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Alice Kim April 11, 2015

Genetic analysis of Yorkie-driven overgrowth: depletion of transcriptional targets

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Abstract

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The Hippo pathway is a conserved signaling pathway for organ growth and development. Current understanding of the Hippo pathway centers on a core of proteins, including a kinase cascade of the Hippo and Warts kinases that transduce extracellular signals from the cell membrane to the nucleus to modulate gene transcription. Studies have revealed that mutations in genes encoding key Hippo components can lead to uncontrolled cell proliferation in humans and flies, making the Hippo pathway a prime suspect in many types of tumorigenesis. The Hippo pathway has been found to be deregulated in human cancers, such liver, colorectal, lung, and ovarian cancer, primarily in two nuclear components YAP1 and TAZ, which are the human homologs of Yorkie (Yki). Studying the deregulation of Yki in *D. melanogaster* may thus bring insight into the deregulation of the Yki homologs YAP1 and TAZ in human cancers. In the work described herein, two key gaps in our knowledge involving the deregulation of Yki in the Hippo pathway will be investigated: (1) the extent to which Yki interacts functionally with the Taiman (Tai) protein to affect gene expression, and (2) the identity of the full set of genes that are transcriptionally induced by Yki and Tai. Tai had been found to not be absolutely required for overgrowth in *ex* mutant wing cells, and CG83212 transcript had been found to be a key transcript of Yki:Tai that may contribute to Yki-driven overgrowth.

Genetic analysis of Yorkie-driven overgrowth: depletion of transcriptional targets

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Table of Content

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I. Background

The Hippo pathway is a conserved signaling pathway for organ growth and development. During organ growth and development, cells do not have a set number of divisions, which provides flexibility to respond to the particular needs of a tissue, but requires precise cell signaling to determine how much cells should divide (Halder *et al*. 2011). In circumstances of high cell density, epithelial cells are adhered to one another and Hippo 'signaling' is off, restricting proliferation. However in circumstances of low cell density, the lack of adhesion between cells initiates Hippo signaling, allowing proliferation. The deregulation of Hippo signaling can lead to over proliferation, which was first observed in the fruit fly *Drosophila melanogaster* (Justice *et al*. 1995). The observed phenotype resembled the rolls of excess tissue on the hide of a hippopotamus, which inspired the name of the "Hippo" pathway. The Hippo pathway involves multiple proteins that form a signaling circuit and are conserved across multicellular eukaryotes. Much of what we know about the molecular mechanisms and cell biological effects of the Hippo pathway has been defined by studies in the fruit fly *D. melanogaster*. *D. melanogaster* has been historically a useful genetic model to investigate the function of genes with clear homologs in humans, and the Moberg Lab uses *D. melanogaster* to study Hippo signaling for this reason.

Current understanding of the Hippo pathway centers on a core of proteins, which constitute a kinase cascade, that transduce extracellular signals from the cell membrane to the nucleus to modulate gene transcription (Halder *et al*. 2011). The cascade comprises of the kinases, Hippo and Warts. Hippo was the first kinase discovered in the pathway and removing it leads to the excess tissue folds that gave the gene and pathway its name. Many proteins upstream and downstream of the kinase cascade have been discovered, but the work described herein focuses on three previously discovered proteins in the Hippo pathway: Yorkie (Yki), Expanded (Ex), and Taiman (Tai). Ex is an upstream cytoskeletal-associated protein that enhances Hippo/Warts kinase activity that leads to the phosphorylation of Yki, preventing Yki from entering into the nucleus (**Figure 1a**). However, decreased activity of Hippo/Warts kinase activity, such as loss of Ex, Yki remains unphosphorylated and is allowed to enter the nucleus. Once in the nucleus, Yki associates with the Scalloped (Sd) DNA binding protein, and the Yki:Sd heterodimer acts as a sequence-specific transcriptional activator to promote transcription of genes that drive cell proliferation and survival (**Figure 1b**). The Tai protein functions with the

ecdysone hormone receptor and has been found to bind with Yki (Moberg Lab, unpublished), but the role of Tai in Yki-driven overgrowth still needs to be investigated.

Under normal circumstances, the Hippo pathway limits cell proliferation and tissue growth during development by keeping Yki in the cytoplasm. Studies have revealed that mutations in genes encoding key Hippo components can lead to uncontrolled cell proliferation in humans and flies (Barron and Kagey *et al*. 2014), making the Hippo pathway an attractive therapeutic target in many types of tumors. However, molecular analysis of common human cancers has revealed few somatic or germline mutations in Hippo genes, leading to the hypothesis that the Hippo pathway is deregulated, not by mutations in Hippo genes, but by mutations that affect pathways that intersect (or 'crosstalk') with the Hippo pathway (Harvey *et al*. 2013).

Many pathways that are deregulated in human cancers are being tested for useful molecular targets, and molecular features of tumors are becoming the cornerstone of targeted therapies intended to suppress tumor growth and progression (Urruticoechea *et al*. 2010). The rationale for molecular targeting is supported by studies on the phenomenon of 'oncogene addiction,' meaning a cancer has become dependent on a single factor that normal cells are not as reliant upon, and that can be exploited as an 'Achilles heel' of the cancer (Weinstein *et al*. 2008). Searching for molecular targets in the Hippo pathway that suppress tumor growth and progression could lead to the discovery of a cancer's 'Achilles heel' and a new effective therapy.

Since the mechanism of deregulation of the Hippo pathway is not well understood in human cancers, investigations continue. Evidence of altered Hippo pathway activity primarily in elevated levels of its two nuclear components YAP1 and TAZ, which are the human homologs of Yki (Johnson and Halder *et al*. 2013), has been found in a number of types of human cancer types (reviewed in Barron and Kagey, 2014) including liver, colorectal, lung, and ovarian cancer. As a consequence, many groups are now working to develop small molecule inhibitors of the Yki homolog YAP1 for use in cancer therapies (Errico *et al*. 2014). Studying the deregulation of Yki in *D. melanogaster* may thus bring insight into the deregulation of the Yki homologs YAP1 and TAZ in human cancers. In the work described herein, two key gaps in our knowledge involving the deregulation of Yki in the Hippo pathway will be investigated: (1) the extent to which Yki interacts functionally with the Tai protein to affect gene expression, and (2) the identity of the full set of genes that are transcriptionally induced by Yki and Tai. My contribution

to ongoing work in the Moberg Lab has been to carry out two sets of experiments to address elements of each of these gaps in knowledge.

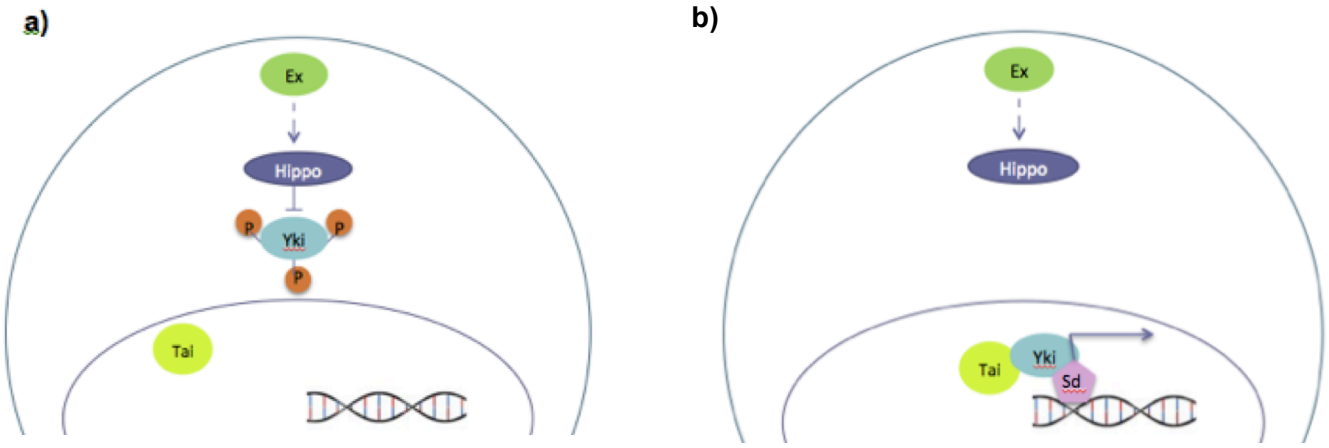

Figure 1. A simplified schematic of the Hippo pathway with the proteins Expanded (Ex), Yorkie (Yki), and Taiman (Tai). a) Expanded leading to the inhibition of Yorkie through phosphorylation. b) Taiman bound to the unphosphorylated Yorkie co-transcription factor

II) Experimental Approach

Experiment 1**:** The first experiment is to test whether a transcriptional coactivator protein called Taiman (Tai), which other researchers in the lab have shown to bind to Yki, is required for Yki-driven overgrowth of the adult fly wing. Tai is a homolog of the nuclear receptor coactivator (NCOA) family of p160 transcriptional regulators (also named SRC-1,2,3). Among the NCOA family, Tai bears most sequence homology to the NCOA3 protein, which was first named Amplified in Breast Cancer-1 (AIB-1) due to its overexpression in a subset of breast cancers (Lahusen *et. al* 2009).

Hypothesis 1**:** We hypothesize that Taiman (Tai) loss prevents Yki from being able to efficiently turn 'on' its target genes, and therefore lower rates of proliferation and growth. I will test this hypothesis by measuring the size of adult wings lacking the Yki-inhibitor Expanded (Ex) versus those lacking Ex and Tai simultaneously.

Experiment 2**:** The second experiment is to use RNA interference (RNAi) transgenes or mutant alleles to test the requirement for "candidate" Yki-target genes in an enlarged-eye phenotype produced by hyperactive Yki. The list of candidates to be tested is taken from a completed RNA-sequencing analysis of the entire transcriptome (about 13,000 RNAs) performed by the Moberg Lab. This analysis identified about 555 RNAs that are induced by Yki in fly cells, about 160 of which were also dependent upon Tai.

Hypothesis 2**:** We hypothesize that depletion of transcriptional targets of Yki:Tai, that are also required for Yki over-proliferation, will suppress a model of Yki-driven overgrowth in the adult eye.

III) Preliminary Data

Relevant to Experiment 1

One piece of critical data in support of my experimental approach in section two is the physical interaction between Tai and Yki. The Tai:Yki interaction is supported in a co-immunoprecipitation analysis of wild type Tai and wild type Yki, and was shown to require two (2) proline-proline-x-tyrosine (PPxY) motifs in Tai that are predicted to interact with the WW domains of Yki (**Fig. 2**; Moberg lab, unpublished).

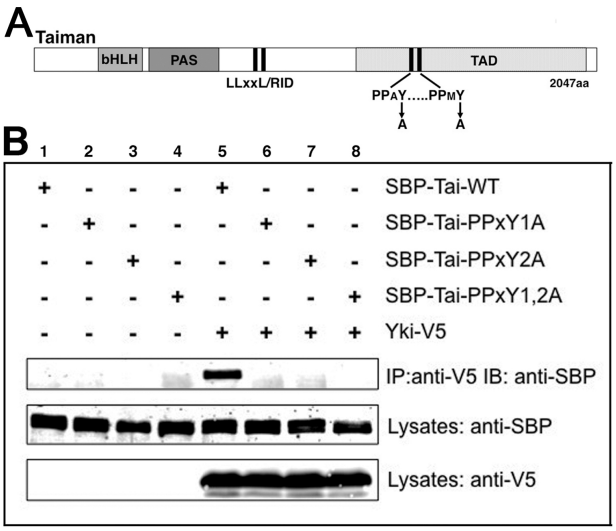

Figure 2. Taiman interacts physically with Yorkie. (A) Domain structure of the Tai protein (bHLH = basic helix-loop-helix, PAS = Period-Arnt-Similar; RID = receptor interaction domain; TAD = transactivation domain). The approximate locations of PPxY motifs and their Y-to-A mutant forms are indicated. (B) Co-immunoprecipitation (coIP) analysis of lysates of S2 cells expressing streptavidin binding peptide (SBP)-tagged Tai (wildtype or mutant forms with Y-A mutations in either PPXY motif or both) and V5-tagged wild type Yki. Figure adapted from Zhang et al, (2015) in revision at *Dev. Cell*.

Relevant to Experiment 2

One piece of critical data in support of my experimental approach in section two is a heat-map summary of the RNA expression patterns in cells with Yki-overexpression with a dependence on Tai (**Fig. 3**, Moberg lab, unpublished)

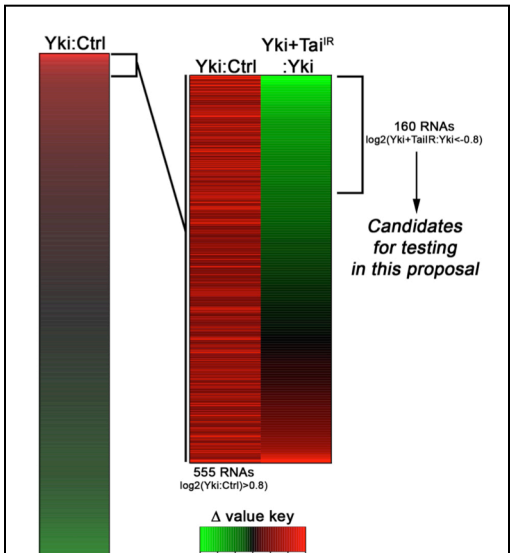

Figure 3. HEAT map summary of RNA sequencing analysis of Yki-induced and Tai-dependent genes. A scaled HEAT map showing expression patterns of >9,000 RNAs in Yki overexpressing versus Control cells (Yki:Ctrl). The top 555 RNAs, selected for a greater than 2-fold induction (log2Δ>0.8) were further filtered for dependence on Tai (Yki+Tai-IR:Yki) by selecting for a drop in expression of greater than 2-fold (log2Δ<-0.8). The genes analyzed in this proposal were selected from this final set of 160 RNAs. Figure adapted from Zhang et al, (2015) in revision at *Dev. Cell*.

IV) METHODS

Experiment 1

Wing phenotype predictions. Ex acts as an indirect inhibitor of Yki, so cells lacking Ex (ie. *ex* mutant) are expected to overgrow. Consistent with this hypothesis, *ex* mutant wings overgrow and are larger than wildtype controls. Tai physically associates with Yki, and is hypothesized to be required in Yki-driven overgrowth. Cells lacking Tai (ie. *tai* mutant) are expected to undergrow and suppress the effects of excess Yki activity. Consistent with this hypothesis, *tai* mutant wings are smaller than control wildtype wings. We aim to test whether double mutant wing cells (lacking both *ex and tai*) overgrow like *ex* mutants or undergrow like *tai* mutants. The phenomenon in which one phenotype is dominant to another opposite phenotype is termed 'epistasis' and generally indicates that one factor acts downstream of the other in a common pathway. We hypothesize that Tai acts downstream of Ex in the Hippo pathway.

Wing genotypes. The genotypes were created using a *Ubx-Flp* source of FLPase to drive mitotic recombination in the wing primordium. By 'flp-ing' over a cell lethal mutation (*cl*) we were able to generate adult wing composed mainly of cells of the indicated genotypes :

control (*FRT*)	(+/+)
expanded mutant	(*ex-/ex-*)
taiman mutant	(*tai-/tai-*)
expanded and *taiman* mutant	(*ex-/ex- tai-/tai-*)

Wing quantification. Pictures of male and female wings were taken on a digital CCD camera at identical magnification. Wing areas were measured and quantified with Photoshop.

Experiment 2

Crossings. Seventeen RNAi (RNA interference) transgenic stocks or mutant alleles corresponding to the genes listed in **Table 1** were selected from the 160 genes identified in the RNA-seq data (see **Fig. 2**). These fly stocks (17) were ordered from the Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu/) (**Table 1**). Each of these stocks was then crossed individually to a stock overexpressing activated Yki in the developing eye (*GMR>YkiS168A*). A phosphorylation site at serine 168 was mutated to alanine, preventing Yki phosphorylation at that site and allowing Yki entrance into the nucleus. The F1 progeny in which expression of the gene

of interest was reduced were then scored for the severity of eye overgrowth. A schematic of the crossing schemes, with controls, is below.

(1) Experiment: gene depletion in background of Yorkie hyperactivity

five males	x	3 virgin females
RNAi line 1	x	GMR-S168A
Heterozygous mutant allele 1	x	GMR-S168A
…		
w^{1118}	x	GMR-S168A

(2) Control: gene depletion in background of GMR-Gal4

five males	x	3 virgin females
RNAi line 1	x	GMR-Gal4, UAS
Heterozygous mutant allele 1	x	GMR-Gal4, UAS
…		
w^{1118}	x	GMR-Gal4, UAS

Crosses in background of w^{1118} (an ethyl methanesulfonate allele of the *white* gene) were used as positive controls. Crosses in background of GMR-Gal4 (without Yki hyperactivity) were to control for effects of gene depletion by itself.

Table 1. RNAi transgene stocks or mutant alleles selected from RNA-sequencing data of cells with Yrki-overexpression and Tai dependence. RNAi fly stocks were ordered from Bloomington (see corresponding stock numbers).

Stock number	Allele/gene	Notes
16109	$CG31145^{A405}$/TM3	Putative secreted kinase
29610	UAS-Dh44-R2 RNAi	GPCR receptor
6447	UAS-RhoGAP100F RNAi	Rho GTPase activating protein at 100F
6446	UAS-RhoGAP100F RNAi	as above
13692	$CG6509^{KG00748}$ /Cy0	Guanylate kinase-like
3109	gd^{7} / FM3	Peptidase *gastrulation defective*

15265	$CG30089^{KG10444}$ / Cy0	Protein coding gene
16858	$CG32813^{EY07727}$	Putative Myb-SANT DNA binding protein
27030	UAS-Dde RNAi	Decarboxylase
31218	UAS-Patsas RNAi	S-palmitoyltransferase
36610	UAS-CG13741 RNAi	Protein coding gene
33079	$dIIp8^{M100727}$	Insulin-like peptide 8
35237	UAS-CG7458 RNAi	General substrate transporter
51500	UAS-CG6765 RNAi	Metal ion binding
51462	UAS-Ddc RNAi	Decarboxylase
36881	UAS-SoYb RNAi	Sister of Yb
36872	UAS-CG17658 RNAI	MYND-type zinc finger

V) Results

Experiment 1

Experiment 1 was to test the hypothesis that Taiman (Tai) loss may prevent Yki from being able to efficiently turn 'on' its target genes, and therefore lower rates of proliferation and growth. Data shows that there was a significant increase in wing area of ex mutant (*ex-/ex-*) compared with control (FRT) wings (p-values: female=3.1×10^{-9}, male=0.003, t-test). There was a significant decrease in wing area in tai mutant (*tai-/tai-*) compared with respective control (FRT) wings (p-values: female=3×10^{-11}, male=2.5×10^{-8}) (**Figs. 4 and 5**). However, there was not a significant difference in wing area between the double mutant (*tai-/tai- ex-/ex-*) and control (FRT) males and females (p-value=0.85 ,t-test) (**Figs. 4 and 5**).

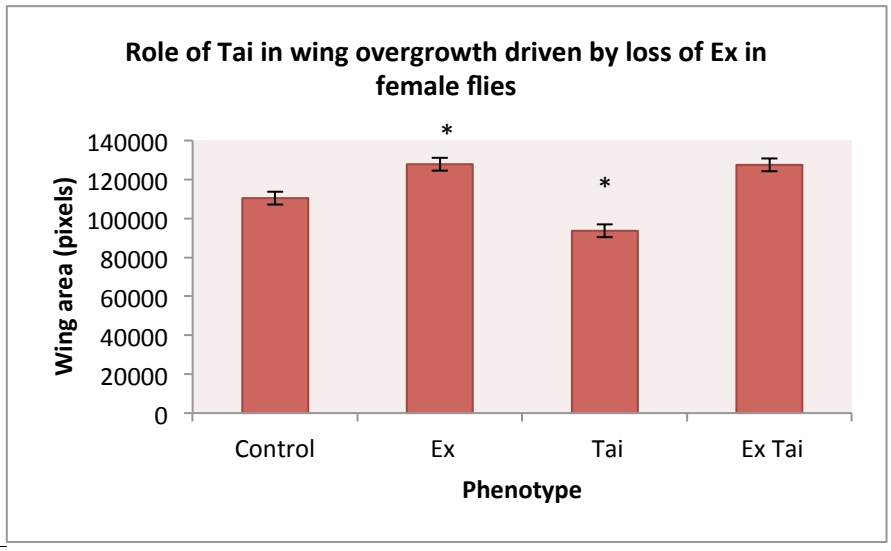

Figure 4. Wing area of female *D. melanogaster* measured in pixels across genotypes: wildtype, expanded mutant, tai mutant, and expanded tai mutant (n=15, 7, 8, 12)

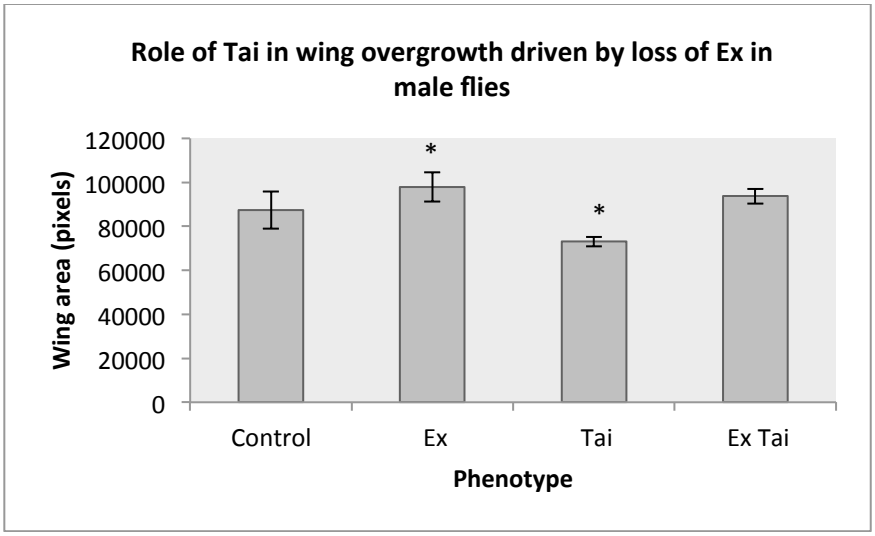

Figure 5. Wing area of male *D. melanogaster* measured in pixels across genotypes: wildtype, expanded mutant, tai mutant, and expanded tai mutant (n=15, 7, 8, 8)

Experiment 2

Experiment 2 was to use RNA interference (RNAi) transgenes or mutant alleles to test the requirement for "candidate" Yki-target genes in an enlarged-eye phenotype produced by expression of hyperactive Yki. After testing all candidates in the crossing scheme shown above (see *Experiment 1* section), I found suppression of eye overgrowth among the progeny of tester females (GMR-Gal4,UAS-yki^{S168A}) crossed with males carrying a P-element insertion in the 5' end of the CG32813 gene (*CG32813^{EY07727}*) (**Fig. 5**). Importantly, there is not an apparent difference in eye area between 'GMR-Gal4 only' flies and GMR-Gal4 in the background of heterozygosity for CG32813 (CG32813^{EY07727}/+). Increasing sample size will increase confidence in statistical significance.

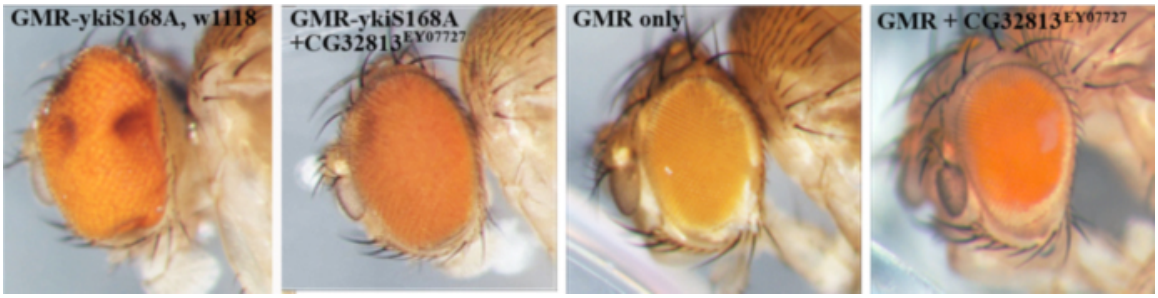

Figure 6. Lateral views of adult female *D. melanogaster* eyes of the indicated genotypes. Note that control GMR-yki^{S168A},w1118 eyes are larger and bulge out of the plane of the image more

than GMR-ykiS168A,CG32813^{EY07727}/+ eyes. Note that CG32813^{EY07727}/+ heterozygosity does not appear to have an effect on control GMR eyes.

The effect of heterozygosity for the CG32183 allele was also clearly evident in head-on views of adult fly eyes of the same genotypes (**Fig. 7**).

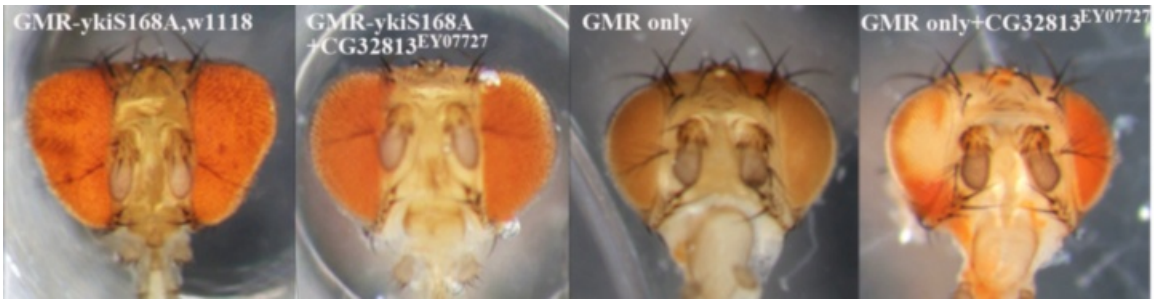

Figure 7. Head-on views of adult female *D. melanogaster* eyes of the indicated genotypes. Note that control GMR-yki^{S168A},w1118 eyes are larger laterally than GMR-ykiS168A,CG32813^{EY07727}/+ eyes. Note that CG32813^{EY07727}/+ heterozygosity appeared to have no effect on control GMR eyes (the white patch of eye in the far right image is an artifact of extended immersion in ethanol).

Importantly, both eye area and width were significantly decreased by heterozygosity for the CG32813 allele (p-value=0.001, 0.0004, t-test) (**Figs. 8** and **9**).

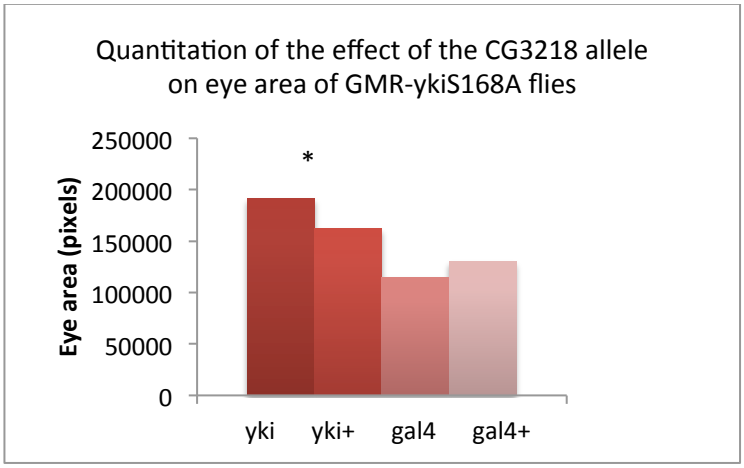

Figure 8. Lateral-view eye area of female *D. melanogaster* measured in pixels among the indicated genotypes: 'Yki' (GMR-yki^{S168A},w^{1118}), 'Yki+CG32813' (GMR-ykiS168A,CG32813^{EY07727}/+), 'Gal4 alone' (GMR-Gal4,w^{1118}), 'Gal4+CG32813' (GMR-Gal4, CG32813^{EY07727}/+). Significant difference in eye area between 'Yki' and 'Yki+CG32813' (p-value=0.001, t-test). (n=8, 10, 1, 1)

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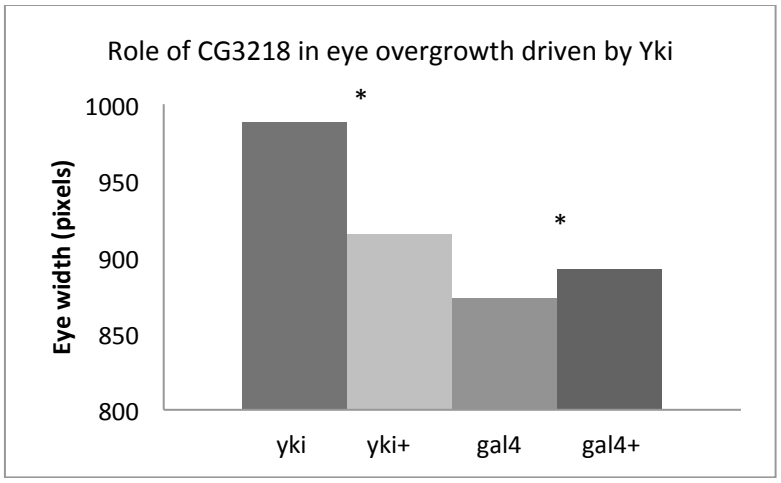

Figure 9. Head-on view eye width of adult female *D. melanogaster* measured in pixels among the indicated genotypes: 'Yki' (GMR-yki^{S168A},w^{1118}), 'Yki+CG32813' (GMR-ykiS168A,CG32813^{EY07727}/+), 'Gal4 alone' (GMR-Gal4,w^{1118}), 'Gal4+CG32813' (GMR-Gal4, CG32813^{EY07727}/+). There was a significant difference in eye width between 'Yki' and 'Yki+CG32813' (p-value=0.0004, t-test). (n=8, 10, 1, 1)

VI) Discussion

The role of Taiman was investigated in *Ex* driven overgrowth in adult wings of *Drosophila,* and double mutant wing cells (lacking both *ex and tai*) was found to overgrow like *ex* mutants, which suggests Tai is not absolutely required for overgrowth in *ex* mutant wing cells (**Figs. 4 and 5**). The dominance of the *ex* mutant phenotype also suggests that Tai acts downstream of Ex in the Hippo pathway. However, *ex* mutant cells are expected to have hyperactive Yki that enters nucleus and acts as a co-transcription factor for proliferative genes, and Tai was hypothesized to support Yki in turning on proliferative genes. Therefore, if Tai provided significant support to Yki, *ex* and *tai* double mutant wing would be expected to be smaller than an *ex* mutant wing. However, the *ex* and *tai* double mutant wing was not significantly different in area than an *ex* mutant wing (**Figs. 4 and 5**). This finding was not surprising, because there are gaps in knowledge of the Hippo pathway. Two explanations are offered here and can be further investigated in the future: (1) Yki is supported by many proteins other than Tai to drive overgrowth, or (2) overgrowth of an *ex* mutant cell can be driven by other means than Yki. Though for reasons not yet known, Tai was not found to be essential in Ex-driven overgrowth, but Tai had been found to physically associate with Yki. Therefore, the role of Tai in Yki-driven overgrowth was investigated.

Among the many transcripts (about 13,000) produced with the aid of Yki, about 160 transcripts were produced in a Tai dependent manner (**Figs. 3**). Seventeen candidate transcripts produced by the Yki:Tai complex were investigated for a role in proliferation in Yki-driven overgrowth. Given the Moberg's lab unpublished data showing that the mRNA of the CG32813 gene is induced by Yki, I proposed that CG32813 was worthy of further study as an important target of Yki in fly cells. My genetic data implicate the product of the CG32813 gene as a potential downstream effector of the Hippo transcriptional coactivator protein Yki. The relevance of the CG32812 gene downstream of Yki is supported by the ability of the CG32813 allele to dominantly (i.e., -/+) suppress Yki-driven proliferation in *D. melanogaster* eyes (**Figs. 6 and 7**). Crossing females with overgrown eyes (hyperactive Yki expression) and males with mutant CG32813 allele produced significant suppression of eye overgrowth in both area and width (p-values=0.001, 0.0004, t-test, **Figs. 8 and 9**). Furthermore, the eye cells are no longer piled up on domes or in folds (GMR Gal4, UAS-Yki^{S168A}), but the cells are aligned to make up a smooth surface of the eye (CG32813^{EY07727} w^{67c23}) (see **Fig. 6**). There was not a large enough sample size

to define the significant difference in eye area between the controls (females with the Gal4, UAS system crossed with male w^{118} or mutant CG23813). However, if there were not significant difference between the controls, this would support that that CG32812 is expressed downstream of Yki, and has specific role in Yki-driven overgrowth.

There are three pieces of evidence of the relevance of the CG32813 gene to human cancers: (1) the depletion of the CG32813 transcript through a heterozygous mutation led to the suppression of over-proliferation (2) the depletion of the CG32813 transcript through a heterozygous mutation led to the suppression of tissue disorganization, and (3) the homology of the CG32813 protein to the human Myb protein (Sala *et. al* 1999). Over proliferation and tissue disorganization are common characteristics of human cancer (Barron *et al*. 2014). Sustaining proliferative signaling is one characteristic that can lead to tumorigenesis, and tissue disorganization can contribute the development of metastatic cancer (Royer *et al*. 2011). Another piece of evidence is revealed in the CG32813 protein's Myb/SANT-like DNA-binding domain that allows the protein to bind to DNA and histones (**Fig. 10**).

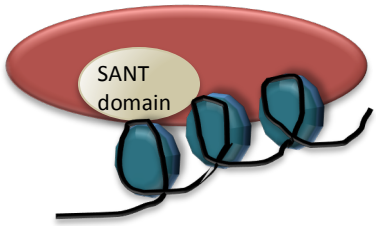

Figure 10. The model shows a **SANT**-domain (**S**witching-defective protein 3 (Swi3), **A**daptor 2 (Ada2), **N**uclear receptor co-repressor (N-CoR), **T**FIIIB) within a DNA/histone binding protein like CG32813 binding to histones wrapped with DNA.

The Myb/SANT-like domain is found among proteins encoded by the Myb oncogene and nuclear receptor co-repressors (Ko *et al*. 2008). The homology of the DNA-binding domain of the CG32813 protein to the human Myb protein supports the possible relevance of CG32812 gene in cancer, because the Myb oncogene has been found to be amplified in hereditary breast cancer (Kaurenemi *et al*. 2000). This finding contributes further evidence that the Hippo pathway plays a role in cancer and further supports the relevance of the Hippo pathway as a source of molecular targets for anti-cancer therapy. Currently, a small-molecule inhibitor of YAP1, the Yki vertebrate homolog, is being tested as a potential therapy, but inhibitors of downstream factors, such as CG38312, may potentially serve as therapeutic target as well.

My work provides a better understanding of the Hippo pathway and identifies CG32813 as a key transcriptional target of Yki, with a possible role in controlling cell proliferation and tissue organization. Future studies should include (1) increase sample size of control crosses in Experiment 2 to support that the CG32813 transcript is downstream from Yki (2) use a different method of CG32813 transcript depletion to support the suppression of proliferation is not specific to the heterozygous mutation of the CG32813 gene, but the depletion of the transcript itself, and (3) determine where the CG38312 protein binds across the genome, and what its targets may be using chromatin-immunoprecipitation studies.

VII) References

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