGCA
GTGACGCGCGCTGGCAACAGAATTTTGCTTTCTGGATGGAAAAAGCGATTATTG

CACCAGAAGttaagtggatgtaatagcagcaatcgatattgtttttttttttttttttttttttttaataaatcggaagcg

gtctgcttcccgctgtaagaagagtctgcttgctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
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