Novel newt regeneration genes regulate Wingless signaling to restore patterning in *Drosophila* eye

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Running Title: Newt genes regulate Wg signaling to recue eye mutant phenotype

Key words: *Drosophila*, Eye, Newts, Wingless, Cell death, Growth, Regeneration

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Abstract

A fundamental process of regeneration, which varies among animals, recruits conserved signaling pathways to restore missing parts. Only a few animals like newts can repeatedly regenerate lost body parts throughout their lifespan that can be attributed to strategic regulation of conserved signaling pathways by newt's regeneration tool-kit genes. Here we report the use of a genetically tractable *Drosophila* eye model to demonstrate the regeneration potential of a group of unique protein(s) from newt (*Notophthalmus viridescens),* which when ectopically expressed can significantly rescue missing photoreceptor cells in a *Drosophila* eye mutant. These newt proteins with signal peptides motifs exhibit non-cell-autonomous rescue properties and their regeneration potential even extends into later stages of fly development. Ectopic expression of these newt genes can rescue eye mutant phenotype by promoting cell proliferation and blocking cell death. These novel newt genes downregulate the evolutionarily conserved Wingless (Wg)/Wnt signaling pathway to promote rescue. Modulation of Wg/Wnt signaling levels by using antagonists or agonists of Wg/Wnt signaling pathway in eye mutant background where newt gene(s) is ectopically expressed suggests that Wg signaling acts downstream of newt genes. Our data highlights the regeneration potential of novel newt proteins that regulate conserved pathways to trigger a robust regeneration response in *Drosophila* model with weak regeneration capability.

Introduction

A urodele amphibian, newts belongs to *Salamandridae* family (Weisrock et al., 2006). The newts are the only four-legged vertebrates that exhibits a remarkable capability to regenerate tissues like limbs, tail, heart, lens, spinal cord, brain, and retina throughout its life time (Mehta and Singh, 2019a; Sanchez Alvarado and Tsonis, 2006). This regeneration potential/ ability of the newt is due to their ability to reprogram and dedifferentiate the terminally differentiated cells to trigger regeneration response (Mehta and Singh, 2019a). Such an exceptional regeneration ability of newts has been attributed to unique gene(s) that may have evolved to form the regeneration tool box (Bryant et al., 2017; Casco-Robles et al., 2018; Elewa et al., 2017; Evans et al., 2018; Keinath et al., 2015; Kumar et al., 2007; Matsunami et al., 2019; Mehta and Singh, 2019a; Nowoshilow et al., 2018; Sanor et al., 2020; Smith et al., 2009; Smith et al., 2019). Earlier, a newt gene Prod1, which encodes a transmembrane receptor, was found to be critical for maintaining proximo-distal identity (pattern memory) during newt limb regeneration (da Silva et al., 2002; Echeverri and Tanaka, 2005; Kumar et al., 2007; Mehta and Singh, 2019a). Homologs of Prod1 are yet to be identified outside of salamanders (Garza-Garcia et al., 2009). Similarly, the transcript of a novel newt gene *newtic1* was found to be significantly enriched in a subset of erythrocytes which formed an aggregate structure called erythrocyte clump. These *newtic* expressing erythrocytes promote limb regeneration (Casco-Robles et al., 2018). Surprisingly, our understanding of underlying molecular genetic mechanism(s) responsible for promoting lifelong regeneration of missing structures and/or rescue of pattern defects in newts is far from complete. Some strategies to determine their regeneration potential are to introduce these unique genes in genetically tractable model(s) that lack regeneration potential exhibited by newts such as mammals, *Drosophila* etc. Since the genetic machinery is conserved across the species, the *Drosophila* model has been successfully used for such cross species studies to model human disease and determine their underlying molecular genetic mechanism(s) (Hughes et al., 2012; Sarkar et al., 2018; Sarkar et al., 2016; Singh and Irvine, 2012; Tare et al., 2011).

Previously, a novel family of five protein members was identified in a
comprehensive transcriptomic analysis from *Notophthalmus viridescens* (red-spotted
newt). These proteins are expressed in adult tissues and are modulated during lens
regeneration (Looso et al., 2013). The three-dimensional structure models of these
novel newt proteins by *ab initio* methods suggested that these proteins could act as ion
transporters and/or involved in signaling (Mehta et al., 2019). These unique newt genes
were introduced in genetically tractable *Drosophila melanogaster* (fruit fly) model by
transgenic approaches to test their regeneration potential. Such transgenic approaches
were not feasible in *N. viridescens*, therefore, *Drosophila* served as a suitable system to
address these queries. The transcriptomic analysis was performed in the transgenic
Drosophila that are expressing newt genes. Based on these studies, graded expression
of 2775 transcripts was reported in transgenic fly where one of the five newly identified
newt genes was ectopically expressed using the GAL4/UAS binary target system
(Brand and Perrimon, 1993; Mehta et al., 2019). Among these 2775 transcripts, genes
involved in the fundamental developmental processes like cell cycle, apoptosis, and
immune response were highly enriched suggesting that these foreign genes from newt
were able to modulate the expression of *Drosophila* gene(s) (Mehta et al., 2019; Mehta
and Singh, 2019b).

Drosophila, a hemimetabolous insect, serves as a versatile model organism to
study development and disease due to presence of fully sequenced and less redundant
genome, presence of homologs or orthologs for human disease related genes, a shorter
life cycle and economy to maintain fly cultures (Bier, 2005; Lenz et al., 2013; Singh and
Irvine, 2012). *Drosophila* eye has been extensively used to understand the fundamental
process of development and model neurodegenerative disorders (Gogia et al., 2020a;
Kumar, 2018; Sarkar et al., 2018; Sarkar et al., 2016; Singh and Irvine, 2012; Tare et
al., 2011; Yeates et al., 2019). The rich repository of genetic tools available in fly model,
and the fact that eye is dispensable for survival, makes *Drosophila* eye suitable for
screening genes from other animals that can promote growth and regeneration. The
compound eye of the adult fly develops from a monolayer epithelium called the eye-
antennal imaginal disc, which develops from an embryonic ectoderm (Cohen, 1993;
Held, 2002). The imaginal disc, a sac-like structure present inside the larva, comprise of

two different layers: the peripodial membrane (PM) and the disc proper (DP) (Kumar,

2020b). The DP develops into retina whereas the PM of the eye-antennal imaginal disc

contributes to the adult head structures (Haynie and Bryant, 1986; Kumar, 2020b;

Milner et al., 1983). In third instar larval eye imaginal disc, a wave of synchronous

retinal differentiation moves anteriorly from the posterior margin of the eye disc, and is

referred to as Morphogenetic Furrow (MF) (Kumar, 2013, 2020a; Ready et al., 1976).

The progression of MF transforms the undifferentiated cells into differentiated

photoreceptor cells. The adult compound eye comprises of 800 unit eyes or ommatidia

(Kumar, 2013, 2018, 2020a; Ready et al., 1976). The ommatidial cells in the compound

eye are grouped together into two chiral forms, which are arranged in mirror image

symmetry along the Dorso-Ventral (DV) midline called the equator. Ventral domain is

the default state of the entire early eye imaginal primordium (Singh and Choi, 2003),

and onset of the expression of the dorsal selector gene *pannier* (*pnr*), establishes the

dorso-ventral (DV) lineage in the eye (Maurel-Zaffran and Treisman, 2000; Singh and

Choi, 2003; Tare et al., 2013). It has been reported that *Lobe* (*L*) is a cell survival gene

required for ventral eye development and growth, and acts upstream to the Wg

signaling pathway (Singh et al., 2006).

The *wg* gene, a *Drosophila* homolog of Wnt, serves as a ligand, and encodes a

secreted signaling protein that acts as a morphogen (Baker, 1987; Bejsovec, 2013;

Swarup and Verheyen, 2012). The transcriptional effector of the Wg/β-catenin signaling

is *Drosophila* T-cell Factor (dTCF), which upon activation by Armadillo (Arm, human

homolog β-catenin) leads to the transcription of Wg target genes. In the absence of

ligand Wg, a destruction complex comprised of Adenomatous polyposis coli (Apc), and

Axin degrades Arm and thus prevents it to form a complex with dTCF. The pathway is

activated by binding of Wg ligand to its co-receptors Frizzled (Fz) and Arrow (Arr). Upon

activation, Fz binds to Dishevelled (Dsh) and Arr, which binds to Axin and thus

inactivating the destruction complex. This results in the translocation of Arm (β-catenin)

from the cytosol to the nucleus where Arm binds to dTCF and activates transcription of

Wg target genes (Bejsovec, 2006). In the eye-antennal disc, Wg acts as a negative

regulator of the eye development (Ma and Moses, 1995; Treisman and Rubin, 1995). In

early third instar eye-antennal imaginal disc, *wg* expresses on the ventral margin.

Ectopic expression of Wg triggers cell death of photoreceptor cells causing eye defects

(Baker, 1987; Cavodeassi et al., 1999; Singh, 2012; Singh et al., 2006). Previously it

has been shown that Wg pathway was upregulated in developing eye of L^2 background.

This perturbation in Wg pathway causes loss of ventral eye in 100% of flies of L^2/+

background (Gogia et al., 2020a; Singh et al., 2006; Singh et al., 2011). Therefore,

timing and level of *wg* expression in this region is crucial for formation of a normally

patterned eye. Here, we demonstrate using a unique gene pool of newt genes (5 newly

identified newt genes) to rescue *Drosophila L^2* mutant pattern defect*.*

Misexpression of these novel newt gene in *Drosophila* downregulate Wg in L^2

mutant background, resulting in significant restoration of missing photoreceptor cells in

the developing *Drosophila* eye. Ectopic expression of these genes restore eye mutant

phenotypes by promoting cell proliferation, and downregulating cell death. Furthermore,

these eye mutant phenotype rescues by newt genes are non-cell-autonomous and are

all along the developmental stages *viz.,* early as well as later time points of

development.

Results

We employed *Drosophila* eye model to test the regeneration potential of these five
unique newt genes, *candidate 1* (*C1), candidate 2 (C2), candidate 3 (C3), candidate 4*
(C4), and *candidate 5 (C5)*. We isolated the total RNA from newt tail and generated
cDNA to amplify these newt genes, and generated transgenic flies for these five genes
(Figure 1A). We employed GAL4/UAS system (Brand and Perrimon, 1993) to allow
targeted misexpression of these newt transgenes in the fly tissues (Figure 1B). To
confirm if targeted misexpression approach can generate newt proteins in *Drosophila*
(Brand and Perrimon, 1993), we tested localization of the fusion protein. As there are
no antibodies available against these novel newt proteins, so we used V5 epitope
tagged transgenes to generate transgenic flies (Wang et al., 2019). The V5 tag
sequence of about 42 bp long is fused towards the 3' end of the 501 bp long open
reading frame (ORF). We tested all these transgenes using the antibody against V5 tag.
Here we present the expression of one of the transgenes *viz.*, C4 using V5 tag (Figure
S1). Next, to detect if misexpression of these newt genes in *Drosophila* can generate
any developmental defects, we used *tubulin* – GAL4 (*tub* –GAL4) to ubiquitously
misexpress newt genes in flies in the wild-type background (Figure 1B), and found no
defects.

Newt genes can rescue *Drosophila* eye mutants

For our experiments, we used the *Drosophila* eye model as it is not required for the
viability of the fly and it is easy to score the phenotypes. We used *ey*-GAL4 driver
(Hazelett et al., 1998) to misexpress these newt genes in the developing eye imaginal
disc, which develops into an adult eye. The phenotypes of UAS-transgene alone were
tested to rule out any contribution(s) in phenotype due to insertion of the transgene. In
comparison to the control, *ey*-GAL4 (Figure 1C), misexpression of these transgenes *ey*-
GAL4>UAS-*C1* (*ey*>*C1*, Figure 1D), *ey*-GAL4>UAS-*C2* (*ey*>*C2*, Figure 1E), *ey*-
GAL4>UAS-*C3* (*ey*>*C3*, Figure 1F), *ey*-GAL4>UAS-*C4* (*ey*>*C4*, Figure 1G), and *ey*-
GAL4>UAS-*C5* (*ey*>*C5*, Figure 1H), did not affect the eye size (Figure 1I). To

investigate the regeneration potential of these genes, we used eye mutant(s) such as *L*

mutant which exhibits selective loss of ventral half of the eye (Figure 1J). This mutant

was selected as the penetrance of the phenotype of loss-of-ventral eye is 100%.

Interestingly, targeted misexpression of the newt gene(s) in L^2; *ey*-GAL4 background

[(L^2; *ey>C1*; Figure. 1K), (*L^2; ey>C2;* Figure 1L)*, (L^2; ey>C3;* Figure 1M), (L^2; *ey>C4*;

Figure 1N), (L^2; *ey>C5*; Figure 1O)] exhibit significant rescue of the loss-of-ventral eye

phenotype (Figure 1P). For each cross, three independent cultures were established

and two hundred flies for each set (triplicate, 600 flies) were counted to determine

phenotype frequency. The rescue frequency was about 40.83% in C1, 37.7% in C2,

49% in C3, 58.2% in C4, and 38.7% in C5 (Figure S2). Note that the strongest rescues

were observed with C4 transgene (Figure 1P; S2; and Table S1).

Since the eye mutant represent developmental defects in the genetic machinery

involved in early eye development, it raises a question whether the regeneration

potential of these newt genes is restricted to the early time window or even later stages

of eye development. The GMR- GAL4 driver was used to drive expression of

transgenes in the differentiating retinal neurons of larval eye imaginal disc, which

continues all along to the adult eye (Moses and Rubin, 1991). Gain-of-function of *head*

involution defective (*hid*), an executioner caspase of cell death machinery, triggers cell

death (Bergmann et al., 1998; Grether et al., 1995; Hay et al., 1995; White et al., 1994).

Misexpression of *hid* using GMR-GAL4 (GMR>*hid*) results in a "No-eye" or highly

reduced eye phenotype (Figure S3B). However, misexpression of newt transgenes

along with *hid* in the eye (GMR-GAL4>*hid*+*C4*) results in a significant rescue of the "No-

eye" phenotype (Figure S3C). Since these newt genes have signal peptides (Looso et

al., 2013), it is possible that these genes may have non-autonomous effects.

Ectopic expression of newt genes exhibits non-autonomous rescue

To determine if these genes have non-autonomous effect, we misexpressed these

genes within a subset of retinal neuron population in the developing eye field of L^2

mutant and assay their regeneration effect. Misexpression of *C4* using *dorsal-eye -*

GAL4 (*DE* - GAL4) (Figure 1B) directs its expression in the dorsal half of the developing

eye (Morrison and Halder, 2010) (Figure 2A, E). The rationale of this experiment was to

misexpress *C4* in the dorsal half of the eye and test its effect on an eye mutant which

results in the loss of the ventral half of the developing eye (Figure 2). Misexpression of

both GFP (reporter) and newt *C4* in the dorsal domain of the developing eye, which

serves as a positive control (+/+*; DE>GFP+C4)* (Figure 2A, B, B′, B″), exhibits a near

wild-type eye. As a negative control, we tested GFP expression alone in L^2-mutant

background (L^2/+*; DE>GFP)* (Figure 2C, D, D', D"). We found that GFP expression was

restricted to the dorsal half as seen in the wild-type control (Figure 2B, B', B").

Furthermore, we did not see any rescue of *L* mutant phenotype of ventral eye loss

(marked by red dotted boundary, Figure 2B, B′, B″). Misexpression of both GFP+C4 in

the dorsal half of the L^2 mutant eye (L^2/+*; DE>GFP+C4)* exhibits a significant rescue of

the loss-of-ventral eye phenotype as seen in the adult eye, and the eye imaginal disc

(marked by white-dotted boundary, Figure 2E, F, F′, F″). Even though the targeted

misexpression of C4 is restricted to the dorsal half of the developing eye, it was able to

rescue the loss-of-ventral eye phenotype suggesting that newt genes can show non-

cell-autonomous rescue of *Drosophila* eye mutants.

In order to rule out any domain specific restraint, or contribution of a *DE*-GAL4

background, we employed genetic mosaic approach. We generated gain-of-function

clones using Flp out approach (Xu and Rubin, 1993), which result in clones of cells that

misexpress high levels of the transgene and these clones are marked by the GFP

reporter (Figure 2G,H,I,J). We found that the misexpression of these transgenes

rescues the loss-of-ventral eye phenotypes, however these rescues extended beyond

the boundary of the clone. This data validate our prior observation that these newt gene

exhibit non-cell-autonomous rescue due to their secretory nature. Next, we wanted to

investigate the mechanism by which these newt genes can rescue loss-of-ventral eye

phenotype.

Newt genes promote cell proliferation and downregulate cell death

To test the possibility of upregulation of cell proliferation resulting in the rescue of the

missing tissue in the L^2 mutant background, we calculated relative fold change in mitotic

index (MI) of the eye imaginal discs showing rescue of *L* mutant phenotype and

compared it to the control L^2 mutant (L^2; *ey*-GAL4) eye disc (Figure 3A-D). Mitotic index

is the ratio between the number of proliferating cells to the rows of ommatidial cells

(Baonza and Freeman, 2005; Vollmer et al., 2016; Yang et al., 2005). The proliferating

cells are marked by anti-phospho-Histone H3 (PH3) marker (Figure 3A-C, A′-C′ and

Figure S4A-F, A′-F′)**.** Phosphorylation of a highly conserved Serine residue (Ser-10) in

the histone H3 tail is considered a crucial event for the onset of mitosis, and represents

completion of cell cycle (Crosio et al., 2002; Kim et al., 2017). Our results show robust

upregulation of about 5.79 ± 1.5 folds increase in the MI when *C4* is misexpressed in

the L^2-mutant background (L^2; *ey>C4*) with respect to the L^2-mutant (L^2; *ey*-GAL4) only

(Figure 3D). Similar phenotypes were observed with other transgenes (Figure S4 A-G)

and the MI values were *C3*: 5.18 ± 1.10, *C2:* 4.6 ± 1.7, *C1*: 4.3± 1.4, and *C5*: 4.2 ± 0.9

respectively**.** These results show that the newt gene(s) can induce cell proliferation to

restore the missing photoreceptor cells of L^2-mutant eye disc and thus exhibit significant

rescue of the loss-of-ventral eye phenotype as seen in the eye imaginal disc and the

adult eye (shown in white dotted boundary, Figure 3B). The control where no newt gene

is misexpressed, in the L^2 mutant background did not show any rescue of the loss-of-

ventral eye phenotype due to lack of cell proliferation as evident from PH3 staining

(shown in red dotted boundary, Figure 3A).

During the newt lens regeneration both cell proliferation and apoptosis are seen

(Tsonis et al., 2004), which warranted the need to look at the levels of cell death. It has

been shown that the loss-of-ventral-eye phenotype observed in L^2 mutation is due to

induction of both caspase-dependent and caspase-independent cell death (Singh et al.,

2006). Therefore, we tested if these newt genes can block or downregulate cell death to

rescue L^2 mutant phenotype by using the TUNEL (Terminal deoxynucleotidyl

transferase dUTP nick end labeling) assay (Figure 3E-G, and S4 H-M). TUNEL assay is

based on the detection of fragmented DNA ends, which are characteristic of apoptotic

cells (Sarkissian et al., 2014; White et al., 2001). Our results clearly demonstrate strong

decrease in the number of dying cells (0.28 ± 0.04 fold) when newt gene (*C4*) is

misexpressed under the L^2 mutant background (L^2; ey>C4) (Figure 3F, F', H) relative to

the L^2-mutant strain in which newt gene is not misexpressed (L^2; ey-GAL4) (Figure 3E,

E', H). The positive control (ey-GAL4) shows 0.32 ± 0.04 fold downregulation in the rate

of cell death (Figure 3G, G', H) relative to the L^2 mutant (L^2; ey-GAL4). The value is

significantly lower in comparison to the L^2 mutant (L^2; ey-GAL4), but approximately

equivalent to the L^2 mutant where newt gene is misexpressed (L^2; ey>C4) (Figure 3H).

This suggests that newt gene(s) can recue the L^2 mutant loss-of-ventral eye phenotype

to wild-type by preventing excessive cell death of photoreceptor cells (marked in white

dotted boundary, Figure 3 F, F'). We found similar results when other members of novel

newt gene family were misexpressed (Figure S4 H-O). Downregulation in cell death is

observed in C3: 0.36 ± 0.14, C2: 0.38 ± 0.09, C1: 0.39 ± 0.15, and C5: 0.45 ± 0.22

respectively (Figure S4N). We also tested the role of cell death using Dcp1 (Death

caspase-1) staining (Figure S5). Antibody against cleaved Dcp1 marks the dying cells in

Drosophila (Sarkissian et al., 2014). Dcp1 staining exhibits reduction in the number of

dying cells when newt genes are misexpressed in L^2 mutant background (Figure S5 C-

F), compared to when newt genes are not expressed in L^2 mutant background (Figure

S5 B). These results suggest that newt genes upregulate cell proliferation and

downregulate cell death to significantly rescue the L^2 mutant phenotype of loss-of-

ventral eye.

We further validated the role of cell death by blocking caspase dependent cell

death using baculovirus p35 misexpression (Hay et al., 1994). Even though

misexpression of *p35* in L^2 mutant background (L^2/+; ey>p35) exhibits rescue of loss-of-

ventral eye phenotype, the phenotype strength (relative eye size ratio of 1.42 ± 0.29 and

rescue frequency of 22.7%) of L^2/+; ey>p35 was significantly weaker than phenotype

strength (relative eye size ratio = 2.34 ± 0.51 and rescue frequency of 58.2%) of L^2/+;

ey>C4 (Figure S6 C, D, F, G). This data suggests that the newt genes upregulate cell

proliferation and block cell death as well, but cell proliferation may be playing significant

role in rescuing the missing tissues in *the* L^2 mutant eye. It is therefore important to

understand what signal is triggered by these newt genes to promote the rescue.

Newt genes downregulate *wg* expression in developing eye

We screened for the signaling pathway(s) that are involved in the rescue of L^2 mutant

loss-of-ventral eye phenotype.   We identified members of evolutionarily conserved

Wg/Wnt signaling pathway in our high throughput screen (RNA sequencing) where newt

genes were misexpressed in fly tissue (Figure S7).   It is reported that L^2 mutant

phenotype can be rescued by downregulating *wg* (Singh et al., 2006). To determine the

involvement of *wg* in promoting rescue of L^2 mutant phenotype by unique newt genes,

we examined expression levels of Wg in larval eye-antennal imaginal disc (Figure 4A-C,

A′-C′, D). Wg expression is seen at antero-lateral margins of the developing eye

imaginal disc (Baker, 1987; Cavodeassi et al., 1999; Singh et al., 2006). In L^2 mutant

robust ectopic expression of Wg is seen at the ventral eye margins (Figure 4B, B′)

(Singh et al., 2006; Singh et al., 2011). Misexpression of newt gene (*C4*) in L^2 mutant

background downregulates the Wg expression at the ventral margin of eye-antennal

imaginal disc (Figure 4C, C′), as evident from the signal intensity plots (integrative

density) in various backgrounds (Figure 4D). The semi-quantitative western blot,

showed that Wg levels increased 3 folds in L^2 mutant as compared to *ey-GAL4* control

whereas misexpressing *C4* in the L^2 mutant background decreased the Wg expression

by 1.6-fold as compared to L^2 mutant (L^2*; ey*-GAL4) (Figure 4 E, F). Similar trends were

seen with other novel newt genes (C1, C2, C3, and C5) when misexpressed in L^2

mutant background (Figure S8 C-F, C′-F′, G, G′). Thus, the newt genes can

downregulate *wg* expression in *Drosophila* L^2 mutant to partially rescue ventral eye-

loss-phenotype.

To further test our hypothesis, we misexpressed *wg* (the target identified in our

screen) along with *C4* transgene in the L^2 mutant background. As control, we

misexpressed *wg* in the developing eye (*ey>wg*), which result in reduced eye phenotype

(Figure 5 A, B) (Lee and Treisman, 2001; Singh et al., 2012a). Misexpressing *C4* in the

wild-type background where *wg* is ectopically induced (+/+*; ey>wg+C4*), rescues the

reduced eye phenotype (Figure 5 C, D) at a frequency of 16.8% (Figure S9 and Table

S1). Misexpression of *wg* in the L^2/+ mutants eye background (L^2/+*; ey>wg*) completely

eliminate the eye field as seen in the eye imaginal disc and the adult eye (Figure 5E, F)

whereas, misexpressing *C4* along with *wg* in the *L²*/+ mutant eye disc (*L²/+; ey>wg; C4*)

partially rescues the loss-of-ventral eye phenotype (Figure 5 G, H) in 21% flies (Figure

S9 and TableS1). Thus, newt genes can even downregulate Wg expression in the wild-

type (*+/+; ey>wg+C4*; Figure 5 C, D) as well as *L²* (*L²/+; ey>wg; C4*; Figure 5 G, H)

background(s) where *wg* was ectopically co-expressed. *Drosophila* eye to promote

rescue of the loss-of-ventral eye phenotype.

Newt genes negatively regulate Wg/Wnt signaling

To investigate if newt genes regulate expression of *wg* alone or they regulate the Wg

signaling, we tested the antagonists, and agonists of the Wg-signaling pathway. The

rationale was to modulate the levels of Wg signaling and sample its effect on the *L²*

mutant phenotype as well as the *L²* mutant phenotype where *C4* is misexpressed (*L²/+;*

ey> C4) (Figure 6). Increasing the levels of Arm (a vertebrate homolog of β-Catenin, an

agonist of Wg signaling) in the wild-type eye (*ey>arm*) resulted in small eyes (Figure

S10 A, B) and in the *L²*/+ background, (*L²/+; ey>arm)* it results in elimination of the

entire eye field (Figure 6B, C). Misexpressing *C4* along with *arm* in the *L²*/+ mutant

background (*L²/+; ey>arm; C4*) partially rescues the loss-of-ventral eye phenotype

(Figure 6D, E) and the rescue frequency is 22.8% (Figure S11A and Table S1)*.*

Similarly, misexpressing *C4* and *arm* in the wild-type background (*+/+; ey>arm; C4*)

partially rescues the small eye phenotype defect caused by *ey>arm* phenotype (Figure

S10 C, D), and the rescue frequency is 21% (Figure S11A and Table S1). This data

suggests that newt gene can modulate the Wg signaling pathway components to

promote rescue of reduced eye phenotype both in *L²*-mutant as well as the wild-type

background(s). However, the rescue frequency obtained by misexpressing newt gene in

addition to ectopically misexpressing *arm* (*L²/+; ey>arm; C4*, 22.8%) and/or *wg* (*L²/+;*

ey>wg; C4, 21%) (Figure S9, S11A and Table S1) is lower than the rescue frequency

obtained when only newt gene is misexpressed in the *L²*-mutant background (*L²/+;*

ey>arm; C4, 58.2%). This could be due to severity of loss-of-ventral eye phenotype

caused by the combined effect of *L²* mutation and gain-of-function of *wg*, or *arm.*

Blocking Wg signaling by misexpressing *shaggy (sgg)*, an antagonists of Wg

signaling, in the eye (*ey>sgg,* Figure S10E, F) (Hazelett et al., 1998; Singh et al., 2002;

Singh et al., 2006) or along with *C4* in the wild-type background (*+/+; ey>sgg; C4,*

Figure S10 G, H) does not affect the eye size. Misexpression of *sgg* in the L^2/+ mutants

(L^2/+*; ey>sgg*), significantly rescues the loss-of-ventral eye phenotype in 27.3% of flies

(Figure 6F, G, S11B and Table S1) (Singh et al., 2006). Similarly, misexpressing *C4*

and *sgg* in the L^2/+ mutant eye (L^2/+*; ey>sgg; C4*) rescues the loss-of-ventral eye

phenotype (Figure 6H, I) and rescue frequency increased dramatically from 27.3% to

71.5% (Figure S11B and Table S1). The transcription factor dTCF is the downstream

target of Wg signaling, and is downregulated by misexpression of the N-terminal deleted

dominant-negative form of TCF (dTCF^{DN}) (van de Wetering et al., 1997). Misexpression

of dTCF^{DN} alone in the eye (*ey>dTCF^{DN}*, Figure S10I, J) or dTCF^{DN} with *C4* (*+/+; ey>*

dTCF^{DN}; C4, Figure S10K, L) does not affect the eye size. Misexpressing *dTCF^{DN}* in the

eye of L^2/+ mutants (L^2/+*; ey>dTCF^{DN}*) significantly rescues the loss-of-ventral eye

phenotype in 21.8% of flies (Figure 6J, K, S11B and Table S1) (Singh et al., 2006).

Similarly, misexpressing C4 and *dTCF^{DN}* in the eye of L^2 mutants (L^2/+*; ey> dTCF^{DN};*

C4) rescues the loss-of-ventral eye phenotype (Figure 6L, M). As expected rescue

frequency again dramatically increased from 21.8% to 70.6% (Figure S11B and Table

S1).

Our results demonstrate that misexpression of newt genes when Wg signaling is

downregulated (using antagonists of Wg) in L^2 mutant background (L^2/+*; ey> sgg; C4,*

71.5%) or (L^2/+*; ey> dTCF^{DN}; C4*, 70.6%) significantly increased the rescue frequency

of the L^2 mutant phenotype. The rescue frequency obtained is greater than either

misexpressing candidate gene *(L^2/+; ey>C4,* 58.2%) alone or only misexpressing

negative regulators (L^2/+*; ey> sgg*, 27.3%) or (L^2/+*; ey> dTCF^{DN}*, 21.8%). The converse

phenotypes were seen when Wg signaling was upregulated. This strongly suggest that

newt gene(s) interact with the Wg signaling pathway to promote rescue of L^2 mutant

phenotype.

Porcupine (Porc) is involved in post-translational modification of Wg nascent

protein in the producing cell and thus facilitates its transport outside to the receiving cell.

Wg ligand can bind to its receptor on the receiving cell (Manoukian et al., 1995; Swarup

and Verheyen, 2012) thus modulating downstream pathway components (Figure 6A).

We blocked Wg transport from the producing cell using *porc porc^{RNAi}* and sampled its

effect on L^2 mutant phenotype, and L^2 mutant phenotype where C4 is misexpressed.

Misexpression of *porc^{RNAi}* alone (*ey> porc^{RNAi}*) (Figure S10M, N), and along with C4 in

the wild-type background (+/+*; ey> porc^{RNAi}; C4*) (Figure S10O, P) does not affect the

eye size. Misexpressing *porc^{RNAi}* in the L^2 mutant eye (L^2/+*; ey> porc^{RNAi}*) significantly

rescues the loss-of-ventral eye phenotype in 20.8% of flies (Figure 6N, O, S11B and

Table S1) whereas when *C4* is also misexpressed in the eye of L^2 mutants along with

porc^{RNAi} (L^2/+*; ey> porc^{RNAi}; C4*) rescued the loss-of-ventral eye phenotype (Figure 6P,

Q). The rescue frequency increased dramatically from 20.8% to 68.1% (Figure S11B,

and Table S1). Thus, newt genes can modulate levels of Wg signaling to promote

regeneration response in L^2 eye mutants.

Discussion

Notophthalmus viridescens have enormous genome size (~ c × 10^{10} bases), a long

reproductive cycle, and have limited genetic tools that makes it difficult to use this model

to ascertain the molecular mechanism(s) behind the function of its novel genes (Mehta

and Singh, 2019a). One of the strategies can be to introduce these genes into models

with array of genetic tools and where regeneration potential is lower as seen in models

like *Drosophila, Bombyx mori* etc. (Gopinathan et al., 1998; Kango-Singh et al., 2001;

Mehta and Singh, 2019a; Singh et al., 2007). The rationale is to test the efficacy of

these novel newt genes in triggering regeneration response in these model systems

with lower regeneration potential. We and others have introduced human, plant and

other vertebrate genes by transgenic approaches in *Drosophila* (Deshpande et al.,

2019; Gogia et al., 2020b; Hughes et al., 2012; Sarkar et al., 2018; Tare et al., 2011).

These transgenic flies have been utilized for targeted expression of foreign genes in

flies using a targeted misexpression approach (Brand and Perrimon, 1993).   The

successful misexpression of the unique newt genes (vertebrates) in *Drosophila* using

the same targeted misexpression approach has been reported earlier (Mehta et al.,

2019; Mehta and Singh, 2019b).

To test the regeneration potential of these novel newt genes, we took a distinctive

approach of using *Drosophila* model because the signaling pathways, which are

involved in regeneration and/or tissue growth, patterning and development are

evolutionarily conserved across the species (Kango-Singh and Singh, 2009; Mehta and

Singh, 2019a; Wang and Hu, 2020). Even though humans *(Homo sapiens),* which

evolved approx. 200,000 years ago (Stringer, 2016) and are 541 million years apart

from *Drosophila* still share 75% of genes with flies (Pandey and Nichols, 2011)*.* Despite

the gap of 250 million years between newts and *Drosophila*, our studies suggest that

the proteins encoded by unique newt genes are functional in *Drosophila* (Figure 1).

Therefore, it is plausible that the pathways that newt genes can modulate in *Drosophila*

might share parallels with their mechanism of action in newts. We found that the five

newly identified genes from newt, can (i) rescue *L* mutant eye phenotype (early eye

mutant) as well as GMR-*hid* GMR-GAL4 (late retinal degeneration) phenotype, (ii)

promote cell proliferation, and downregulate cell death to restore loss-of-ventral eye
phenotype, and (iii) downregulate Wg signaling (Figure 7). Our studies clearly
demonstrate the regeneration potential of these novel newt genes in triggering rescue
response in *Drosophila* model along the temporal axis.

Newt genes promote cell proliferation to restore *Drosophila* eye mutant
445
phenotype

Regeneration involves the formation of a blastema, a proliferating zone of
undifferentiated cells to restore the missing structures (Brockes and Kintner, 1986;
Mehta and Singh, 2019a; Sanchez Alvarado and Tsonis, 2006). Furthermore, a network
of newt gene(s) trigger proliferation response to promote lens regeneration (Eguchi and
Shingai, 1971; Grogg et al., 2005; Looso et al., 2013; Madhavan et al., 2006) A similar
proliferating response was observed when newt genes were misexpressed in the
Drosophila L^2 mutant eye (Figure 3C, and Figure S4). L^2 mutant flies exhibit a loss-of-
ventral eye phenotype, and misexpressing newt genes causes 4.2-5.8 fold increase in
number proliferating cells (Figure 3, 7 and Figure S4), which clearly mimics the classic
mechanism of epimorphic regeneration in the newt lens.

In our study, we found that newt genes can also modulate cell death in
Drosophila developing eye. When newt genes were misexpressed in the L^2 mutant
background (L^2; *ey>newt gene*), the rate of cell death was lower as compared to the L^2
mutant (L^2; *ey*-GAL4) eye (Figure 3E, F, H and Figure S4H-L, N), but minimally higher
compared to the positive control (*ey*-GAL4) (Figure 3F, G, H and Figure S4I-N). A
minimal rate of apoptosis is observed during the newt lens regeneration (Tsonis et al.,
2004). Our data suggest that newt genes hijack the evolutionarily conserved signaling
pathway(s) in *Drosophila* to regulate cell proliferation, and cell death. It is a strong
possibility that the same pathways may promote regeneration in newts.

Newt genes tightly regulate Wg signaling pathway in *Drosophila*

We screened for the various signaling pathways using the candidate approach as well as compared them to our transcriptomic analysis (Mehta et al., 2019). Among the various genetic modifiers of the L^2 mutant phenotype, we found *wg* as one of the possible candidate. Wg, a member of the evolutionarily conserved Wg/Wnt pathway, has been previously reported to promote regeneration, growth, development etc. (Harris et al., 2016; Schubiger et al., 2010; Singh et al., 2012b; Singh et al., 2018; Smith-Bolton et al., 2009; Yokoyama et al., 2007). Misexpresion of newt genes in *Drosophila* wild-type background modulate both agonists of Wg/Wnt signaling like *frizzled (fz),* and *microtubule star,*(*mts*) and antagonists like *Casein Kinase IIβ* (*CKII*β), and *sinaH* (Figure S7). This modulation in the levels of both antagonists as well as agonists can be a homeostatic reaction shown by the *Drosophila* to normalize expression levels of Wg/Wnt signaling pathway. As a result, changes in the eye growth after misexpressing newt genes in the wild-type was non-significant when compared to the wild-type control (*ey*-GAL4) (Figure 1). We did not observe any lethality, developmental defects etc.

Interestingly, when newt genes were misexpressed in L^2 mutant background, there was significant downregulation of Wg/Wnt expression (Figures 4, 7, and Figure S8), which resulted in rescue of the loss-of-ventral eye phenotype. It is known that overexpressing Wnt, and its receptor Fz promote lens regeneration in newts, whereas blocking Wnt pathway by expressing Dkk1 inhibits lens induction (Hayashi et al., 2006). Therefore, our study may have parallels with the regeneration mechanism in newts.

Furthermore, we found that when Wg pathway components are modulated in *Drosophila* along with the newt genes, it not only rescue the L^2 mutant loss-of-ventral eye phenotype but can also rescue the eye-loss caused by the activation of the Wg signaling pathway in the wild-type background. Hence, our data strongly suggest that the newt genes can modulate Wg signaling pathway in loss-of-ventral eye background. Interestingly, these newt genes did not show any phenotype when they were

496 misexpressed alone suggesting that regeneration response is triggered only to the cues

497 generated due to tissue damage.

Newt proteins exhibit non-cell-autonomous response

Genetic mosaic experimental approach, which resulted in the generation of clone(s) of

retinal neurons expressing high levels of newt genes within the L^2 mutant eye disc.

These experiments showed that clones expressing high levels of newt genes can

rescue the L^2 mutant phenotype, however the rescue was not limited to the clone itself

and spread into the neighboring cells where newt genes were not misexpressed. This

non-cell-autonomous rescue of the missing tissue is possible only if the newt proteins

are getting secreted from one cell to another and/or newt proteins are acting on the

signaling molecules. These newt proteins have signal peptides (Looso et al., 2013),

which can facilitate its transport from one cell to another. Similarly, we have reported

that these genes interact with Wg (Figure 4), which is a morphogen that can have a long

distance effect in the *Drosophila* eye (Zecca et al., 1996a).

It will be interesting to investigate, (a) if newt genes can regulate Wg signaling

pathway to promote regeneration in other tissues of *Drosophila* e.g. wing, leg etc., and

under the background of other congenital mutations (Schubiger et al., 2010; Smith-

Bolton et al., 2009), (b) if these proteins can rescue/regenerate missing tissues/organs

in other animals with low regenerative potentials. The pathways that have roles in

regeneration, growth, development are evolutionarily conserved throughout the animal

kingdom (Mehta and Singh, 2019a). For example, the Wg/Wnt signaling pathway, is

one such evolutionarily conserved pathway that apart from promoting regeneration in

hydra, planaria, newts, zebrafish, etc. has also been found to promote cell proliferation

during regeneration of mammalian muscles, liver, and bone (Polesskaya et al., 2003;

Sodhi et al., 2005; Zhong et al., 2006). Therefore, it raises the possibility that genetically

engineering genes from the highly regenerative animals that show regeneration in a

precisely controlled fashion might systematically regulate evolutionarily conserved

pathways in a foreign host to promote tissue regeneration and/or cell survival.

Materials and methods

Animals

Handling and operations of *Notophthalmus viridescens* (newts) have been described
previously (Mehta et al., 2019; Sousounis et al., 2013). For our studies, *Notophthalmus*
viridescens (newts) were purchased from Charles Sullivan Inc. newt farm. All
procedures involving animals were approved by the University of Dayton Institutional
Animal Care and Use Committee (IACUC; Protocol ID: 011-02). Newts were
anesthetized in 0.1% (w/v) ethyl-3-aminobenzoate methanesulfonic acid (MS222;
Sigma) dissolved in phosphate buffered saline. The surgical procedures were
performed in anesthetized with MS222 newts, and appropriate procedures were
undertaken to alleviate pain and distress while working with newts.

Generation of transgenic flies

The design and generation of transgenic flies (UAS-C1, UAS-C2, UAS-C3, UAS-C4,
and UAS-C5) used in this study have been described (Mehta et al., 2019). To validate
the successful insertion of newt genes in *Drosophila* by targeted misexpression
approach, we generated transgenic fly like UAS-C1-5-V5 with 14 aa long V5-epitope
sequence tagged towards the 3' end of the C4 ORF. The complete gene fragment (5'-
C4-V5-3') was synthesized, and cloned into the XbaI and XhoI restriction sites in
pUAST-attB plasmid. The pUAST-attB plasmid containing both the transgene and donor
sequence (attB) was co-injected along with ϕC31 integrase mRNA into attP-containing
recipient embryos, resulting in site-specific insertion of the transgene at Chromosome III
(Fish et al., 2007; Groth et al., 2004). The integration and expression of transgene was
verified by the western blots using V5 antibody.

Drosophila stocks

In this study, we used transgenic flies UAS-C1, UAS-C2, UAS-C3, UAS-C4, UAS-C5,
and UAS-C1-5-V5). Other fly stocks used in this study are described in Flybase
(http://flybase.bio.indiana.edu). We used _Tub_-GAL4/_S-T_ (Yang et al., 2019)_, L^2/CyO;_
_ey_-GAL4/_ey_-GAL4, L^2/C4; ey_-GAL4/_ey_-GAL4_, UAS-GFP_ (Singh et al., 2005; Singh and
Choi, 2003; Singh et al., 2006). _DE_-GAL4/_CyO; UAS-GFP/Tb_ (Morrison and Halder,
2010), _UAS-wg-GFP_ (Azpiazu and Morata, 1998) , _UAS-arm (Zecca et al., 1996b),_
UAS-sgg (Hazelett et al., 1998)_, UAS-dTCF^{DN}_ (van de Wetering et al., 1997) _, UAS-p35_
(Hay et al., 1994).

Targeted misexpression studies

We used the GAL4/UAS system for targeted misexpression studies (Brand and
Perrimon, 1993). All GAL4/UAS crosses were performed at 25°C. In order to
misexpress the UAS-transgenes, we used _tub_ –GAL4 (Yang et al., 2019) to ubiquitously
misexpress genes_, ey_-GAL4 (Hazelett et al., 1998) to misexpress transgene in the
developing eye, and _DE_-GAL4 (Morrison and Halder, 2010) that drives transgene in the
dorsal domain of the developing eye.

Genetic mosaic analysis

Clones were generated using the FLP/FRT system of mitotic recombination (Xu and
Rubin, 1993). To generate clones of C4 _in L^2_-mutant eye, _yw_ hsFLP; Act>y+>GAL4,
UAS-_GFP_/_CyO_ females were crossed to L^2/CyO;_ UAS-_C4/Tb_ males. C4 is expressed in
all the GFP+ cells of eye-antennal imaginal disc.

Immunohistochemistry

Eye-antenna discs were dissected from wandering third instar larvae and stained
following the standard protocol (Singh et al., 2006). The eye imaginal discs were
dissected in cold 1X solution of Phosphate Buffered Saline (PBS) and fixed using 4% p-
formaldehyde (Electron Microscopy Sciences, Catalog no. 15710). Fixed tissues were

washed using PBS-T [0.2% Triton X-100 (Sigma, Catalog no. T8787) in 1XPBS] and

immunostained using primary antibodies: mouse anti-Phospho histone-3 (1:200)

(Sigma-Aldrich); Rabbit anti- *Drosophila* caspase-1 (DCP-1) (1:100) (Sigma-Aldrich);

rat anti-Elav (1:100) (Developmental Studies Hybridoma Bank); mouse anti-Wg (1:100)

(Developmental Studies Hybridoma Bank), and goat anti-Hth (a gift from H. Sun and R.

Mann). Secondary antibodies (Jackson Laboratories) used were goat anti-rat IgG

conjugated with Cy5 (1:200); donkey anti-rat IgG conjugated to FITC (1:200); donkey

anti-rat IgG conjugated to Cy5 (1:200), donkey anti-mouse IgG conjugated to Cy3

(1:200); donkey anti-goat IgG conjugated to Cy3 (1:200), and goat anti-rabbit IgG

conjugated with Cy5 (1:200). The discs were mounted in Vectashield (Vector labs,

H1000) and imaged using Olympus Fluoview 1000 microscope.

Western Blot

In this study, two different western blots were run. For blot 1, protein samples were

prepared from ($n = $ ~10) the wandering third instar larvae of transgenic fly UAS-C4-V5

as experimental sample, and *ey*-GAL4 was used as control. For blot 2, protein samples

were prepared from ($n = $ ~25) the adult heads of *ey*-GAL4, L^2*; ey*-GAL4 and L^2*; ey>C4*.

Protein samples were isolated by standardized protocol (Gogia et al., 2017), ran on

10% gel, and then transferred onto nitrocellulose membranes. For blot 1, the membrane

was then incubated overnight at 4°C with primary antibodies: 1:15000 rabbit anti-V5

antibody (SIGMA, Cat. # V8137), and 1:12000 mouse anti-α-Tubulin antibody (SIGMA,

Cat. # T5168). Followed by 1h incubation with secondary antibodies: horseradish

peroxidase conjugated goat anti–rabbit IgG (1:5000 dilution) secondary antibody, and

goat anti-mouse IgG-HRP (1:5000 dilution) (Santa Cruz Biotechnology, Cat. Sc-2005).

For blot 2, the membrane was then incubated overnight at 4°C with primary antibody

1:12000 mouse anti-α-Tubulin antibody (SIGMA, Cat. # T5168). Followed by

1h incubation with secondary antibody: horseradish peroxidase conjugated goat anti-

mouse IgG-HRP (1:5000 dilution) (Santa Cruz Biotechnology, Cat. Sc-2005). The blot

was then stripped and then incubated overnight with primary antibody 1:1000 mouse

anti-Wingless antibody (DSHB, Cat. # 4D4). Followed by 1h incubation with secondary

antibody 1:5000 horseradish peroxidase conjugated goat anti-mouse IgG-HRP (Santa

Cruz Biotechnology, Cat. Sc-2005). The signal in both the blots were detected using
Super Signal West Dura Extended Duration Substrate (ThermoFisher Scientific, Cat.
#34076). Images were captured using the UVP Imaging System. Wg levels were
quantified and normalized by using Licor Image Studio lite 5.2 software and this was
followed by statistical analysis.

TUNEL assay for detection of cell death

Apoptosis was detected by using Terminal deoxynucleotidyl transferase-mediated
dUTP nick-end labeling (TUNEL) assay (Tare et al., 2011; White et al., 2001). TUNEL
assay is based on the detection of fragmented DNA ends, which are characteristic of
apoptotic cells. A deoxynucleotidyl transferase (TdT) is used to add fluorescently
labeled nucleotides to free 3'-OH DNA ends in a template-independent manner. The
modified nucleotides can then be detected by fluorescence microscopy. Eye-antennal
discs, after secondary-antibody staining were blocked in 10% normal goat serum in
phosphate buffered saline with 0.2% Triton X100 (PBT) for an hour. After blocking,
samples were incubated in 0.1 M sodium citrate (Fisher Scientific) and 10% TritonX-100
for 30 minutes in dark at 65°C. Samples were washed and incubated in TUNEL dilution
buffer (Roche Diagnostics, Catalog no. 11966006001) for 10 minutes at room
temperature. Followed by labeling with 50% TUNEL labeling solution for 30 minutes
(Roche Diagnostics, Catalog no. 12156792910) in dark at room temperature, and finally
samples were subjected to TdT enzyme solution (Roche Diagnostics) for 2 hours at
37^{0}C. After which samples were washed with PBS-T and mounted in Vectashield
(Vector labs, Catalog no. H1000).

Bright Field imaging

Adult flies were selected and were transferred to -20^{0}C for two hours, followed by
removal of legs and wings. The legs and wings were removed in order to get a clear
view of the compound eye. The Bright field pictures of adult heads were taken using a
Zeiss Apotome Imager Z1 (Wittkorn et al., 2015). Eye size was measured using ImageJ
software tools (http://rsb.info.nih.gov/ij/). To normalize the eye size values and neglect

any variations caused by overall different head size we took ratio of eye size to the head

size for each individual picture. Then relative ratio was calculated for all the treatments,

and the value reported is the relative fold change for each experimental treatment

compared to the control.

Statistical analysis

Statistical analysis was performed using student's t-test for independent samples.

Sample size used for the calculation was five in number (n=5), otherwise specified.

Statistical significance was determined with 95% confidence ($p < 0.05$).

Acknowledgments

We thank Bloomington *Drosophila* Stock Center (BDSC) for *Drosophila* strains, and the
Developmental Studies Hybridoma Bank (DSHB) for antibodies. We would like to thank
to Katia Del Rio-Tsonis, Labib Rouhana, Madhuri Kango-Singh, Meghana Tare, Neha
Gogia, Shilpi Verghese, Pothitos Pitychoutis, Deepika K Sodhi for critical comments on
the manuscript. We also thank Justin Kumar, Y. Henry Sun, and Kyung Ok Cho for gift
of fly strains and antibodies. Confocal microscopy was supported by core facility at
University of Dayton.

Funding resources

655 A.S. is supported by NIH1R15GM124654-01 from NIH, Schuellein Chair Endowment
656 Fund and STEM Catalyst Grant from the University of Dayton.

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Figure legends:

Figure 1. Novel newt genes rescues L^2 mutant's loss-of-ventral eye phenotype.

(A, B) Schematic representation of generating transgenic *Drosophila* carrying novel

newt (*Notophthalmus viridescens*) candidate genes (*C1, C2, C3, C4,* and *C5*).

Bright field image of a (C) wild type adult eye as positive control (*ey*-GAL4), (D-H) adult

eye phenotype when *ey*-Gal4 drives expression of newt transgenes (D) UAS-*C1*

(ey>C1), (E) UAS-*C2* (*ey>C2*), (F) UAS-*C3 (ey>C3)*, (G) UAS-*C4 (ey>C4)*, and (H)

UAS-*C5 (ey>C5)*. (I) The bar graph shows there is no significant (ns) change in eye size

between control and where ey-GAL4 drives expression of newt transgenes (*ey>C1-C5*).

Bright field image of a (J) L^2 mutant (*L^2; ey*-GAL4*)* adult eye where there is preferential

loss-of- ventral eye phenotype, (K-O) L^2 mutant (*L^2; ey*-GAL4*)* background where newt

transgene (K) UAS-*C1 (L^2; ey >C1)*, (L) UAS-*C2 (L^2; ey >C2)*, (M) UAS-*C3 (L^2; ey >C3)*,

(N) UAS-*C4 (L^2;ey>C4)*, and (O) UAS-*C5 (L^2;ey>C5)* are expressed in the developing

eye using ey-GAL4. (P) L^2 mutant adult eye is half the adult eye size of the *ey*-GAL4

which serve as positive control. The bar graph clearly displays the significant increase in

the adult eye size of L^2-mutant background where unique newt genes were

misexpressed compared to the adult eye size of the L^2-mutant only (*L^2; ey*-GAL4).

Eye size was measured using ImageJ software tools (http://rsb.info.nih.gov/ij/). Sample

size used for statistical analysis was five (n=5). Statistical analysis was performed using

student's t-test for independent samples. Statistical significance was determined with

95% confidence ($p<0.05$). Error bars show standard deviation, and symbols above the

error bar signify as: ns is non-significant p value, * signifies p value <0.05, ** signifies p

value <0.005, *** signifies p value <0.0005 respectively. In this study *ey* is used as a

driver to misexpress *newt genes* in the developing *Drosophila* eye. All the images are

displayed in same polarity as dorsal domain-towards top, and ventral domain-towards

bottom. Scale bar= 100 **μm.**

See also Figure S1, S2, and S3 and Table S1

Figure 2. Misexpression of newt genes exhibits non-cell-autonomous rescue. (A,

B, B′, B″) Misexpression of *C4* in the dorsal half using *DE*-GAL4 driver results in (A)

Adult eye and (B, B′, B″) developing third instar larval eye imaginal disc (as positive

control). Note that Green fluorescence protein (GFP) (green) reporter marks the DE-

GAL4 driver domain and proneural marker Embryonic lethal abnormal visual system

(Elav) (red) marks the retinal fate. (B′) is a single channel confocal image showing GFP

expression, and (B″) is a single channel confocal image showing Elav expression. (C,

D, D′, D″) L^2 mutant eye where there is no misexpression of newt genes (L^2/+*;*

DE>GFP) (as negative control). (C) Adult eye bright field image and (D, D′, D″) third

instar eye disc of L^2 mutant (L^2/+*; DE*>GFP) exhibiting loss-of-ventral eye phenotype

and red dotted line marks the boundary of eye. GFP reporter (green) marks domain of

DE-GAL4 expression domain in L^2 mutant background. . (E, F, F′, F″) Misexpression of

C4 in dorsal eye domain of L^2 mutant (L^2/+*; DE*>GFP; C4) exhibits non-cell autonomous

rescue of loss-of-ventral eye phenotype in (E) Adult eye (white dotted boundary) and

(F′, F′, F″) third instar eye disc. White dotted boundary marks the significantly rescued

loss-of-ventral eye phenotype.   (G-J) Genetic mosaic "Flp out" somatic clones of

(G,G′,G″) newt gene C4 in the developing eye disc wild-type, (H,H′,H″) GFP in L^2

background (L^2/+*; hsflp*>GFP, served as the negative control) and (I,I′,I″ & J,J′,J″) newt

gene *C4* clones in L^2 mutant (L^2/+*; hsflp*>GFP*;C4*) background. All clones are marked

by GFP reporter (green). Note that misexpressing *C4* rescue missing photoreceptor

cells in a non- cell-autonomous manner, as photoreceptor cells rescue marked by Elav

(in red) extends outside the boundary of the clone marked by GFP (in green). (J, J′, J″)

exhibits the magnified view of the *C4* misexpressing clone (green) in L^2/+*; hsflp*>GFP*;*

C4 background. All the images are displayed in same polarity as dorsal domain-towards

top, and ventral domain-towards bottom. Scale bar= 100 **μm,**

Figure 3. Newt genes promote cell proliferation and downregulate cell death to

promote rescue of L^2 mutant phenotype of loss-of-ventral eye. (A-D) PH-3 staining

(blue), as a marker to calculate cell proliferation and mitotic index, in (A) L^2 mutant eye

disc (red dotted boundary marking eye boundary), (B) L^2 mutant eye disc where C4

gene is misexpressed (L^2; *ey*>C4), and (C) Wild type (*ey*-GAL4) eye disc which serve

as control. A'-C') single channel confocal images demonstrating PH-3 stained nuclei. (B)

White dotted boundary represents the partially rescued loss-of-ventral eye phenotype.

The zone of interest that was considered to count number of dividing cells is marked in

yellow dotted boundary. (D) Bar graph showing relative increase in the mitotic index

(MI) when newt genes are misexpressed in L^2 mutant background. MI is calculated as

ratio of cells dividing (stained by PH-3 in blue) to total rows of ommatidial cells (stained

by Elav in red). There is no significant change in MI between the L^2 mutant, and wild-

type (*ey*-GAL4) eye disc, but comparatively there is robust increase in the MI inL^2;

ey>C4 background. (E-H) Terminal deoxynucleotidyl transferase-mediated dUTP nick-

end labeling (TUNEL) staining as a marker for cell death assay. TUNEL staining

(shown in green) marks the dying nuclei in eye imaginal disc of (E) L^2 mutant, (F) L^2

mutant where C4 gene is misexpressed (L^2; *ey*>C4), and (G) Wild type (*ey*-GAL4) eye

disc. Red dotted boundary represents ventral-eye-loss area, and white dotted boundary

represent the rescued loss-of-ventral eye region. (E'-G')The tunnel positive cells are

counted within the zone of interest marked within yellow dotted boundary as shown in

single filter confocal images. (H) bar graph showing relative decrease in the cell death

when newt gene is misexpressed under the L^2-mutant background. The downregulation

in cell death is comparable to the cell death happening in the normal wild type

background

Statistical analysis was performed using student's t-test for independent samples.

Sample size used for the calculation was five in number (n=5). Statistical significance

was determined with 95% confidence (p<0.05). All bar graphs show relative fold change

in cell proliferation and cell death as average between 5 samples. Error bars show

standard deviation, and symbols above the error bar ** signify p value <0.005,   ***

signify p value <0.0005, and ns signify p value > 0.05 respectively. All the images are

displayed in same polarity as dorsa domain-towards top, and ventral domain-towards

bottom. Scale bar= 100 **μm**

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Figure 4. Newt genes downregulate Wg expression to rescue loss-of-ventral eye

phenotype. (A-D) Immunohistochemistry showing Wg modulation. (A-C) Eye disc

showing Wg staining in green, and Elav in red (A′-C′) are single channel confocal
images showing Wg expression. The green window is the area of interest that is utilized
to calculate (D, D′) integrative density (intensity plot). The bar graph represent the Wg
intensity at the ventral margin (within the green box) in (A, A′) wild type fly (*ey*-GAL4),
(B, B′) *L²*-mutant fly, (C, C′) Misexpression of newt gene (*C4*) in the *L²* mutation
background. Note that *C4* misexpression downregulates Wg expression. (E, F) Western
blot (WB) to semi quantitate modulation of Wg expression levels. (E) The blot shows the
expression of Wg protein (C4-V5) in *Drosophila melanogaster.* The samples were
loaded in the following sequence: Lane 1- *ey*-GAL4, Lane 2- *L²; ey*-GAL4, Lane 3- *L²;*
ey>C4. Alpha tubulin is used as a loading control. Molecular weight of Wg is 46kD, and
alpha tubulin is 55 kD. The samples were treated with anti-Wg antibody, and anti-α
tubulin antibody. (F) Bar graph representing as a relative Wg level, is a measure of
signal intensity of the bands, which clearly demonstrates that newt genes when
misexpressed under the *L²*-mutant background downregulates Wg levels. Statistical
analysis was performed using student's t-test for independent samples. Statistical
significance was determined with 95% confidence (p<0.05). All bar graphs show
integrative density as a scale to measure Wg intensity for each sample represented as
the average between 5. Error bars show standard deviation, and symbols above the
error bar * signify p value <0.05, and ** signify p value <0.005 respectively. All the
images are displayed in same polarity as dorsal domain-towards top, and ventral
domain-towards bottom. Scale bar= 100 **µm**

1030

1031 **See also Figure S7, S8**

1032

1033 **Figure 5. Newt gene regulates ectopically misexpressed Wg both under the wild**
1034 **type background, and under the *L²*-mutant background.**

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1036 (A-H) Wg is ectopically misexpressed in the *Drosophila* developing eye. (A,B) wild type
1037 background (+/+*; ey>wg-GFP*). (C, D) Newt gene and Wg are misexpressed under the
1038 wild type background (+/+*; ey>wg-GFP;C4*) showing increase in the eye size compared
1039 to (+/+*; ey>wg-GFP*). Comparatively represented as both (A, C) bright field adult eye

picture, and (B, D) third instar eye-antennal imaginal disc confocal image. (E, F) Wg is misexpressed under the L^2–mutant background (L^2/+; *ey>wg-GFP*) that lack newt gene. L^2–mutant small eye phenotype becomes more severe. (G, H) Newt gene and Wg are misexpressed under the L^2-*mutant* background (L^2/+; *ey>wg-GFP; C4*). There is a significant rescue in the eye-loss phenotype. Comparatively represented as both (E, G) bright field adult eye picture, and (F, H) third instar eye-antennal imaginal disc confocal image. The GFP as reporter is in green that signifies ectopic misexpression of Wg in the developing eye. Elav staining is shown in red. All the images are displayed in same polarity as dorsal domain-towards top, and ventral domain-towards the bottom. Scale bar= 100 **μm**

See also <u>Figure S9</u>

Figure 6. Modulating positive and negative regulators of Wg affect *L* mutant phenotype. (A-Q) Schematic presentation of Wg signaling pathway showing various members of the canonical pathway. (B-E) Activating Wg signaling in L^2 mutant eye background by misexpression of (B, C) *arm* alone (L^2/+; *ey>arm*), (D, E) *arm* and *C4* (L^2/+; *ey>arm; C4*). Note that *C4* misexpression with *arm (L^2/+; ey>arm; C4)* in the eye rescues the L^2/+; *ey>arm* reduced eye phenotype. (F-M) Blocking Wg signaling in L^2 mutant eye background by misexpression of (F, G) *sgg* alone (L^2/+; *ey>sgg*), (J, K) $dTCF^{DN}$ alone (L^2/+; *ey> $dTCF^{DN}$*) results in rescue of L^2 loss-of-ventral eye phenotype. However, in L^2 mutant background misexpression of *C4* along with (H, I) *sgg* (L^2/+; *ey>sgg; C4*) and (L, M) $dTCF^{DN}$ (L^2/+; *ey> $dTCF^{DN}$; C4*) significantly rescue the L^2/+; *ey>sgg* and L^2/+; *ey> $dTCF^{DN}$* partial loss-of-ventral eye phenotype.

(N-Q) Blocking transport of Wg morphogen in L^2 mutant eye background by misexpression of (N, O) $porc^{RNAi}$ alone (L^2/+; *ey> $porc^{RNAi}$*), (D, E) $porc^{RNAi}$ and *C4* (L^2/+; *ey> $porc^{RNAi}$; C4*). Note that L^2/+; *ey> $porc^{RNAi}$* exhibits weak rescue of loss-of-ventral eye phenotype whereas *C4* misexpression with *sgg (L^2/+; ey> $porc^{RNAi}$; C4)* in the eye rescues the L^2/+; *ey> $porc^{RNAi}$* small eye phenotype. All the images are displayed in same polarity as dorsal domain-towards the top, and ventral domain-towards the bottom. Scale bar= 100 **μm**

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1072 **See also Figure S10, and S11**

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Figure 7. Model of newt genes function during development. These novel newt

genes (C1-C5) can negatively regulate evolutionarily conserved Wg /Wnt signaling

pathway in *Drosophila*, promote cell proliferation, and block cell death, which together

results in the rescue of L^2 mutant loss-of-ventral eye phenotype. This regenerative

ability of these novel newt genes extend from early eye development to late larval eye

and adult eye.

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Mehta *et al.*, 2021, Figure 1

Generating transgenic flies

Tissue sample collected & total RNA extracted → Newt Candidate gene cloned & 5 transgenic flies generated

UAS-C1
UAS-C2
UAS-C3
UAS-C4
UAS-C5

A

Misexpressing newt genes using GAL4/UAS binary system

♂*tub*-GAL4 X ♀UAS-Ca   ♂*ey*-GAL4 X ♀UAS-Ca   ♂*DE*GAL4 X ♀UAS-Ca

F1 — Newt gene misexpressed ubiquitously
F1 — Newt gene misexpressed in whole eye
F1 — Newt gene misexpressed only in dorsal domain of eye

B

ey>GAL4 — C — 100μm
ey>C1 — D — 100μm
L²;ey-GAL4 — J — 100μm
L²;ey>C1 — K — 100μm

ey>C2 — E — 100μm
ey>C3 — F — 100μm
L²;ey>C2 — L — 100μm
L²;ey>C3 — M — 100μm

ey>C4 — G — 100μm
ey>C5 — H — 100μm
L²;ey>C4 — N — 100μm
L²;ey>C5 — O — 100μm

I — Relative fold change — ns ns ns ns ns — ey-GAL4, ey>C1, ey>C2, ey>C3, ey>C4, ey>C5

P — Relative fold change — *** ** *** *** ** *** — L²;ey-GAL4, L²;ey>C1, L²;ey>C2, L²;ey>C3, L²;ey>C4, L²;ey>C5, ey-GAL4

Mehta *et al.*, 2021, Figure 3

Mehta *et al.*, 2021, Figure 6