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Effects of *Domino* Mutations on Cell Proliferation in the *Drosophila* Eye

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

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Abstract
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Domino is a critical component of the Notch signaling pathway, which regulates cell proliferation. *Domino* has also been implicated as a *Notch*-independent regulator of cell proliferation in the *Drosophila* eye. Recently, it was reported that *domino* may be pleiotropic for cell proliferation in the wing, such that both *domino* loss-of-function and gain-of-function mutations enhance nicking in the wing margin. In the present study, *domino* mutant strains, over-expression strains, and RNAi strains were crossed to an eye hyperproliferation strain (*GMR-YkiS168A*) in order to determine whether *domino* behaves the same way in the eye as it has previously been reported to behave in the wing. *Notch* loss-of-function and gain-of-function mutants were also crossed to the eye hyperproliferation strain. Offspring of the crosses were analyzed for visually-scoreable phenotypic differences, differences in penetrance, and for statistically significant differences in eye size compared to controls. The offspring from both sets of crosses were compared to each other in order to determine whether phenotypes created by the different *domino* strains might be due to Domino's interactions with Notch.

We found that two *domino* RNAi strains and one *domino* over-expression strain showed suppression of the hyperproliferation eye phenotype, but that the remaining strains did not show consistent, statistically significant differences either in eye size or in the penetrance of the hyperproliferation phenotype compared to control crosses. *Notch* loss-of-function strains also showed suppression of proliferation when crossed to w^{1118} (wild-type) or *GMR-GAL4* (over-expression of the enhancer GAL4 in the eye) flies. *Notch* gain-of-function mutations were lethal when crossed to *GMR-YkiS168A*. When *Notch* loss-of-function mutants were crossed to *GMR-YkiS168A*, the resulting offspring had different eye phenotypes than those of the *GMR-YkiS168A x domino* experimental strains, suggesting that Domino's effects on cell proliferation may be independent of Notch.

It is not possible to definitively conclude that *domino* behaves the same way in the eye tissue as it does in the wing tissue. Our results that *domino* loss-of-function and *domino* gain-of-function suppress hyperproliferation in the eye, however, are congruent to previous observations that *domino* RNAi and *domino* over-expression strains both enhance a hypo-proliferation phenotype in the wing.

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Acknowledgements

I would like to thank Dr. Yedvobnick for his guidance and support, and Matt Kwon for teaching me the technical aspects of fruit fly experiments. I would also like to thank my committee members, Dr. Moberg and Dr. Beck, for their feedback on the project.

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Introduction

The Notch signaling pathway is evolutionarily conserved and is used by both invertebrate and vertebrate organisms, but has been studied in detail mainly in invertebrates (*Drosophila melanogaster*, *Caenorhabditis elegans*, sea urchins, etc.) (Artavanis-Tsakonas et. al. 1999). This pathway is critical to embryonic development from the early stages onward, and is responsible for regulating gene expression in the developing organism (Gerhart 1999). Notch has been implicated in the regulation of cell proliferation, cell differentiation, and cell death (Kopan 2012). Among many other processes, it is specifically responsible for regulating neurogenesis, retina development, feather bud development, the generation of somites, the generation of blood cells, and oligodendrocyte differentiation (Gerhart 1999).

The *Notch* gene encodes a type 1, single pass transmembrane receptor. Depending on the organism, there are up to three paralogs of the gene; *D. melanogaster* possesses only one copy of the *Notch* gene, for example, but humans possess four copies (Kopan and Ilagan 2009). As shown in Figure 1, key components of the Notch signaling pathway include the Notch transmembrane protein, the Delta-like ligand (a transmembrane protein embedded in the membrane of a different cell), and the CSL transcription factor. The signaling process begins when the extracellular portion of the Notch receptor comes into contact with the Delta-like ligand. The cell containing the Delta-like ligand then begins the process of endocytosis while the ligand and the Notch receptor are still in contact, which pulls on the Notch receptor (Wang 2011). This triggers the cleavage of the extracellular domain of the Notch receptor from the membrane by ADAM10, which in turn triggers the cleavage of the intracellular domain by γ-secretase (Kopan 2012). The intracellular Notch domain then travels to the nucleus, where it binds as part of the CSL transcription factor complex and thereby activates its target genes (Lai

2004). Other components of the active CSL complex include Mastermind, SKIP, and a histone acetyl transferase (HAT) (Kopan 2012).

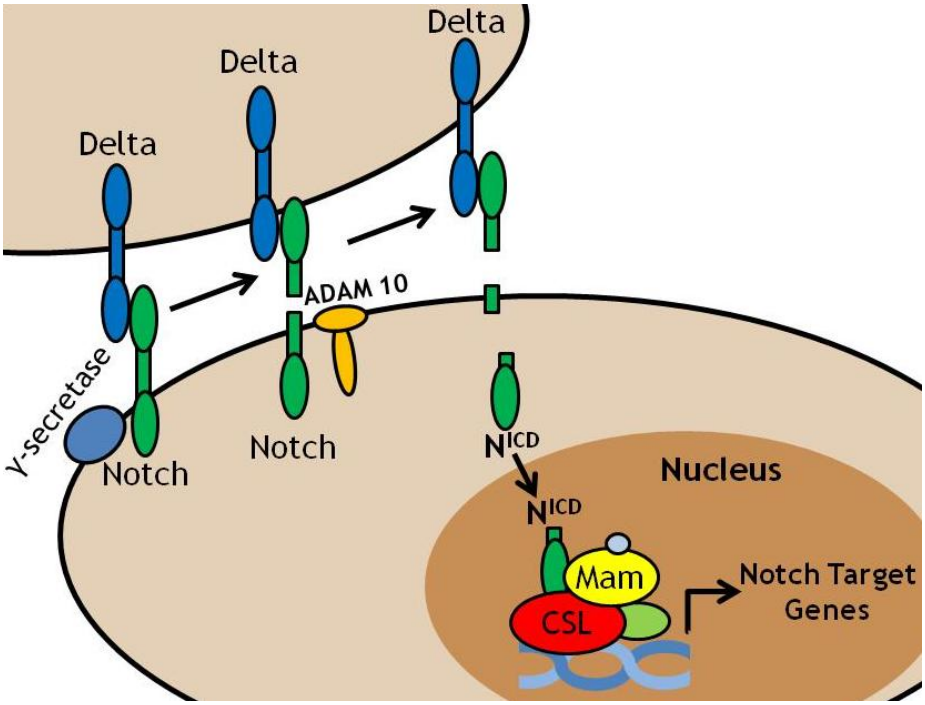

Figure 1. Schematic of the Notch signaling pathway, as described in the previous paragraph. N^{ICD} is the Notch intracellular domain, Mam is Mastermind, and CSL is the CSL transcription factor.

Some prominent human diseases involving Notch include Alagille syndrome, spondylocostal dysostosis, and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). Alagille syndrome is caused by an autosomal dominant loss-of-function mutation in the *Jagged* gene, which normally encodes a transmembrane ligand for the Notch receptor, and results in malformation of the liver, heart, eye, skeleton, and lower organs. Spondylocostal dysostosis is the result of disturbances in the process of somite patterning, which Notch regulates, and its symptoms are rib fusions and deletions and dwarfism. CADASIL is associated with migraines, strokes, dementia, and early death. It is caused by a mutation in the *Notch* gene that results in the Notch protein remaining in the cell membrane and the death of vascular smooth muscle cells in the arteries. (Gridley 2003)

Components of the Notch pathway have historically been found by using genetic screens with invertebrates, especially fruit flies. In one example of this technique, a driver gene (such as *GAL4*) is inserted into a line of flies via a genetic cross, creating a recombinant chromosome that can over express random target genes (Hall et al. 2004). To locate other potential members of the pathway, the offspring over-expressing the target genes are crossed with lines that express altered versions of other genes (such as a shortened version of Mastermind, a co-activator of the CSL transcription factor (Bray 2006)), and the resulting offspring are examined and scored for phenotypes that differ from those of the parents (Hall et. al. 2004). One gene found using this technique was *domino,* which results in severe wing notches when combined with the dominant transgene *MamH,* which encodes a shortened and mutant version of Mastermind (Hall et al. 2004).

Domino was first discovered in a screen looking for genes related to the *Drosophila* immune system. This screen was performed by looking for the absence of hemoctyes in different strains of flies which had random genes knocked out through the insertion of a transposon containing LacZ. One of the genes discovered was named *domino* because it resulted in the formation of two spots of LacZ on the lymph gland of third instar larvae. (Braun et. al. 1997). *Domino* has subsequently been found to encode an ATPase that is a subunit of the Tip60 chromatin remodeling complex (Ruhf et. al. 2001, Kusch et. al. 2004). The Tip60 complex, along with the SAGA remodeling complex and the Nipped-A protein, is responsible for the proper function of Mastermind and for assembling the Notch activator complex (Gause et. al. 2006). Loss-of-function mutations in *domino,* therefore, affect the formation of the Notch activator complex and reduce the expression of Notch target genes. This presumably results in the phenotype observed in the Hall genetic screen.

Analysis of *domino* has shown that it contains 14 exons and may be alternatively spliced to form transcripts for two different proteins, DominoA (all 14 exons) and DominoB (11 exons). DominoA and DominoB are identical in sequence up until the point in the 11th exon, where splicing of the original transcript occurs to form the transcript for DominoB. DominoA thus has a large poly-glutamine domain and a nuclear localization signal near its C-terminus that DominoB does not have. DominoA is initially found in the developing central nervous system and peripheral nervous system, but its expression later becomes restricted to specific areas of the brain or the eye tissue precursors. DominoB, on the other hand, is expressed in almost all cell nuclei during early development in the blastoderm, and then becomes localized to the lymph nodes, brain, salivary glands, and imaginal discs. (Ruhf et. al. 2001)

Domino has been linked to cell proliferation defects in various tissues, and is believed to be involved in chromatin remodeling. During the early stages of *Drosophila* embryonic development, Domino is supplied by the mother's cells. *Domino* mutations in female germ line cells result in the death of the embryo, as zygotic transcription of *domino* does not begin until later in development. In cases where there is a loss-of-function *domino* mutation in an embryo but the mother's germ line cells express *domino* normally, the developing fly can survive until the first or second instar, or later stages in the case of weak alleles. There is some evidence that *domino* acts as an epigenetic chromatin repressor, as its loss-of-function mutations enhance the effects of loss-of-function mutations in Polycomb group (PcG) genes, which are associated with epigenetic repression. (Ruhf et al. 2001)

Recent work by Lu et. al. has shown that *domino* loss-of-function enhances the effects of over-expression of the gene *E2F*, and suppresses the effects of loss-of-function of *CyclinE* in the eye tissue (Lu et. al. 2007). Because both *E2F* and *CyclinE* are involved in the positive

regulation of cell proliferation (Lu et. al. 2007), this data indicates that *domino* has a negative effect on cell proliferation in the eye tissue. Other experiments, however, show that Domino has a positive impact on cell proliferation in the wing tissue through its interactions with Notch (Hall et. al. 2004, Kwon et. al. 2013). Previous work in the Yedvobnick lab has shown that both over-expression of Domino through *UAS* strains and under-expression of Domino through knock-out or RNAi strains result in the enhancement of a wing-nicking phenotype derived from a cell proliferation defect (Kwon et. al. 2013). This indicates that *domino* may be pleiotropic for cell proliferation in the wing tissue (Kwon et. al. 2013). It is unclear from the results of Lu et. al. (2007), however, if *domino* is also pleiotropic for cell proliferation in the eye tissue because the study only examined the effects of *domino* loss-of-function mutants.

The following study is bipartite, starting in the wing and then continuing into the eye. One component investigates wing modifier genes found to interact with *domino* that were identified in the Kwon et. al. (2013) genetic screen. This screen was performed by crossing thousands of strains with transposon insertions to the strain *C96-domR*, which has a *domino* RNAi transgene expressed only in the wing margins. This *domino* RNAi expression resulted in nicking in the anterior wing margins; the screen looked for any strains that could either enhance or repress this phenotype (Kwon et. al. 2013). In the present study, 13 *domino* modifier strains found in this screen were analyzed for *domino*-independent effects on cell proliferation. These strains were crossed to the strain *C96-UAS-Rbf280*, which has a cell proliferation defect that results in wing nicking. Offspring resulting from these crosses were then analyzed for enhancement or suppression of the wing nicking phenotype. Those strains that interacted with the *C96-UAS-Rbf280* wing phenotype were judged to be *domino*-mutation independent.

The second thesis component investigates the effects of *domino* on cell proliferation in

the eye in an attempt to determine whether *domino* affects cell proliferation in the eye the same way that Kwon et. al. (2013) suggests that it affects proliferation in the wing. The strain *GMR-YkiS168A*, which has a cell proliferation mutation that results in hyperproliferation and loss of cell death in the eye, was crossed to *domino* mutant strains. These strains included *domino* gain-of-function mutants, *domino* knock-out mutants, and mutants expressing *domino* RNAi transgenes. Results indicating that all three types of *domino* mutant strains suppressed hyperproliferation in the eye would indicate that *domino* has related effects on proliferation in the wing tissue and in the eye tissue.

The strain *GMR-YkiS168A* was then crossed to strains that had either loss-of-function or gain-of-function *Notch* mutations in an attempt to determine whether or not *domino's* effects on proliferation might be due to interactions with *Notch*. If strains with loss-of-function *Notch* mutations resulted in the same phenotype as loss-of-function *domino* mutations and strains with gain-of-function *Notch* mutations resulted in the same phenotype as gain-of-function *domino* mutations, it would indicate that *domino* affects cell proliferation through interactions with Notch. A negative result would indicate that Domino and Notch function are not closely linked in proliferation, as measured by this hyperproliferation assay.

Methods

Strain	Genotypic Effect
w^{1118}	Wild-type
UAS-RBF280/C96-GAL4	Over-expression of hyperactive RBF (Retinoblastoma Factor) in the wing margins; over-expression of GAL4 in the wing margins
GMR-YkiS168A	Over-expression of hyperactive Yki (Yorkie) in the eye; over-expression of GAL4 in the eye
GMR-GAL4	Over-expression of GAL4 in the eye
nd^{1}	*Notch* loss-of-function
UAS-N^{ACT}	Over-expression of constitutively active Notch when in combination with GAL4
UAS-MamH	Over-expression of dominant-negative Mastermind when in combination with GAL4
EP Strains	See Table 2.
Domino Mutant Strains	See Table 3.

Table 1. Strains used in the genetic crosses and their genotypic effect. RBF is involved in the down-regulation of cell proliferation (Xin et. al. 2002), Yorkie is involved in increasing cell proliferation and decreasing cell death (Huang et. al. 2005), and Mastermind is a critical component of the Notch signaling pathway (Helms et. al. 1999).

i) Genetic crosses

To collect virgin female flies from a tester strain, a stock vial is cleared of all eclosed flies and is placed either at 18°C for no more than 24 hours or at room temperature for no more than 8 hours. Because the flies do not become sexually mature during this period, any female flies collected and separated from males would remain virgin. The collected flies are then allowed to mature at room temperature for at least two days before being crossed to males in order to increase the fertility of the cross.

Crosses are set up by placing six virgin females from a tester strain in the same vial as four males from an experimental strain. All ten flies are passed on to a new vial after 5 days and are discarded after 10 days. The offspring of the cross begin to emerge from pupa cases as adults in approximately 11 days from the start of the cross.

For the crosses involving *Notch* mutant strains, there were a few exceptions to the procedure described above. Because *notchoid* is carried on the X chromosome, crosses involving

notchoid mutants used virgin flies that were collected from the nd^1 strain instead of the *GMR-YkiS168A* or *GMR-GAL4* strains; this ensured that all male offspring of crosses with the nd^1 strain received the *notchoid* mutation. The other crosses with *Notch* mutant strains were performed similarly, with the tester strain serving as the source of virgin females, so that all the sets of crosses would have male parents of similar genetic background. (Normally, all of the female parents would have a similar genetic background, but that was not feasible in this case.)

Because the first cross between the N^{ACT} strain and *GMR-YkiS168A* was lethal, the second cross and all associated controls were placed at 18° C, where the *GMR* driver was less active, in order to get living offspring. Parental flies from the N^{ACT} strain were flies passed onto new media a second time (instead of being discarded after 10 days), and placed at 18° C after three days at room temperature. Only the last vial was placed at 18° C; the original vial and the vial from the first pass remained at room temperature.

ii) *Drosophila* strains

a) The Domino Modifier Genetic Analysis

The genetic analysis looking for *domino* modifiers with independent effects on cell proliferation used *UAS-Rbf280/Cy; C96-GAL4/Hu* as the tester strain. This strain contains *UAS-Rbf280*, a super-active form of Rbf that lacks four cyclin-dependent kinase phosphorylation sites, has *UAS* enhancer-binding regions, and has a *C96-GAL4* driver, the enhancer for the *UAS* system (*GAL4*) expressed under a promoter active only in the wing margins (*C96*). The protein Rbf280 is thus expressed only in the wing margins and is over-expressed. Over-expression of Rbf280 in the wing margins results in the blockage of E2F target genes (Xin et. al. 2002), which in turn results in a cell proliferation defect. This defect manifests phenotypically as nicks in the wings

(with a penetrance of approximately 30%), where portions of tissue are missing (Kwon et. al. 2013).

The *UAS-Rbf280/Cy; C96-Gal4/Hu* tester strain was crossed with thirteen experimental strains, each containing an enhancer-promoter (*EP*) transposon hop. The *EP* transposons consist of a series of *UAS* sequences upstream of a promoter region, and were artificially introduced into the genome using a plasmid. When an *EP* transposon hops around the genome, it can result in a gain-of-function mutation if it inserts into the region before a gene or a loss-of-function mutation if it either inserts into the gene or inserts backwards into the promoter region. The Yedvobnick lab previously conducted a genetic screen that identified thirteen of these mutations affecting three classes of *domino* modifiers (either transcription factors, RNA metabolism factors, or factors affecting growth and autophagy), and 12 have recently been reported (Kwon et. al. 2013). The strains containing these mutations were those used in the present study (Table 2).

EP Strain	Modifier Class	Mutation Type	Gene Mutated
EP 226	3	GOF	*atg9*
EP 558	2	LOF	*pabp2*
EP 573	3	LOF	*lk6*
EP 593	2	GOF	*Tudor-SN*
EP 918	2	GOF	*SmD3*
EP 939	1	LOF	*EcR*
EP 1000	1	LOF	*lola*
EP 1037	3	GOF	*wdb*
EP 1202	3	LOF	*atg1*
EP 1538	1	LOF	*lola*
EP 1561	1	GOF	*emc*
EP 1630	1	GOF	*lilli*
EP 1646	2	LOF	*pum*

Table 2. Modifier type, mutation type, and gene affected for each of the 13 *EP* strains. Class 1 modifiers are transcription factors, class 2 modifiers are RNA regulators, and class 3 modifiers are factors involving growth and autophagy. For more information, see Kwon et. al. 2013.

Because not all crosses took place at the same time, each set of crosses between the *EP*

strains and the tester strain was paired with a cross between the tester strain and w^{1118}, which took place simultaneously. This cross served as a control for environmental effects that could alter wing nicking percentages.

Crosses that yielded either a significant percentage of nicked wings or a severe phenotype in comparison to the paired w^{1118} control cross were repeated to obtain additional numbers. Two crosses of the tester strain with the *EP* strain were performed simultaneously, under the same conditions as the original cross.

b) Test for Environmentally Caused Variations in Eye Size

This set of crosses was designed to determine whether or not competition for resources could affect the size of emerging flies and to determine if there was a large amount of size variation between offspring resulting from the same cross and of the same sex. w^{1118} flies were placed at high (12 females and 8 males), medium (6 females and 4 males), or low (3 females and 2 males) density and allowed to breed. The resulting offspring were separated by sex and were quantitatively scored for body size, head size, and wing size. Flies from very high density stock vials were also measured.

c) Genetic Crosses with GMR-YkiS168A and Domino Mutant Strains

The tester strain for the genetic crosses with *domino* mutant strains was *GMR-YkiS168A*, which contains a hyperactive form of Yorkie, a *UAS* region in front of *yorkie*, and the driver *GMR-GAL4*, which leads to over-expression of Gal4 (and thus to over-expression of YorkieS168A) in the eye tissues. When Yorkie becomes hyperactive and over-expressed, it results in increased cell proliferation and decreased cell death (Huang et. al. 2005). The eyes of

the flies in this strain are correspondingly large, and have a rugged, lumpy appearance.

The experimental strains for this cross were a variety of *domino* loss-of-function and gain-of-function mutant strains (Table 3). The strain $cycE^{AR95}$, which has a *CyclinE* loss of function mutation, served as a control for the *GMR-YkiS168A* model system. Because functional CyclinE is necessary for cell division, if the system is working as intended, crossing *GMR-YkiS168* to $cycE^{AR95}$ should result in offspring that have less cell proliferation in the eyes than the *GMR-YkiS168A* strain does. The remaining strains either were *domino* loss of function mutants (dom^{1}, dom^{2371}, dom^{3}), expressed *domino* RNAi transgenes (*UAS-domR, UAS-domRNAi-TRIP, UAS-domRNAi),* or over-expressed Domino through the *UAS-GAL4* system (*UAS-DomB, UAS-DomA*). $Dom^{2371}rev$ was a revertant for the mutation that caused *domino* loss of function in dom^{2371}, and served as a control for phenotypic effects that may have been the result of the generation of the mutation instead of the result of the loss of function *domino* mutation itself. A cross between the *GMR-YkiS168A* strain and w^{1118} served as a control for the effects of YkiS168A; each subset of crosses that took place at a different time had its own *GMR-YkiS168A* x w^{1118} control cross. Crosses between *GMR-GAL4* and the strains with *UAS* enhancer regions were set up to examine whether there might be an observable eye phenotype resulting from over-expression of *domino* or *domino* RNAi transgenes.

Domino loss-of-function mutant lines are typically created by inserting transposons relatively close to the start of the gene, knocking out expression of both forms of Domino. Dom^{1} corresponds to an insertion one base-pair downstream of the intron 1 splice site, dom^{3} derives from an internal transposon excision (Ruhf et. al. 2001), and dom^{2371} corresponds to an insertion into the upstream region of the gene. *Domino* over-expression lines are created by the insertion of cDNA for either DominoA or DominoB and a *UAS* element into the fly genome. *Domino*

RNAi strains are created by the insertion of one or more copies of a *domino* RNAi transgene into a chromosome. Most strains used in these crosses were created elsewhere and were ordered by the laboratory. Others had been created by the Yedvobnick lab previously for use in other experiments.

d) Genetic Crosses with GMR-YkiS168A and Notch Mutant Strains

The tester strain for these crosses, *GMR-YkiS168A*, was the same as in the previously described crosses with *domino* mutant strains. The experimental strains were *notchoid* (nd^1), a strain which carries a hypomorphic Notch allele; *UAS-*N^{ACT}, which contains a *UAS* promoter region and a gain-of-function *Notch* construct (N^{ACT} encodes only the intracellular domain of the Notch protein, which makes the Notch signaling pathway active even in the absence of external cell signals); and *UAS-MamH*, which contains a *UAS* promoter region and encodes a dominant negative form of Mastermind (this protein contains a Notch-binding domain, but is missing the domains that bind to components of the Notch activation complex). Control crosses were *GMR-YkiS168A* x w^{1118}, w^{1118} alone, nd^1 x w^{1118}, and *GMR-GAL4* x all tester strains. The *GMR-GAL4* crosses control for the expression of the GAL4 protein, which would lead to over-expression of N^{ACT} and MamH in the *UAS-*N^{ACT} and *UAS-MamH* strains, respectively.

Genetic Modification	Balancer	Effect
UAS-domR	*Sb*	*domino* RNAi
UAS-DomB	*Cy*	DominoB over-expression
dom^{1}	*Cy*	*domino* loss of function
dom^{3}	*Cy*	*domino* loss of function
dom^{2371}	*Cy*	*domino* loss of function
$dom^{2371}rev$	*Cy*	Normal Domino function
UAS-DomA (c3)	n/a	DominoA over-expression
UAS-DomA (c2)	n/a	DominoA over-expression
$cycE^{AR95}$	*Cy*	*cyclinE* loss of function
UAS-domRNAi-TRIP	n/a	*domino* RNAi
UAS-domRNAi (c3)	n/a	*domino* RNAi
UAS-domRNAi (c2)	n/a	*domino* RNAi

Table 3. List of *domino* mutant strains. The genetic modification, balancer (if any), and result of the genetic modification are listed for each of the strains used.

iii) Data collection

a) Scoring for Wing Nicks

After emerging as adults, the offspring resulting from the *domino* modifier analysis experiments were scored for wing nicks. Flies were anesthetized with carbon dioxide gas and separated by sex. Each wing was scored individually, with a score of 1 accorded to any wing with any type of nicking, and with a score of 0 accorded to wings without nicks. Flies containing balancers (*Humeral*, *Stubble*, *Curly*) were not scored. Representative wing images were obtained by anesthetizing flies with carbon dioxide and freezing them at -80˚C for approximately 1 minute. Wings were clipped from flies and mounted on a slide with Euparol. Images were obtained with a digital camera and a light microscope, and were put into grayscale and sharpened using Adobe Photoshop.

b) Scoring for the Yorkie Eye Phenotype

Eclosed flies were anesthetized with carbon dioxide gas, frozen at -80˚C for at least 1 minute, separated by sex, and scored for the presence of hyperproliferation of eye tissues. Because the degree of hyperproliferation is difficult to determine at a glance, eyes with any type of hyperproliferation were given a score of 1, and eyes with no discernible hyperproliferation were given a score of 0. Flies containing balancers were not scored. Ten representative images of the eyes were taken from a frontal view, and another ten were taken from a side view. For the *GMR-YkiS168A*/*domino* mutant strain crosses, image sets were collected from both male and female flies, for a total of 40 images for each cross. For the *GMR-YkiS168A*/*Notch* mutant strain crosses, representative images were taken only from male flies because female flies from the *GMR-YkiS168A* x nd^1 cross are heterozygous for the recessive *notchoid* allele.

Each representative image contained a scale and was taken using a digital camera attached to a light microscope. Images were analyzed using ImageJ, a program allowing users to assign a set distance to a number of pixels and thereby measure the area of objects in a digital photograph. From the side view, a single measurement of the total eye area was taken for each picture. For the frontal view, measurements of the area of each eye were taken. The measurements for frontal view eye areas were averaged for each fly to compensate for one side of the head being mounted on the slide higher than the other (if the head is tilted on the slide, one eye will appear larger in a photograph, and the other eye will appear correspondingly smaller).

iv) Data analysis

Penetrance of the nicked-wing phenotype was calculated for the *domino* modifier genetic

analysis. Significance was determined by using a X^2 test, with the penetrance of the paired control cross serving as the percentage wing nicking expected if a particular *EP* mutation had no effect on cell proliferation.

Penetrance of the rugged, hyperproliferation eye phenotype was calculated for the *GMR-YkiS168A* x *domino* mutant and *GMR-YkiS168A* x *Notch* mutant crosses. Significance was determined by using a X^2 test, with the penetrance of the paired control cross serving as the percentage of hyperproliferation penetrance expected if a particular mutation had no effect on cell proliferation. Because the control cross (*GMR-YkiS168A* x w^{1118}) was 100% penetrant, this analysis was effective only for strains showing loss of the hyperproliferation eye phenotype.

Severity of the hyperproliferation eye phenotypes was determined by performing an ANOVA statistical analysis on the eye size measurements collected using ImageJ. Data from repetitions of the same cross were separated, as attempts to measure all flies of the same sex and cross removed statistical significance from measurements that were significant in individual repetitions and added statistical significance to measurements that had none before. Combining the measurements from male and female flies of the same cross or the same cross and same repetition yielded similar results.

For all analyses, a p value less than or equal to 0.05 was used to denote statistical significance.

Results

i) The *Domino* Modifier Genetic Analysis

Significant wing nicking enhancement was found for *EP* strains *558, 918, 939, 1538*, and *1630* (Fig.2, panels E, H, I, M, and O, respectively) based on both the severity (Fig. 2) and penetrance (Table 4) of wing nicks compared to the control crosses (Fig. 2, panels A, B, and C). Although *EP* strain *1000* (Fig. 2, panel J) displayed an enhanced wing nicking phenotype, the penetrance of wing nicks compared to the control cross were slightly too low to be significant. Significant wing nicking suppression was found for *EP* strain *1202* (Fig. 2, panel L), which had an almost wild type wing phenotype and a dramatically reduced wing nicking penetrance compared to its control crosses.

Increased severity of wing nicking was also found for *EP* strains *593, 1037, 1561*, and *1646* (Fig. 2, panels G, K, N, and P, respectively), but there was no significant wing nicking penetrance for any of these strains. *EP* strain *573* (Fig. 2, panel F) similarly showed wing nicking suppression, but did not have a significantly different wing nicking penetrance. Strains *226* and *1037* (Fig. 2, panels D and K, respectively) had neither significant wing nicking phenotypes nor significant wing nicking penetrance differences compared to control strains.

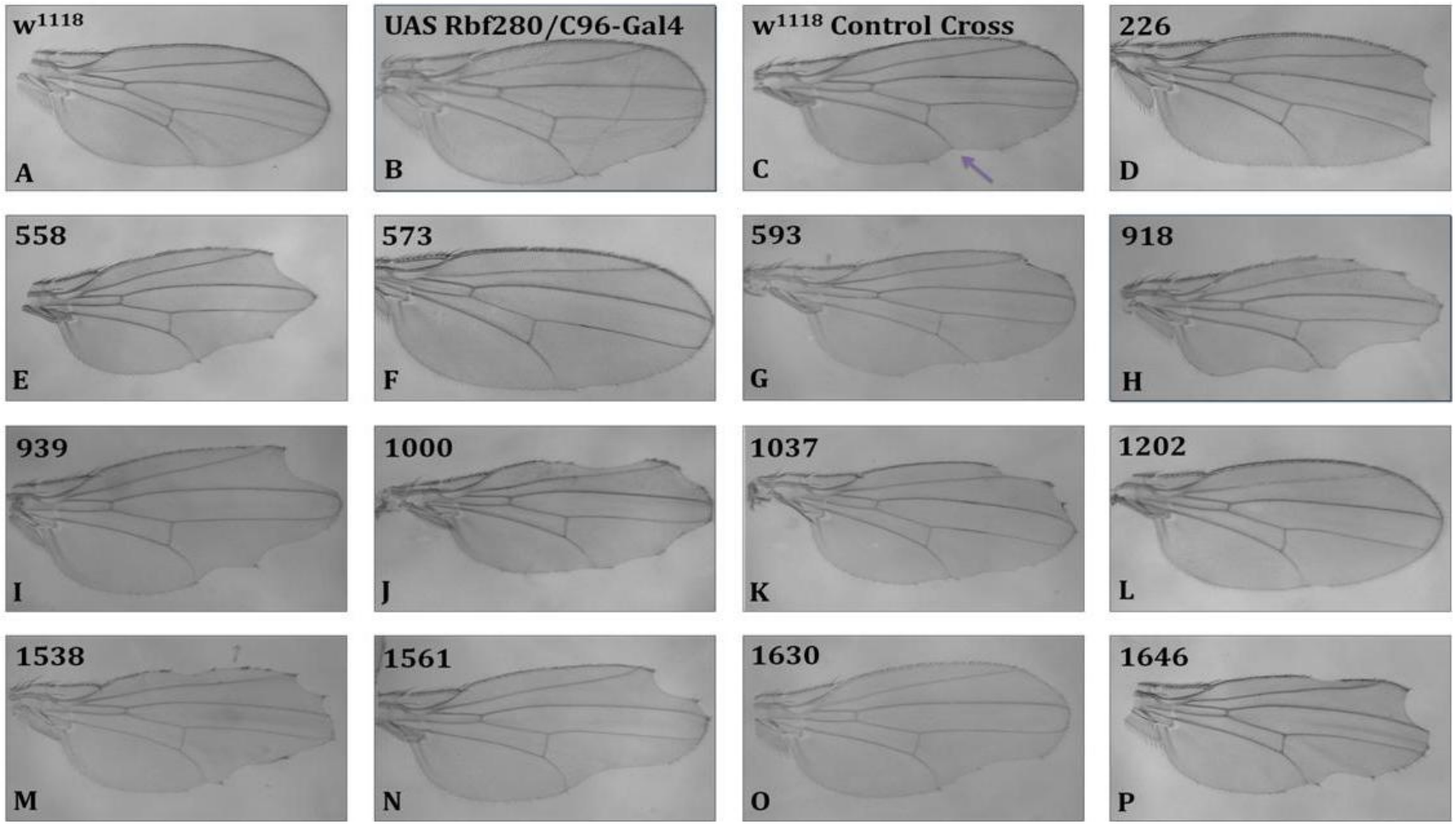

Figure 2. Wing nicking phenotypes for the three wild type or control groups and the 13 *EP* strains. A typical wing nick is indicated by the arrow in panel C. Significantly nicked phenotypes are recognizable by both the presence of nicking on the anterior wing edge and either frequent or large nicks on the remaining wing segments, as can be seen in panels E, G, H, I, J, K, M, N, and P. Also of note are panels F and L, which show a suppression of nicking.

Strain	Control Pairing	Wings Affected	Wings Total	Percentage Affected	X^2 Value	P
***w*^{1118} (Total)**	**N/A**	501	694	72.2	N/A	N/A
EP 226	**1**	60	86	69.8	0.02	0.888
EP 558	**1, 5**	88	88	100.0	11.62	**0.001**
EP 573	**1**	106	134	79.1	1.25	0.264
EP 593	**2**	39	44	88.6	0.01	0.920
EP 918	**7**	98	98	100.0	10.35	**0.001**
EP 939	**2, 5**	74	76	97.4	6.56	**0.010**
EP 1000	**2, 5**	35	36	97.2	3.07	0.080
EP 1037	**3**	25	34	73.5	0.26	0.610
EP 1202	**3, 6**	52	196	26.5	49.93	**<0.001**
EP 1538	**4, 6**	138	150	92.0	5.01	**0.025**
EP 1561	**4**	14	18	77.8	0.41	0.522
EP 1630	**3, 6**	171	186	91.9	15.34	**<0.001**
EP 1646	**4, 6**	80	94	85.1	1.01	0.314

Table 4. The totals and percentages of wings affected by nicking, as well as the X^2 and p values. Control pairing refers to the identity of the *UAS-Rbf280 x* ***w***^{1118} cross that served as a control for each *EP* cross.

	Wings Affected	Wings Total	Percentage Affected
***w*^{1118} 1**	44	62	71.0
***w*^{1118} 2**	14	16	87.5
***w*^{1118} 3**	81	122	66.4
***w*^{1118} 4**	48	52	92.3
***w*^{1118} 5**	41	60	68.3
***w*^{1118} 6**	95	136	69.9
***w*^{1118} 7**	178	246	72.4

Table 5. The totals and percentages of wings affected by nicking for the *UAS-Rbf280 x* ***w***^{1118} control crosses. The control pairing number from Table 4 is indicated in the cross name (***w***^{1118} 1, for example, corresponds to control pairing 1). Where a given *EP* strain's cross had multiple control pairings, the number of wings (total and affected) for both groups was added and was used to compute the X^2 value that determined significance in Table 4. Percentages of wings affected for the control crosses varied, but the average was 72.2%.

ii) Test for Environmentally Caused Variations in Eye Size

Average eye area (the average area of both eyes from a frontal view) was found to have the least variation between flies of the same strain and cross, with standard deviation values at approximately five percent or less of the average value for a cross (Table 6). The values for individual eyes from the frontal view, however, were far more varied, with standard deviation values approximately twice that of those from the average eye area values. Of particular note is that for the *exact same group of flies*, body size varied widely, but average eye size remained relatively uniform.

Overall, female flies had larger eyes and bodies than males from the same level of population density (Table 6). For both males and females, flies from higher population densities had smaller eyes and bodies than flies from lower population densities. Although flies at low density had larger bodies than flies at medium density, the average eye size for low density flies was not greater than that for medium density flies.

Right Eye	Area (mm)	STDEV	CV		Left Eye	Area (mm)	STDEV	CV
Very High Density Female	0.076	0.008	10.22		Very High Density Female	0.069	0.005	7.51
High Density Female	0.094	0.008	8.71		High Density Female	0.079	0.008	9.97
Medium Density Female	0.087	0.017	19.21		Medium Density Female	0.085	0.013	15.81
Low Density Female	0.080	0.008	10.24		Low Density Female	0.083	0.009	11.47
Very High Density Male	0.068	0.007	10.69		Very High Density Male	0.072	0.011	14.70
High Density Male	0.072	0.012	16.10		High Density Male	0.079	0.012	15.18
Medium Density Male	0.088	0.006	6.66		Medium Density Male	0.071	0.005	7.58
Low Density Male	0.077	0.009	11.92		Low Density Male	0.071	0.007	10.16
Average Eye	**Area (mm)**	**STDEV**	**CV**		**Body**	**Area (mm)**	**STDEV**	**CV**
Very High Density Female	0.073	0.003	4.80		Very High Density Female	1.375	0.190	13.83
High Density Female	0.086	0.003	3.74		High Density Female	1.538	0.174	11.34
Medium Density Female	0.086	0.004	4.90		Medium Density Female	1.604	0.292	18.19
Low Density Female	0.082	0.004	5.28		Low Density Female	1.620	0.140	8.61
Very High Density Male	0.070	0.005	7.81		Very High Density Male	1.029	0.134	13.01
High Density Male	0.076	0.004	5.28		High Density Male	1.169	0.148	12.63
Medium Density Male	0.079	0.002	2.50		Medium Density Male	1.283	0.097	7.57
Low Density Male	0.074	0.004	5.57		Low Density Male	1.289	0.123	9.57

Table 6. The averages, standard deviation (STDEV), and Coefficient of Variation (CV) (the standard deviation as a percentage of area) for measurements of the right eye, left eye, right and left eye averaged, and body for male and female flies at different levels of population density. 'Very high density' refers to stock vial flies that developed at the highest density levels; exact levels of density, however, are unknown.

iii) Genetic Crosses with *GMR-YkiS168A* and *Domino* Mutant Strains

w^{1118} eyes are small, evenly rounded, and without lumps or ridges of any sort (Figs. 3A and 4A). Crossing *GMR-YkiS168A* to w^{1118} results in offspring with very large eyes that have lumps/ridges (the *yorkie* eye phenotype). These offspring served as paired controls for the crosses between *GMR-YkiS168A* and the *domino* mutant strains. Because not all crosses occurred at the same time, Table 8's measurement of difference from control refers to the difference in area for either flat (side view) or average eye (frontal view) area measurements between offspring of a given cross and its particular paired control cross. For analyzing the results of two or more different crosses, the best measurement of comparison may therefore be the difference of an average from the average of the paired control.

The strain $cycE^{AR95}$ showed significant repression of the *yorkie* eye phenotype, both visually (Figs. 3 and 4, panel C) and in terms of penetrance, indicating that the *GMR-YkiS168A* crosses are working as intended as a screen for effects on cell hyperproliferation. The statistical size analysis for this strain is less clear, especially given that there is data missing for the second repetition measurements of average eye area (Table 8); male flies follow a general pattern of suppression in terms of eye size, whereas female flies sometimes have mild, but statistically significant, enhancements.

Most strains involving *domino* loss-of-function mutations (Figs. 3 and 4, Row 2) showed little difference from the control cross visually, in terms of penetrance, or statistical size. Although dom^{1} does show statistically significant differences in eye size from the control cross, the overall results are inconsistent, with some measurements showing enhancement and others showing repression (Table 8). The results for the strain $dom^{2371}rev$, a revertant for the dom^{2371} loss of function mutation, show a similar pattern of alternating suppression and enhancement of

eye size, though the values are mostly insignificant. The strain *dom*^{2371} showed significant repression in average eye area for female flies, but not for males, and the strain *dom*^{3} showed inconsistent statistical results for both males and females.

With the exception of the strain *UAS-domR* (and possibly *UAS-domRNAi (c3)*), none of the RNAi strains showed significant differences from the control crosses visually, in terms of statistical size, or in terms of penetrance. *UAS-domR*, on the other hand, appeared to be visually wild-type (Figs. 3 and 4, panel H); the lumps and ridges of the *yorkie* eye phenotype were completely gone. The penetrance of the *yorkie* eye phenotype was also at 0%. Although this strain did not completely restore the wild-type phenotype's eye size, all the crosses with *UAS-domR* showed a significant (or very nearly significant) decrease in eye size compared to control crosses. The strain *UAS-domRNAi (c3)* showed a significant decrease in penetrance compared to control crosses (Table 7), but had no differences from controls in terms of eye size or shape.

DominoA over-expression lines did not significantly and consistently differ from the control crosses by any measurement. Overall, both females and males from *UAS-DominoA* crosses tended to show slight decreases in eye area compared to controls, but males tended to show more cases of slight increases in eye area compared to controls than females did. Of all the crosses with *domino* over-expression lines, those with *UAS-DomB* yielded the most consistent results, with decreases in eye area compared to the *GMR-YkiS168A* x *w*^{1118} control in all measurements; these results, however, were not always significant for measurements of flat eye area.

Offspring resulting from crosses between *GMR-GAL4* flies and strains containing *UAS* transgenes showed virtually no differences from the *GMR-GAL4* x *w*^{1118} control cross (data not shown). All eyes were indistinguishable from wild-type eyes in appearance. The only significant

difference in eye sizes was for females of the *GMR-GAL4* x *UAS-DomB* cross, which had slightly larger average eye areas than controls (an average of 0.015 mm larger than the *GMR-GAL4* x w^{1118} control cross; $p<0.001$); males also had larger average eye areas than controls, but not significantly so (an average of 0.011 mm larger than the *GMR-GAL4* x w^{1118} control cross; $p=0.07$). Of particular note is that for these crosses, there was no additional repetition because of time constraints, and only five flies of each sex were measured (a total of 10 for each cross).

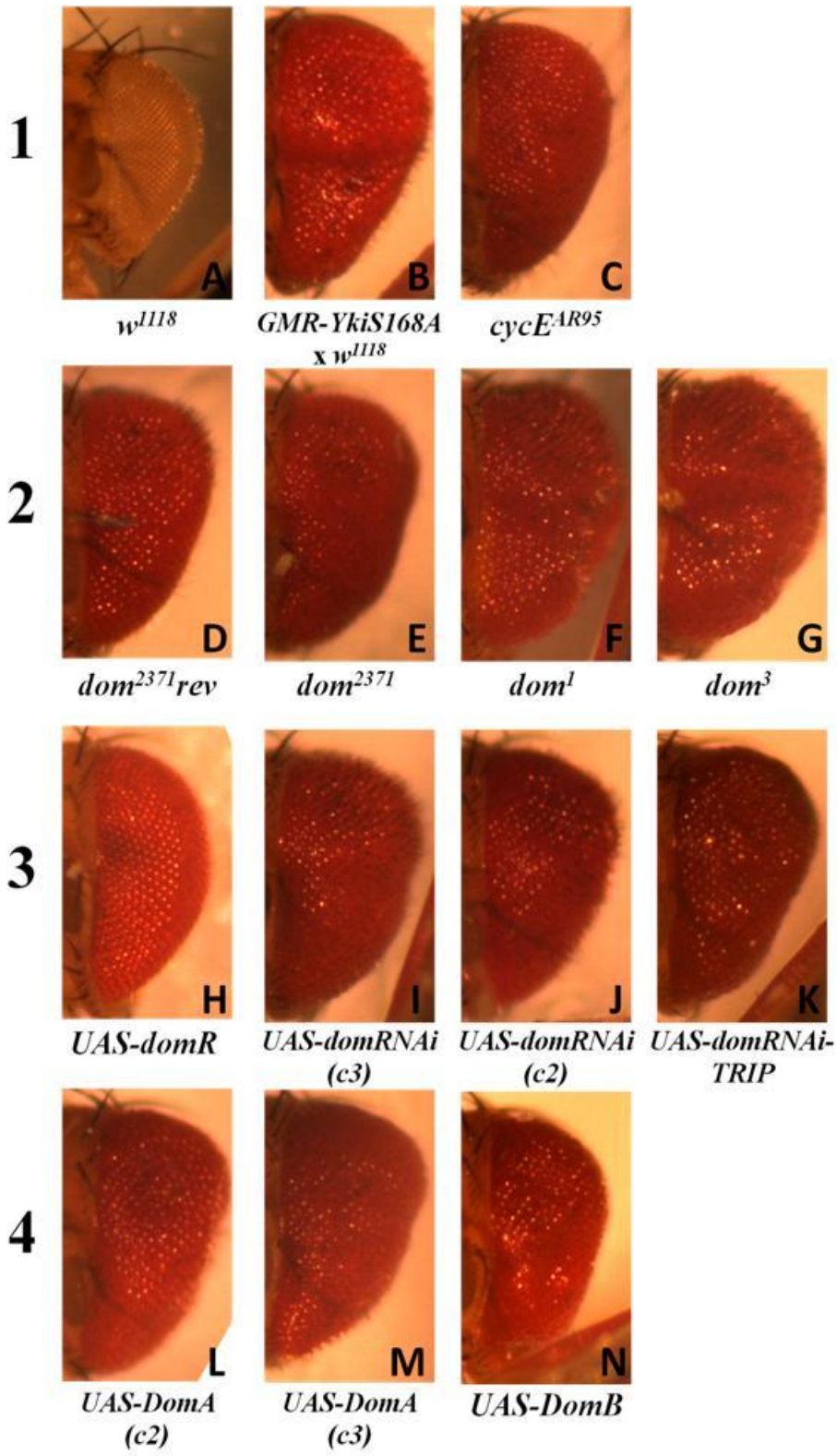

Figure 3. Eyes from the frontal orientation, taken from male flies. Row 1 shows control crosses, row 2 shows crosses with *domino* loss-of-function mutants, row 3 shows crosses with *domino* RNAi strains, and row 4 shows crosses with *domino* over-expression strains. Of particular note is panel H, which shows significant suppression of the *yorkie* eye phenotype.

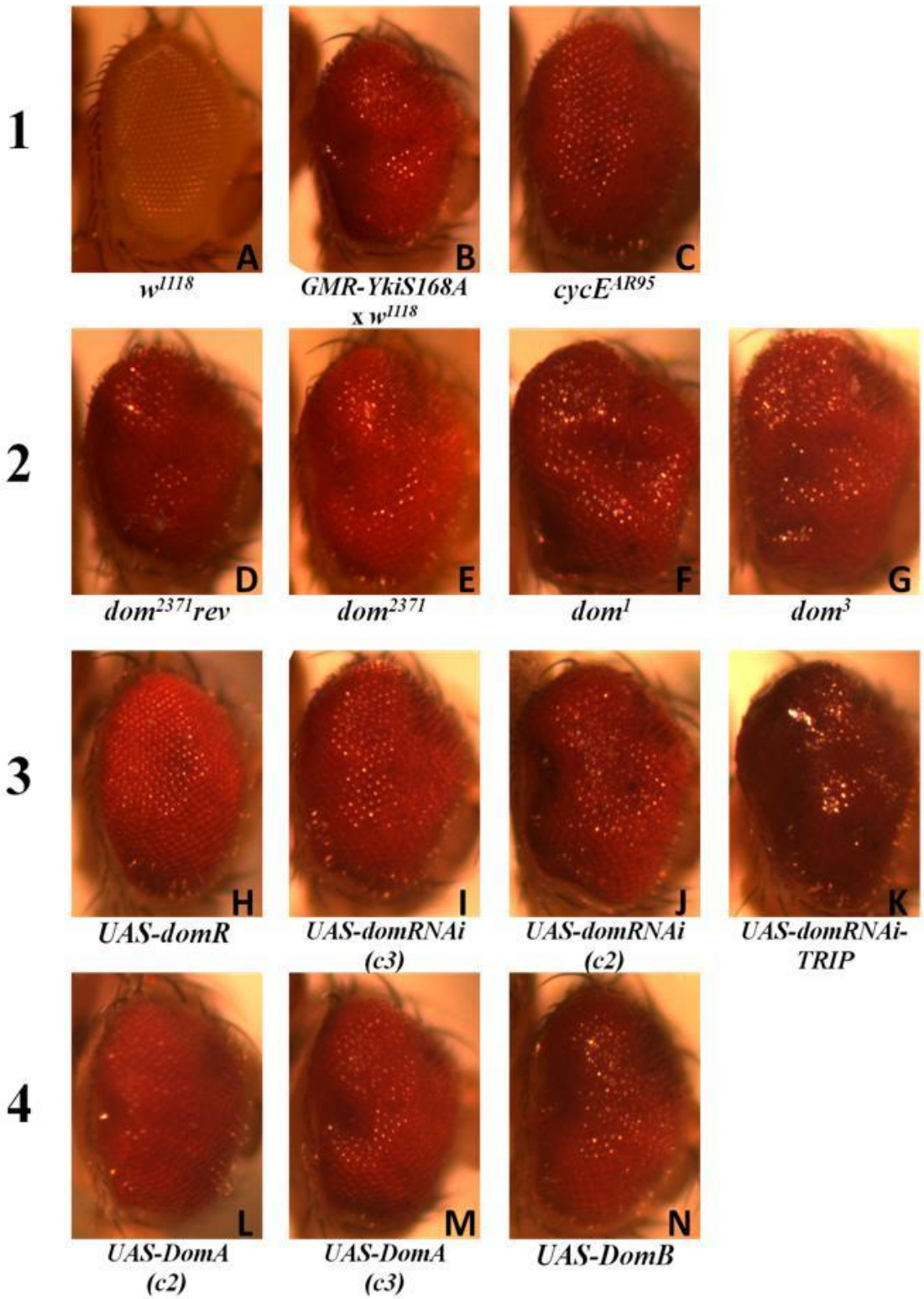

Figure 4. Eyes from the side orientation, taken from male flies. Row 1 shows control crosses, row 2 shows crosses with *domino* loss-of-function mutants, row 3 shows crosses with *domino* RNAi strains, and row 4 shows crosses with *domino* over-expression strains. Of particular note is panel H, which shows significant suppression of the *yorkie* eye phenotype.

Tester Strain	Affected Eyes	Total Eyes	Percent Affected	X^2 Value	P
cycE^{AR95}	84	226	37.168%	89.221	**<0.001**
dom^{2371}rev	196	224	87.5%	3.5	0.0613
dom^{2371}	129	130	99.231%	0.008	0.9287
dom^{1}	204	204	100%	0	1.00
dom^{3}	204	204	100%	0	1.00
UAS-domR	0	217	0%	217	**<0.001**
UAS-domRNAi (c3)	186	306	60.784%	47.059	**<0.001**
UAS-domRNAi (c2)	238	238	100%	0	1.00
UAS-domRNAi-TRIP	468	468	100%	0	1.00
UAS-DomA (c2)	344	344	100%	0	1.00
UAS-DomA (c3)	386	386	100%	0	1.00
UAS-DomB	224	224	100%	0	1.00

Table 7. Penetrance of the *yorkie* eye phenotype (rough, lumpy eyes) in crosses between *GMR-YkiS168A* and 11 different *domino* strains. Control crosses between *GMR-YkiS168A* and *w^{1118}* were 100% penetrant, so this measurement only distinguishes strains that suppress the *yorkie* phenotype.

	Flat Eye Area (Repetition 1)			Flat Eye Area (Repetition 2)			Average Eye Area (Repetition 1)			Average Eye Area (Repetition 2)		
Strain (Female)	**Avg. Area (mm)**	**Diff. from Control (mm)**	**P**	**Avg. Area (mm)**	**Diff. from Control (mm)**	**P**	**Avg. Area (mm)**	**Diff. from Control (mm)**	**P**	**Avg. Area (mm)**	**Diff. from Control (mm)**	**P**
cycE^{AR95}	0.169	-0.009	0.23	0.170	0.026	**<0.01**	0.125	-0.009	**0.04**	0.126	-0.01	
dom^{2371}rev	0.172	-0.032	**<0.01**	0.184	0.003	0.98	0.124	-0.008	0.15	0.135	-0.004	0.55
dom^{2371}	0.183	-0.021	0.05	0.172	-0.010	0.56	0.127	-0.012	**0.01**	0.116	-0.023	**<0.01**
dom^{1}	0.217	0.008	0.81	0.199	0.022	**0.01**	0.149	0.008	**0.04**	0.154	0.018	**<0.01**
dom^{3}	0.189	-0.003	0.99	0.192	0.048	**<0.01**	0.144	0.005	0.59	0.145	0.009	**0.03**
UAS-domR	0.171	-0.038	**0.01**	0.155	-0.049	**<0.01**	0.110	-0.031	**<0.01**	0.109	-0.027	**<0.01**
UAS-domRNAi (c3)	0.175	-0.002	0.98	0.175	-0.002	1.00	0.129	-0.005	0.54	0.131	-0.006	0.57
UAS-domRNAi (c2)	0.179	0.001	1.00	0.176	-0.001	1.00	0.127	-0.007	0.14	0.138	0.002	0.99
UAS-domRNAi-TRIP	0.181	0.003	0.95	0.189	0.011	0.36	0.134	0.000	1.00	0.136	0.000	1.000
UAS-DomA (c2)	0.169	-0.023	**<0.01**	0.162	0.018	**0.02**	0.128	-0.011	**<0.01**	0.125	-0.012	**<0.01**
UAS-DomA (c3)	0.184	-0.008	0.64	0.172	0.028	**<0.01**	0.131	-0.008	**0.04**	0.133	-0.004	0.43
UAS-DomB	0.158	-0.047	**<0.01**	0.172	-0.009	0.39	0.109	-0.027	**<0.01**	0.110	-0.028	**<0.01**
Strain (Male)	**Avg. Area (mm)**	**Diff. from Control (mm)**	**P**	**Avg. Area (mm)**	**Diff. from Control (mm)**	**P**	**Avg. Area (mm)**	**Diff. from Control (mm)**	**P**	**Avg. Area (mm)**	**Diff. from Control (mm)**	**P**
cycE^{AR95}	0.145	-0.011	0.07	0.150	-0.036	**<0.01**	0.108	-0.010	**<0.01**	0.111	-0.007	0.83
dom^{2371}rev	0.159	0.003	0.96	0.156	-0.002	0.97	0.117	0.004	0.44	0.119	-0.005	0.94
dom^{2371}	0.152	-0.003	0.96	0.139	-0.020	0.12	0.112	-0.001	0.98	0.106	-0.018	0.61
dom^{1}	0.165	-0.005	0.76	0.173	0.008	0.32	0.129	0.016	**<0.01**	0.138	-0.011	0.75
dom^{3}	0.161	0.007	0.58	0.163	-0.023	**<0.01**	0.129	0.014	**<0.01**	0.124	0.006	0.93
UAS-domR	0.131	-0.039	**<0.01**	0.143	-0.013	0.07	0.096	-0.016	**<0.01**	0.096	-0.017	**<0.01**
UAS-domRNAi (c3)	0.151	-0.005	0.74	0.153	-0.011	0.10	0.111	-0.007	**<0.01**	0.121	-0.001	1.00
UAS-domRNAi (c2)	0.148	-0.008	0.56	0.154	-0.010	0.15	0.115	-0.003	0.45	0.121	-0.001	0.99
UAS-domRNAi-TRIP	0.159	0.003	0.94	0.165	0.001	1.00	0.119	0.001	0.98	0.123	0.001	0.97
UAS-DomA (c2)	0.149	-0.005	0.82	0.143	-0.043	**<0.01**	0.109	0.035	**<0.01**	0.114	-0.011	0.17
UAS-DomA (c3)	0.155	0.001	1.00	0.154	-0.033	**<0.01**	0.118	0.004	0.80	0.120	0.002	1.00
UAS-DomB	0.151	-0.004	0.88	0.153	-0.006	0.65	0.096	-0.017	**<0.01**	0.103	-0.045	**<0.01**

Table 8. Eye size measurements for all crosses. Flat eye area refers to measurements of eyes from a side view and average eye area refers to measurements taken from both eyes from a frontal view, which were then averaged. Avg. Area is the average eye area measurement for a cross, and Diff. from Control is the difference between the average measurement for a cross and its paired control cross. Because not all crosses took place at the same time, different experimental crosses had different control crosses (*dom^{2371}* and *dom^{2371}rev* always had the same paired control, however). Measurements from both male and female flies are shown.

iv) Genetic Crosses with *GMR-YkiS168A* and *Notch* Mutant Strains

Crosses between *UAS-MamH* and *GMR-GAL4* resulted in offspring with significantly decreased eye area compared to control crosses (w^{1118} and *GMR-YkiS168A* x w^{1118}), both visually (Fig. 5A, panel C) and statistically (Table 9). Crossing *UAS-MamH* to *GMR-YkiS168A* also resulted in decreases in eye area for both the average eye area and flat eye area measurements (Table 9), but none of those measurements was statistically different from the data collected from the *UAS-MamH* x *GMR-GAL4* control cross. A visual analysis, however, showed that offspring from the *UAS-MamH* x *GMR-YkiS168A* cross had very smooth, glossy eyes. The *UAS-MamH* x *GMR-GAL4* control cross offspring also exhibited an eye surface that was glossier than wild type flies, but not to the extent seen with the *UAS-MamH* x *GMR-YkiS168A* cross (Figs. 5A and 5B, panels C and D).

Crosses between nd^{1} and *GMR-YkiS168A* resulted in offspring with increased numbers of ridges compared to the *GMR-YkiS168A* x w^{1118} control cross (Fig. 5B, panel G). On the whole, these flies also had significantly larger eye average areas from the frontal view than w^{1118}, *GMR-YkiS168A x* w^{1118}, and nd^{1} x *GMR-GAL4* control crosses, but there was some variation in significance between different repetitions (Table 9), and size measurements of flat eye area were largely inconsistent for this cross. The nd^{1} *x* w^{1118} and nd^{1} *x GMR-GAL4* crosses both had significantly smaller eye areas than the *GMR-YkiS168A x* w^{1118} or w^{1118} controls (Table 9), and appeared visually wild-type, with no lumps/ridges on the eye (Fig. 5A and 5B, panels E and F).

Under room temperature conditions, the *UAS-*N^{ACT} *x GMR-YkiS168A* cross did not yield any living offspring. The *UAS-*N^{ACT} *x GMR-GAL4* cross fared little better, with all offspring that eclosed dying before their wings unfolded. Placing these crosses at 18°C did not affect this outcome.

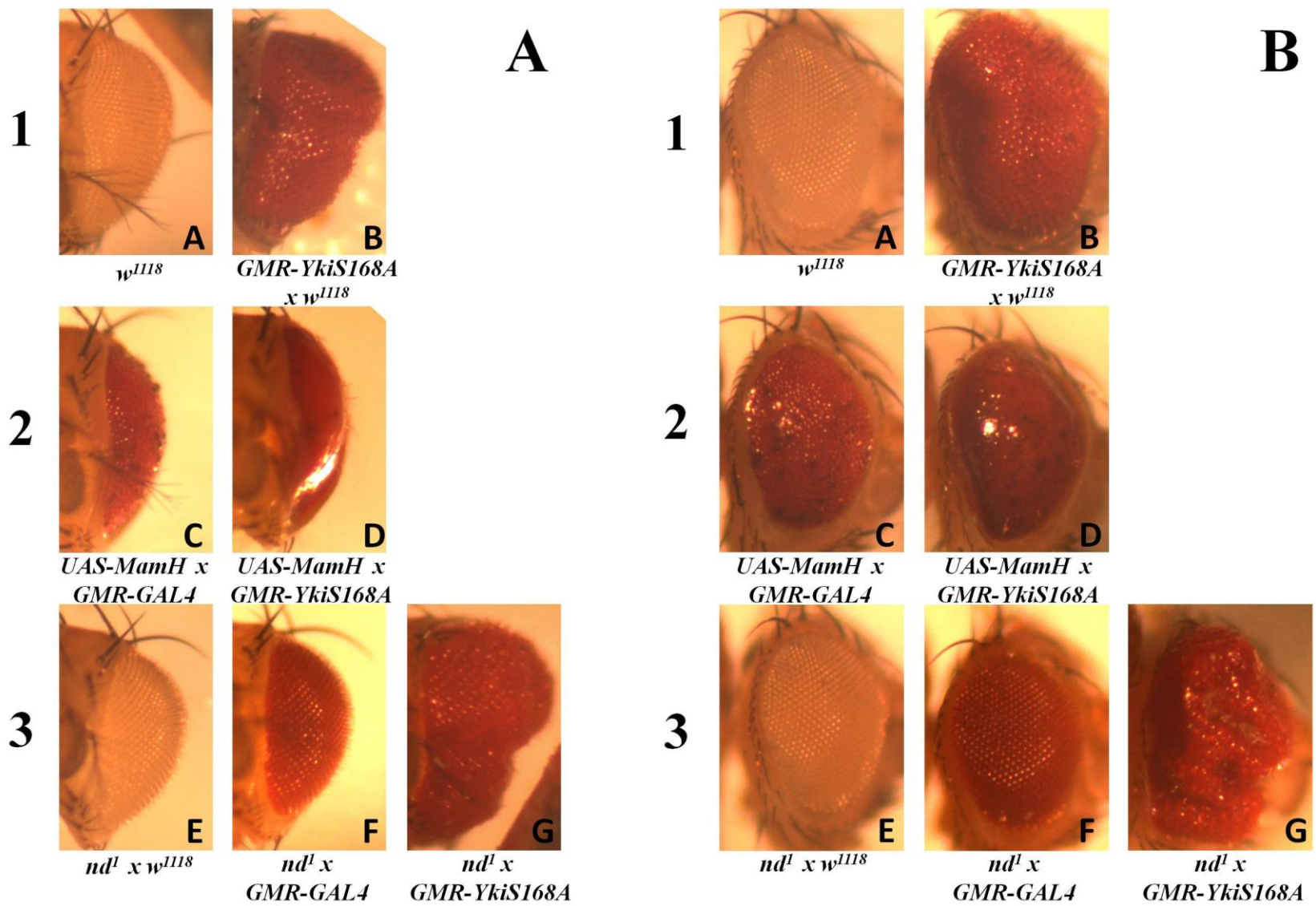

Figure 5. Eyes from the frontal orientation (A) or side orientation (B), taken from male flies. Row 1 shows control crosses, row 2 shows crosses with the strain *UAS-MamH*, and row 3 shows crosses with the strain nd^1.

Flat Eye Area										
Strain		**Avg. Area (mm)**	**Diff. from *w*^{1118}**	**P**	**Diff. from *GMR-YkiS168A* x *w*^{1118}**	**P**	**Diff. from *UAS-MamH x GMR-GAL4***	**P**	**Diff. from *nd*^{1} *x GMR-GAL4***	**P**
UAS-MamH x GMR-GAL4	***Cross 1***	0.106	-0.052	**<0.01**	-0.054	**<0.01**				
	Cross 2	0.101	-0.020	**<0.01**	-0.068	**<0.01**				
UAS-MamH x GMR-YkiS168A	***Cross 1***	0.113	-0.045	**<0.01**	-0.047	**<0.01**	0.007	0.45		
	Cross 2	0.105	-0.016	**<0.01**	-0.064	**<0.01**	0.004	0.80		
nd*^{1} *x GMR-GAL4	***Cross 1***	0.102	-0.056	**<0.01**	-0.058	**<0.01**				
	Cross 2	0.107	-0.015	**<0.01**	-0.063	**<0.01**				
nd*^{1} *x GMR-YkiS168A	***Cross 1***	0.153	-0.005	0.77	-0.007	0.41			0.051	**<0.01**
	Cross 2	0.159	0.037	**<0.01**	-0.011	**0.01**			0.052	**<0.01**
***nd*^{1} *x w*^{1118}**	***Cross 1***	0.103	-0.055	**<0.01**	-0.057	**<0.01**			0.001	1
	Cross 2	0.101	-0.021	**<0.01**	-0.069	**<0.01**			-0.006	0.36
Average Eye Area										
Strain		**Avg. Area (mm)**	**Diff. from *w*^{1118}**	**P**	**Diff. from *GMR-YkiS168A* x *w*^{1118}**	**P**	**Diff. from *UAS-MamH x GMR-GAL4***	**P**	**Diff. from *nd*^{1} *x GMR-GAL4***	**P**
UAS-MamH x GMR-GAL4	***Cross 1***	0.054	-0.064	**<0.01**	-0.063	**<0.01**				
	Cross 2	0.056	-0.018	**<0.01**	-0.063	**<0.01**				
UAS-MamH x GMR-YkiS168A	***Cross 1***	0.057	-0.062	**<0.01**	-0.061	**<0.01**	0.002	0.99		
	Cross 2	0.057	-0.016	**<0.01**	-0.061	**<0.01**	0.002	0.98		
nd*^{1} *x GMR-GAL4	***Cross 1***	0.068	-0.050	**<0.01**	-0.049	**<0.01**				
	Cross 2	0.071	-0.005	0.43	-0.049	**<0.01**				
nd*^{1} *x GMR-YkiS168A	***Cross 1***	0.126	0.010	**<0.01**	0.011	**<0.01**			0.060	**<0.01**
	Cross 2	0.120	0.046	**<0.01**	0.002	0.96			0.051	**<0.01**
***nd*^{1} *x w*^{1118}**	***Cross 1***	0.068	-0.050	**<0.01**	-0.049	**<0.01**			0.000	1.00
	Cross 2	0.067	-0.007	0.08	-0.051	**<0.01**			-0.002	0.97

Table 9. Eye size measurements for all crosses. Flat eye area refers to measurements of eyes from a side view and average eye area refers to measurements taken from both eyes from a frontal view, which were then averaged. Avg. Area is the average eye area measurement for a cross, and Diff. from Control (here, the control is *w*^{1118}, *GMR-Yki* x *w*^{1118}, *UAS-MamH x GMR-GAL4*, or *nd*^{1} *x GMR-GAL4)* is the difference between the average measurement for a cross and its paired control cross. Only male flies were measured. All flies from a single repetition (either Cross 1 or Cross 2) shared the same controls.

Discussion

Domino has been shown to be of critical importance to the *Notch* signaling pathway, such that loss-of-function for a single *domino* allele dramatically enhances wing nicking in flies with loss-of-function *Notch* mutations (Hall et. al. 2004, Kwon et. al. 2013). This indicates that Domino is necessary for cell proliferation, as Notch function is closely linked to cell proliferation control (Guruharsha et. al. 2012). Domino has also been shown to have more direct effects on cell proliferation, and appears to repress E2F targets (Lu et. al. 2007). *Domino* loss-of-function mutations, furthermore, have been shown to suppress *cyclinE* loss-of-function mutations (Lu et. al. 2007), indicating that Domino is involved in reducing cell proliferation. These contradicting observations are the subject of the present study; understanding Domino and Notch function, specifically how Domino and Notch interact, is essential to the understanding of how cell proliferation and differentiation occur.

Genetic Crosses with *C96-Rbf280* and *Domino* Wing Modifier Strains

The *domino* wing modifier genetic analysis identified several genes that have *domino*-mutation independent effects on cell proliferation, as evidenced through interactions with the proliferation-defective *C96-Rbf280* strain. Strains 558, 918, 939, 1000, 1202, 1538, and 1630 showed evidence of enhanced or suppressed wing nicking phenotypes and penetrance, and thus are good candidates for proteins involved in cell proliferation independently of *domino*. The remaining strains, which did not show significant phenotypes, are not likely candidates, although they (along with the other *EP* strains) had previously been shown to interact with *domino* (Kwon et. al. 2013). These data are consistent with the hypothesis that a subset of *domino* modifiers may act primarily via a proliferation effect.

Although some of the strains may contain altered versions of proteins affecting transcription or translation of many genes and proteins, others may encode specific regulators of a cell proliferation pathway. Further experimentation will be necessary to determine which, if any, of these strains contain specific regulators of *domino* in a cell proliferation pathway. Alternatively, *domino* may instead be required for expression of particular modifiers.

Test for Environmentally Caused Variations in Eye Size

Prior work has linked *domino* to cell proliferation in the eye (Lu et. al. 2007), so additional studies were directed to this tissue instead of to the wing**.** Because the *Drosophila* eye was a relatively new model system for the Yedvobnick lab, we performed several preliminary crosses on wild-type flies to test how environmental effects impacted eye phenotype. The average eye size of flies stopped increasing after being placed at sufficiently low density, as we observed offspring of crosses at medium density were not greatly different than those of crosses at low density (Table 6). This indicates that availability of food or space was not a significant factor in any of the experimental crosses that took place later. Although body size varied widely among flies of the same sex at the same population density, eye size did not, indicating that the eye size is resistant to environmental effects. Because male and female flies differed in eye and body size at all population densities, males and females were separated during the statistical analysis of eye size (Table 8).

Genetic Crosses with *GMR-YkiS168A* and *Domino* Mutant Strains

Previous studies have established that Notch positively regulates cell proliferation (Artavanis-Tsakonas et. al. 1999), and that Domino positively regulates Notch signaling (Hall et.

al. 2004, Kwon et. al. 2013). However, it was only recently found that Domino associates with E2F and that E2F target genes in the eye tissue and may have Notch-independent effects on cell proliferation (Lu et. al. 2007). The Yedvobnick lab has recently found that *domino* mutations result in increased wing nicking penetrance and increased loss of wing tissue when crossed to the strain *C96-Rbf280* (Kwon et. al. 2013). Because both *domino* loss-of-function and gain-of-function mutations produced this effect, there is evidence that *domino* is pleiotropic for effects on cell proliferation (Kwon et. al. 2013). The lab, however, has not examined the effects of *domino* mutations in the eye, where Lu et. al. (2007) reported that *domino* mutation enhanced cell proliferation. It is unclear, therefore, whether *domino* behaves the same way in eye tissue as it does in wing tissue, or if *domino* instead has tissue-specific effects. The crosses between the *GMR-YkiS168A* strain and *domino* mutant strains were designed to re-examine whether alteration of Domino levels affects cell proliferation. Another goal of this project was to examine the effects of *domino* loss-of-function and gain-of-function mutations in the eye in order to determine whether *domino* has eye-specific or wing-specific effects.

The measurement of average eye area from the frontal view of the head was by far the most reliable measurement of eye size. Because both eyes are visible, any rotations of the head that expose more of one eye to the camera correspondingly reduce the visible portion of the other eye. Taking the average of the areas of both eyes therefore gives a fairly accurate measurement of the area of a single eye. The side view eye area measurements (flat eye area) were more accurate than measurements of a single eye from the frontal view (data not shown), but because only one eye could be measured, having the head out of alignment with the camera could induce an error in measurement that could not be compensated for; this may be partially responsible for some of the inconsistencies in the flat eye area measurements. For cases where the measurements

of average eye area and flat eye area both show enhancement or suppression, however, it can be assumed that the flat eye area measurement is fairly accurate.

Of the eleven *domino* experimental strains, only three had definitive, significant phenotypic effects, and of those three, two were *domino* RNAi strains that suppressed the effects of *yorkie*; the other was the DominoB over-expression strain. Finding an effect for *domino* RNAi strains, but not for other *domino* mutant strains raises questions. There are several explanations for this result. Crosses involving *domino* loss-of-function mutant strains would result in offspring with one wild-type *domino* allele, and it is possible that a single functional copy of *domino* was enough to allow cell proliferation to proceed. It is also possible that mutant Domino protein levels are somewhat variable, but because this was a purely genetic study, there was no way to test for *domino* expression or for expression of any *domino* targets. Alternatively, the different *domino* mutant strains may be fine, but *domino* alleles may have different effects in different tissue types at different points of development, leading to the formation of distinctive phenotypes in the wing (Kwon et. al. 2013), but not in the eye. *Domino* may have a greater effect on the wing than on the eye because it is expressed under the *C96* promoter during a phase of cell proliferation in the wing (Kim et. al. 2006), but, under the *GMR* promoter, it is not expressed in the eye tissue during a period of comparable growth (Li et. al. 2012).

Another explanation is that *domino* has a pleiotropic effect on cell proliferation; there is considerable evidence that Domino positively regulates cell proliferation through its interactions with Notch (Kwon et. al. 2013), but it has more recently been found that Domino may directly regulate cell proliferation in a negative fashion (Lu et. al. 2007). Under this model, different levels of *domino* expression would result in different effects on cell proliferation depending on whether Domino interacted more with Notch or instead negatively regulated cell proliferation.

The results from the crosses between *GMR-YkiS168A* and various *domino* strains support this model. Both *domino* loss-of-function mutations (through RNAi) and *domino* gain-of-function mutations (through the *UAS/GAL4* driver system) resulted in decreases in eye area, which implies that *domino* may be acting through different pathways to affect cell proliferation. If this were not the case, and *domino* was operating on cell proliferation through only one pathway, we would expect to see *domino* loss-of-function mutations having the opposite effect of *domino* gain-of-function mutations. It is unclear, however, how reliable it is that *domino* gain-of-function mutations resulted in decreases in cell proliferation in the eye, as only the crosses between *GMR-YkiS168A* and *UAS-DomB* showed this effect consistently and significantly.

Previous experiments in the Yedvobnick lab also show this pattern; crossing flies with *C96-Rbf280* to those with either loss-of-function *domino* mutations (*UAS-domR*, dom^{1}, or dom^{3}) or *domino* gain-of-function mutations (*UAS-DomB*) lead to enhancement of the wing nicking phenotype typical of the hypo-proliferation *C96-Rbf280* strain (Kwon et. al. 2013). This is equivalent to *domino* loss-of-function mutations and *domino* gain-of-function mutations both suppressing a hyperproliferation phenotype in the eye. These results could indicate that *domino* has a role in distinct, multi-protein complexes, possibly involving both regulation of the Notch pathway and the repression of E2F targets.

An additional possibility involves a negative effect of *domino* over-expression that actually mimics *domino* loss-of-function. This could occur if unusually high Domino concentrations result in abnormal amounts of Domino binding to normal partners, or possibly binding to novel partners inappropriately. Further experiments are needed to determine which proteins bind to Domino and how those proteins interact to form a complex.

There is also some concern as to why some RNAi strains produced an eye effect, but

others did not. One explanation is that the other *domino* RNAi strains were not as strong as the two that produced effects; either there were not enough copies of the RNAi transcript to generate a sufficient response, or the particular RNAi transgene was ineffective in targeting the *domino* transcript. The *domino* RNAi strain that produced the most dramatic phenotypic effects, *UAS-domR*, actually had four copies of the *domino* RNAi transgene. Similarly, because *domino* over-expression strains each produced a particular form of Domino, they may not have produced enough of the right form of Domino at the right time to generate a significant effect.

Genetic Crosses with *GMR-YkiS168A* and *Notch* Mutant Strains

In a further attempt to determine whether Domino's proliferation effects in the eye were due to interactions with Notch, *Notch* mutant flies were crossed to the *GMR-YkiS168A* tester strain. The *UAS-MamH x GMR-GAL4* and *UAS-MamH x GMR-YkiS168A* crosses both resulted in offspring that had significantly smaller eyes than both the w^{1118} and *GMR-YkiS168A* x w^{1118} control crosses. The *UAS-MamH* x *GMR-YkiS168A* cross also resulted in flies that significantly differed from the *GMR-YkiS168A* x w^{1118} control in that they had a smooth, glossy appearance similar to that of flies possessing the *Notch* mutant gene *facet-glossy*. The eyes in *facet-glossy* flies have lenses separated by shallow troughs when compared to normal eyes, and the lens tissue within those troughs is poorly defined as belonging to one facet or another, giving eyes a smooth appearance (Cagan and Ready 1989). This phenotype is due to changes in cell fate, which is influenced by Notch signaling, and is also similar to that of flies where *Notch* signaling is interrupted through a temperature sensitive mutation early in pupal development (Cagan and Ready 1989). This indicates that MamH also interferes with Notch signaling during eye development in pupal flies, as seen previously (Helms et. al. 1999).

The *nd*^{1} x *GMR-YkiS168A* cross resulted in offspring that showed a visual enhancement of the *yorkie* phenotype compared to the *GMR-YkiS168A* x *w*^{1118} control cross. This cross also had larger eyes than the *GMR-YkiS168A x w*^{1118} controls, although there was variation in the significance of these measurements, especially for the measurements from the side view. The *nd*^{1} x *GMR-GAL4* and *nd*^{1} x *w*^{1118} crosses, on the other hand, actually showed significant decreases in eye size compared to the *w*^{1118} control cross. This indicates that the *nd*^{1} mutation was knocking down *Notch* expression and reducing cell proliferation, as it has been shown to do in other tissues (Kwon et. al. 2013), but that the *yorkie* eye assay might not have been sensitive enough to detect the change. Alternatively, Notch and Yorkie may be interacting, producing an enhanced proliferation phenotype in offspring of the *nd*^{1} *x GMR-YkiS168A* cross, but not those of the *UAS-MamH x GMR-YkiS168A* cross. Previous studies, which show that a *Mam* truncation and *nd*^{1} produce different effects although both mutations cause the loss-of-function of the Notch pathway, support this (Kankel et. al. 2007).

In crosses between *Notch* loss-of-function mutant strains and *w*^{1118} or *GMR-GAL4*, decreases in *Notch* signaling resulted in decreased cell proliferation, as expected. This observation lends some credence to the idea that *domino* loss-of-function mutations suppress cell proliferation through suppressing *Notch* signaling, but does not explain how *domino* gain-of-function mutations also suppress cell proliferation, as observed in the wing (Kwon et. al. 2013). The Lu et. al. (2007) paper, however, shows that *domino* affects E2F target genes and thus does directly suppress cell proliferation.

Conclusion

The data we have collected so far support our hypothesis that *domino* is pleiotropic for cell proliferation in the eye, such that both *domino* loss-of-function and gain-of-function mutations would suppress hyperproliferation. However, the data is not sufficient to determine whether or not that is the case. We found that two *domino* RNAi transgenes caused significant suppression of the *GMR-YkiS168A* hyperproliferation eye phenotype. This data is analogous to the data from Kwon et. al. (2013), which showed that *domino* RNAi transgenes enhanced a hypo-proliferation wing phenotype. We also found that one of the *domino* over-expression strains, *UAS-DomB*, suppressed the *yorkie* hyperproliferation eye phenotype, which is analogous to *domino* over-expression enhancing hypo-proliferation in the wing, as seen in Kwon et. al. (2013).

Our results from crosses between *GMR-GAL4* or w^{1118} and *Notch* loss-of-function strains showed that *Notch* loss-of-function mutations, like *domino* loss-of-function mutations, reduced the amount of cell proliferation. The differences in appearance between the offspring of crosses between *GMR-YkiS168A* and *domino* or *Notch* experimental strains, however, suggest that Domino's effects on cell proliferation may be independent of Notch. For crosses between *UAS-MamH* and *GMR-YkiS168A*, we found that offspring had a glossy eye phenotype and decreased eye size compared to w^{1118} or *GMRYkiS168A x* w^{1118} controls. We also found that crosses between nd^{1} and *GMR-YkiS168A* resulted in offspring with an enhanced hyperproliferation eye phenotype, suggesting that Notch and Yorkie may interact. That we did not see enhancement of the *yorkie* phenotype in offspring of the *UAS-MamH x GMR-YkiS168A* cross indicates that nd^{1} and *UAS-MamH* cause loss-of-function of the Notch signaling pathway differently, or that Mam

and Notch can have independent functions, as has been suggested previously (Kankel et. al. 2007).

Overall, our eye study results suggest that Domino has Notch-independent effects on cell proliferation, and that Mastermind may have Notch-independent effects on cell fate. Our wing study results suggest that a subset of the strains analyzed in the *domino* genetic analysis may have Domino-independent effects on cell proliferation (Fig. 6). Based on the effects of the *domino* wing modifier analysis, and whether a mutation was loss-of-function or gain-of-function, it is likely that SmD, Atg1, and Lilli have negative effects on cell proliferation, and that EcR, Pum, and Lola have positive effects on cell proliferation.

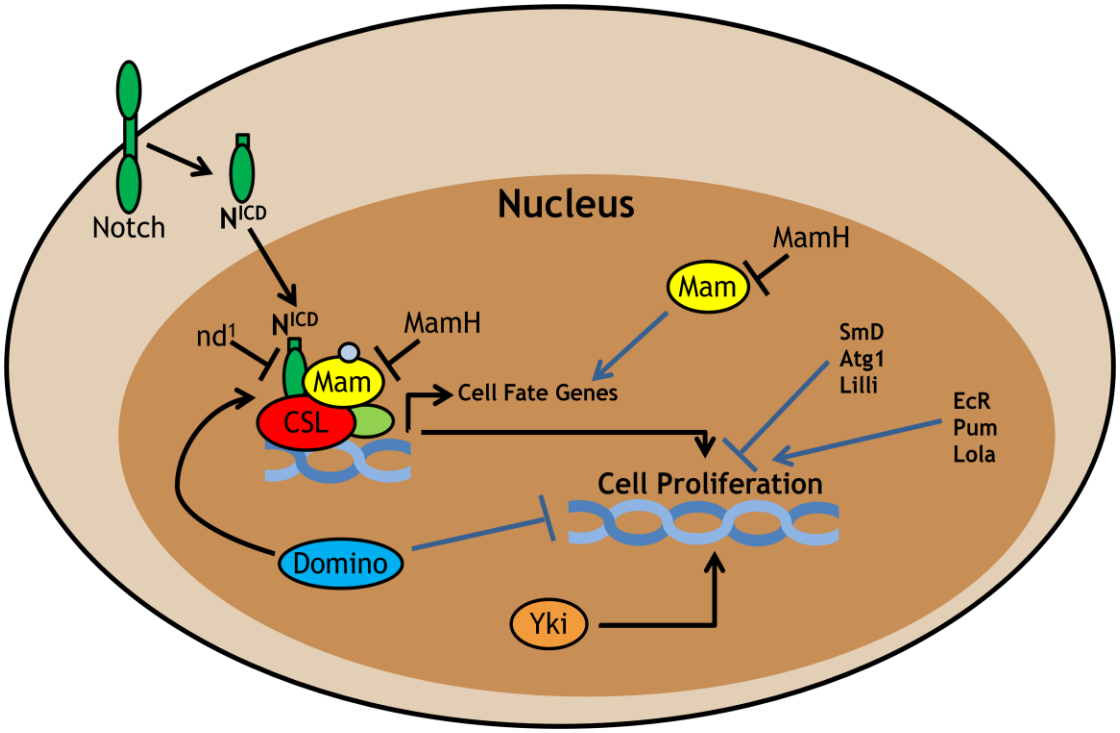

Figure 6. Our proposed model of protein interactions in the cell. Black arrows show interactions that have been demonstrated in previous studies. Blue arrows are proposed pathways based on data from this study. Yki is Yorkie, Mam is Mastermind, and N^{ICD} is the Notch intracellular domain. Lines that end with a perpendicular "T" designate negative interactions.

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