Validating the Function of Bin3 as a Potential Tumor Suppressor that Antagonises the Function of the oncoprotein Yorkie

A thesis submitted towards partial fulfillment of
BS-MS Dual degree programme
By

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Certificate

This is to certify that this dissertation entitled "*Validating the Function of Bin3 as a Potential Tumor Suppressor that Antagonises the Function of the oncoprotein Yorkie*" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents the research carried out by Pravallika Govada, IISER Pune undermy supervision during the academic year 2015-2016.

Dr. L. S. Shashidhara

Date: 27/3/16

Declaration

I hereby declare that the matter embodied in the report entitled "*Validating the Function of Bin3 as a Potential Tumor Suppressor that Antagonises the Function of the oncoprotein Yorkie*" are the results of the investigations carried out by me at the at the Department of Biology, Indian Institute of Science Education and Research (IISER), Pune, under the supervision of Dr. L. S. Shashidhara, Biology, IISER, Pune and the same has not been submitted elsewhere for any other degree.

Pravallika Govada Date: 27 / 03/ 2016

Abstract

Evidence suggests that cancer is a multistep process. Of these processes, activation of oncogenes and loss of tumor suppressors play a significant role in tumorigenesis. Yorkie/YAP signalling pathway has been well-studied and implicated in organ size control and tumorigenesis. However, there might be several unidentified genes which act as tumor suppressors that regulate the oncogenic function of Yorkie. To identify such genes, we performed a large scale-RNAi screen using *Drosophila melanogaster* as a model organism. In this screen, we identified *bin3* as a potential tumor suppressor wherein, Yorkie overexpression leads to overgrowth of wing imaginal discs and loss of *bin3* enhances this phenotype. The characterisation of these overgrown wing discs was performed to confirm several well-described hallmarks of cancer such as replicative immortality, evasion of apoptosis and metastatic nature. To understand the underlying pathway that leads to tumorigenesis, a hypothesis was proposed which investigates the role of Bin3 as a tumor suppressor that regulates the levels of Dpp target genes by regulating the levels of active Cdk9. Genetic rescue experiments and immunostainings were performed to validate the involvement of Cdk9. Furthermore, we have observed that there is increased nuclear localization of Yorkie when *bin3* is knocked down, mechanism for which needs further investigation. Validation of these results in mammalian system is of future interest.

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Acknowledgements

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Introduction

Hallmarks of Cancer

Cancer in simple terms is a multistep process, which involves deregulation of checkpoints that regulate cell proliferation. To describe the complexity of a cancer microenvironment and tumor progression, Hanahan and Weinberg have proposed a model that involves eight hallmarks of cancer (Hanahan et al. 2011). These hallmarks are sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, deregulating cellular energetics and avoiding immune destruction. The interplay between these hallmarks is what defines the tumor microenvironment and cancer progression. The complex interactions between the hallmarks of cancer promotes tumorigenesis and the knowledge of these complex interactions facilitates the discovery of efficient drugs to treat cancer (Figure 1).

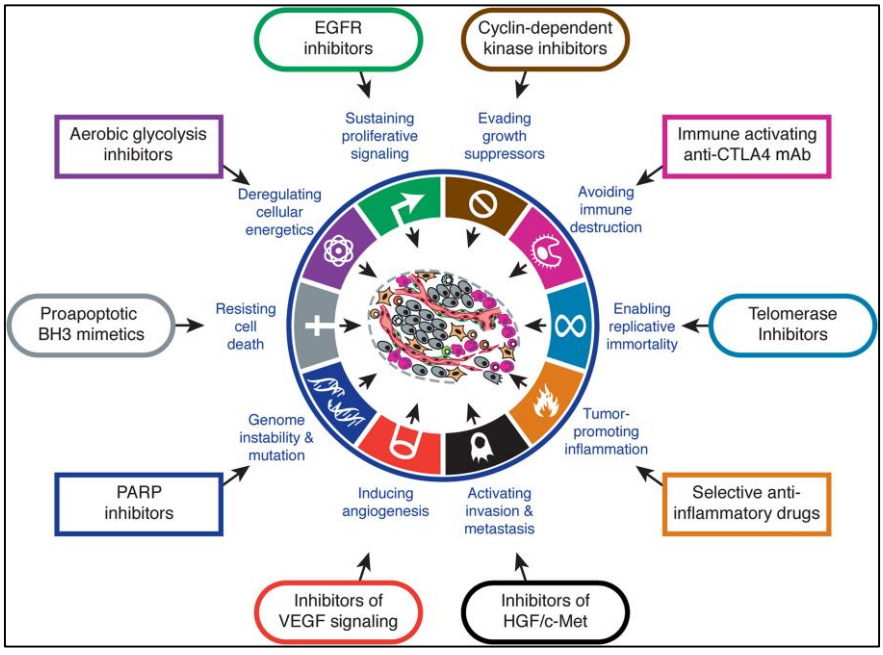


Figure 1. The hallmarks of cancer

The eight hallmarks of cancer include sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, deregulating cellular energetics and avoiding immune destruction. Apart from these aforementioned hallmarks there are enabling characteristics as well; genomic instability and tumor-promoting inflammation that help in the acquisition of the hallmarks of cancer (Hanahan et al. 2011).

Controlling Organ Size through Regulation of Yorkie

In *Drosophila melanogaster,* organ size is controlled by several pathways like Dpp pathway, Notch pathway and Wingless pathway where the key players in regulating the organ size include Dpp, Notch and dMyc (Baonza & Garcia-Bellido 2000; Moreno et al. 2002; De La Cova et al. 2004). However, of the pathways that regulate organ size Hippo pathway; also known as SWH (Salavador-Warts-Hippo) pathway has been well-studied (Zhao Li, L., Lei, Q. and Guan, K. L. 2010; Xu et al. 1995; Keder et al. 2015; Justice et al. 1995; Johnson & Halder 2014; Huang et al. 2005; Bennett & Harvey 2006; Badouel et al. 2009). The chief component of Hippo pathway is 'Hippo'; a kinase, which regulates both cell proliferation and apoptosis. Hippo, through regulation of Yorkie localization, negatively regulates cell proliferation and thus, organ size (Oh & Irvine 2010; Oh & Irvine 2008; Ikmi et al. 2014).

Regulation of Yorkie localization can either be a phosphorylation dependent event or phosphorylation independent event (Figure 2). Hippo, a Ste-20 like protein has a PPxY motif, which enables it to bind to the WW motif of Yorkie (Zhao Li, L., Lei, Q. and Guan, K. L. 2010). Formation of 'Hippo-Yorkie' complex results in inactivation and cytoplasmic retention of Yorkie. This is a phosphorylation independent regulation (Fomby & Cherlin 2011; Oh & Irvine 2010). Other components of Hippo pathway, such as Warts and Expanded regulate Yorkie localization independent of phosphorylation. Although Warts and Expanded belong to a different family of proteins (NDR-kinase family and FERM-domain containing proteins, respectively), they have PPxY motifs which facilitates the interaction of Yorkie and thus, cytoplasmic retention of Yorkie (Fomby & Cherlin 2011; Oh & Irvine 2010).

Phosphorylation dependent regulation of Yorkie is facilitated via Warts. Warts phosphorylates Yorkie at Ser168 site and promotes association of Yorkie with 14-3-3 proteins and results in cytoplasmic retention (Fomby & Cherlin 2011; Oh & Irvine 2010).

Active form of Yorkie is dephosphorylated and localized to the nucleus (Das et al. 2016; Fomby & Cherlin 2011; Chen & Verheyen 2012; Ikmi et al. 2014; Oh & Irvine 2009; Oh & Irvine 2008). Interaction of Yorkie with other transcriptional co-activators such as Scalloped and pMad promotes nuclear localization of Yorkie and

activation of genes that promote cell proliferation (Wartlick et al. 2011; Oh & Irvine 2011).

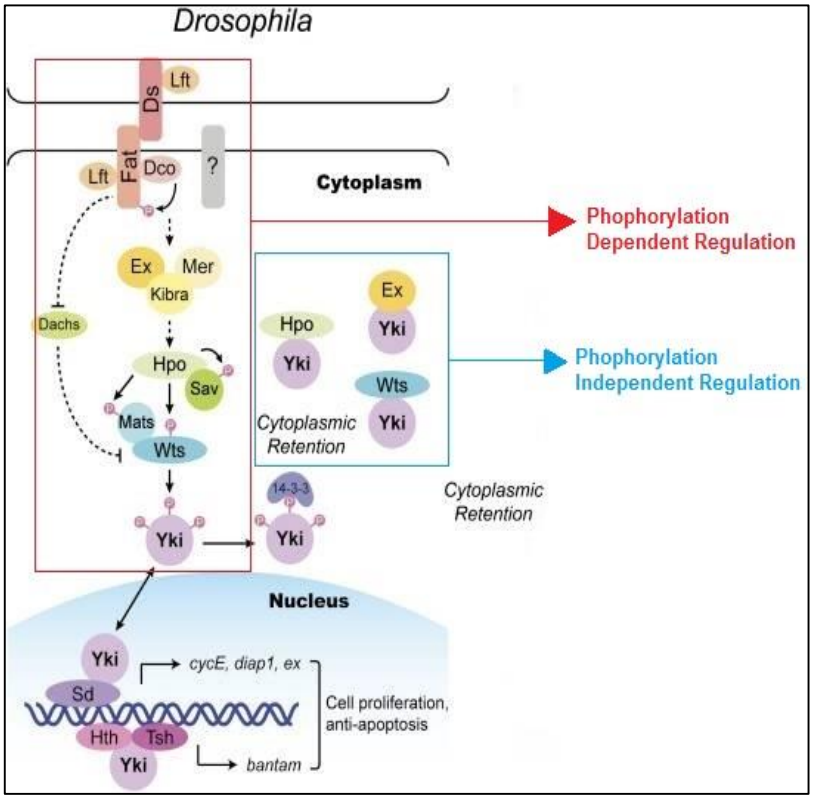


Figure 2: Regulation of Yorkie

Phosphorylation dependent regulation of Yorkie involves Fat-Daschous interaction. Binding of Fat with Daschous results in activation of Hippo pathway. Fat negatively regulates Dachs resulting in derepression of Warts, while activating other components of Hippo pathway such as Expanded and Merlin. Expanded and Merlin phosphorylate Salvador and Mats, which in turn phosphorylates and activates Warts. Phosphorylated Warts deactivates Yorkie. Phosphorylation independent regulation of Yorkie involves direct binding of Hippo, Expanded and Merlin with Yorkie (Zhao Li, L., Lei, Q. and Guan, K. L. 2010).

Hippo Pathway in Cancer

Hippo pathway is generally regarded as a tumor suppressor pathway as it regulates both cell proliferation and apoptosis to regulate organ size. Evidence suggests that mutations in Hippo results in increased cell number and cell size (Wu et al. 2003). Mutations in other components of Hippo pathway such as Salvador and Warts show similar results wherein overgrowth of tissue is observed (Justice et al. 1995; Tapon et al. 2002). Overexpression of Yorkie results in tissue overgrowth that resembles the phenotype of loss of function mutations of Hippo, Salvador and Warts. (Figure 3)

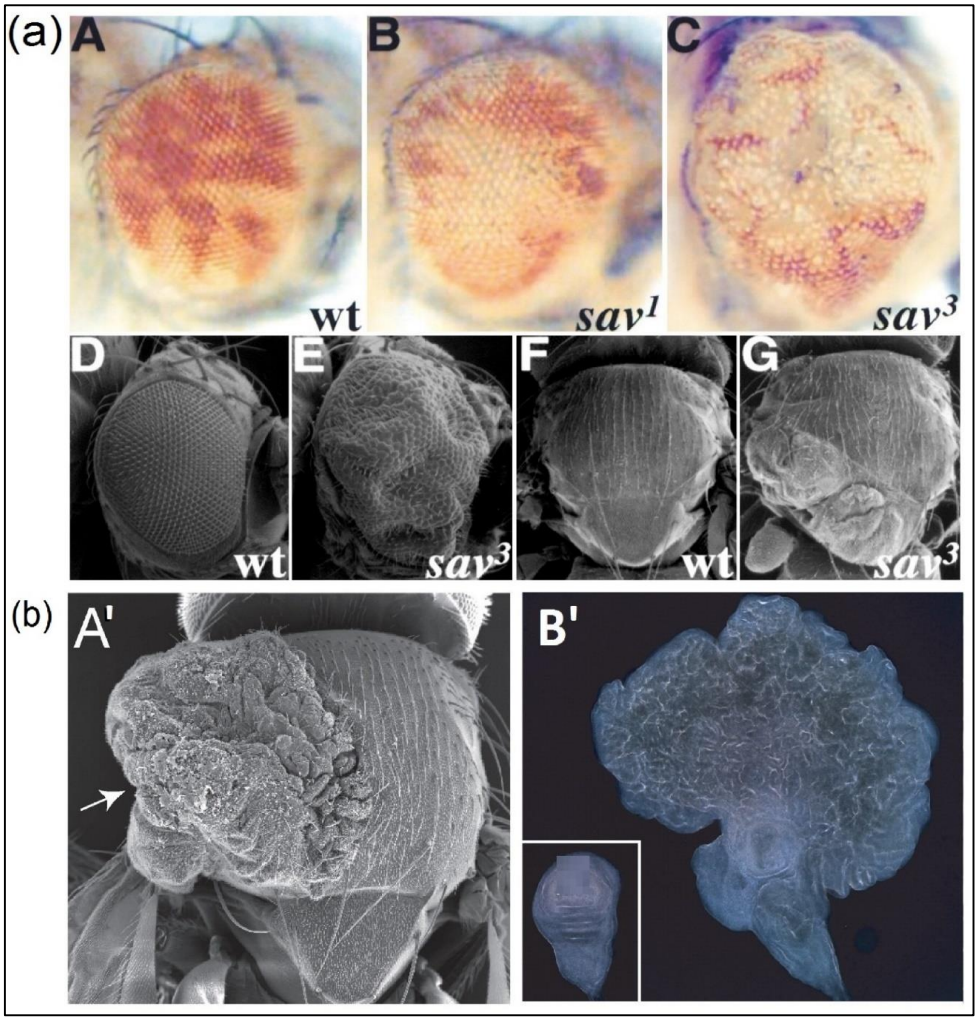


Figure 3: Role of Salvador and Warts as tumor suppressors and role of Yorkie as an oncogene
(A) Overgrown tissue (white ommatidia) in adult eyes in the homozygotic clones of sav^1 allele (B) and sav^3 allele (C) (Adapted from (Tapon et al. 2002)). (D-G) Scanning electron micrographs depicting the overgrown tissue in eyes (E) and notum (G) as opposed to the wild type eyes (D) and notum (F) (Adapted from (Tapon et al. 2002)). (b) Scanning electron micrographs depicting the overgrown tissue in the adult notum (A') and wing imaginal disc (B') (Adapted from (Huang et al. 2005)).

Cooperative Tumorigenesis

Activation of oncogenes or downregulation of tumor suppressors is not sufficient to drive cancer progression. Recent studies indicate that the crosstalk between the two processes is necessary for neoplastic tumor growth. Doggett. *et al* (2011), have provided the evidence for such a crosstalk between downregulation of scribbled and overexpression of Ras (Donoughe et al. 2011). Ras overexpression alone or scribbled downregulation alone results in hyperplastic growth of tissue, whereas scribbled downregulation in combination with Ras overexpression results in neoplastic growth of tissue.

Another evidence of cooperative tumorigenesis is provided by (Herranz et al. 2012). Here again, EGFR overexpression alone or downregulation of Socs36E alone does not result in neoplastic overgrowth. But, a cooperation between the two processes results in neoplastic tumors.

These studies have provided 'Epithelial Transformation Model' (Herranz et al. 2012; Donoughe et al. 2011) to investigate the crosstalk between the processes that might be necessary during the cancer progression.

Using a similar model, we initiated an RNAi screen to identify genes that might give rise to neoplastic tumors. In this screen we used Yorkie overexpression as the background that leads to hyperplastic tumors. Using RNAi lines provided by VDRC, several genes were knocked-down (Table 1) in the background of Yorkie overexpression. The crosses were setup (described in Materials and Methods) and were later screened for GFP positive giant larvae of the following genotype:

$$\frac{ap\ \mathrm{Gal4, UAS-GFP}}{UAS-RNAi}, \frac{\mathrm{UAS-Yki}, tubGal80^{ts}}{+}$$

Table 1: Consolidated Data of RNAi-Screen with Yorkie as an oncogene

The table indicates the number of genes identified as positives in an ongoing screen. The positives are retested and confirmed as described in Materials and Methods (The total number of crosses setup here indicates the number of crosses setup before this project was taken up).

	Yorkie as Background
No. of genes screened in total	334
No. of positives	11
Percentage of positives	3

Through this screen I had earlier identified several tumor suppressor genes (reports as part of 7th and 8th semester projects) that antagonize function of Yorkie (Table 2).

Table 2: List of positives identified as potential tumor suppressors
Of the screened genes (334) approximately 3% percent have been identified as potential tumor suppressors that antagonize oncogenic function of Yorkie. The identification of Hippo (CG 11228) as a tumor suppressor in this screen validates the model, as Hippo is a well-known negative regulator of Yorkie.

Trans ID	CG Number	Gene Name
100197	CG 7586	Macroglubulin complement related
101090	CG 8276	Bicoid interacting protein 3
101524	CG 4379	Protein Kinase A
101887	CG 9653	Brinker
103412	CG 12079	NADH dehydrogenase 30kDa subunit
103416	CG 8174	SR Protein Kinase
103944	CG 4071	Vacuolar Protein Sorting 20
104169	**CG 11228**	**Hippo**
104804	CG 12073	5-Hydroxytryptamine receptor 7
105201	CG 1130	Scratch
108888	CG 9181	Protein tyrosine phosphatase 61F

Out of the identified positives, the role of few genes (highlighted in blue) as regulators of cell proliferation has been validated previously in other studies (Anon n.d.; Tchankouo-Nguetcheu et al. 2014; Fomby & Cherlin 2011; Martín et al. 2004). However, literature lacks the evidence which indicates the role of the aforementioned genes as tumor suppressors that antagonize oncogenic Yorkie. Of these genes, Bicoid interacting protein 3 (Bin3) has been shown previously to play a role in development and has been well-characterized as a methyltransferase that regulates transcriptional and translational processes (Singh et al. 2011). Since, '*bin3*' and Yorkie both are involved in processes that regulate transcription, *'bin3'* was chosen as the gene of interest and a pathway was hypothesized to investigate the role of Bin3 as a tumor suppressor that regulates the levels of Dpp target genes through formation of Yorkie-pMad complex.

Role of *bin3* in development

In a custom two-hybrid selection Bin3 was identified as a protein that interacts with Bicoid and hence, the name 'Bicoid interacting protein 3' (Zhu & Hanes 2000). Based on the protein sequence, the possible role of Bin3 in the development was investigated. In this study it was identified that Bin3 has a SAM (S-adenosyl-L-

methionine) motif; a sequence common in proteins that methylate DNA, RNA, protein or small lipid molecules.

In 2008, the role of MePCE; the human ortholog of Bin3 as a methyltransferase responsible for methylation of RNA, but not proteins, was established (Krueger et al. 2008). In that study, it was identified that MePCE forms a complex with 7SKsnRNA (by methylating its 5' end) and two other proteins, Hexim1 and Larp7. This methylation event is necessary to stabilize 7SKsnRNA which promotes the binding of Hexim1 and Larp7. Binding of Hexim to 7SK-MePCE complex promotes Cdk9 association with the stable complex and results in transcriptional regulation by sequestering Cdk9.

Unlike MePCE (689aa), Bin3 (1368aa) has several extended regions that have no known function (Cosgrove et al. 2013). However, both MePCE and Bin3 have a conserved AdoMet domain (also known as SAM motif), which led to the investigation of role of *bin3* as a methyltransferase in *Drosophila melanogaster* as well (Cosgrove et al. 2013; Nguyen et al. 2012; Singh et al. 2011; Zhu & Hanes 2000). Singh et al. (2011) have reported a role for Bin3 in *Drosophila* embryonic development. Bin3 binds to 7SKsnRNA and methylates 5' end of 7SKsnRNA, facilitating the interaction of Hexim, Larp1, PABP and Ago2 proteins. This forms a stable RNA-protein complex to facilitate translational repression of *caudal* via Bicoid. Therefore, Bin3 acts at the level of both RNA and DNA in order to control development of *Drosophila*.

But, role of Bin3 in cancer or in regulation of organ size has not yet been investigated. This study attempts to identify role of Bin3 in tumor progression with Yorkie as the oncogenic background.

Hypothesis

This study tests the hypothesis (Figure 4) that increased tumor size when *bin3* is downregulated in the background of Yki over-expression is due to decreased activity of Hexim and increased levels of active Cdk9. Active Cdk9 in turn enhances the levels of pMAD-Yki complex (Alarcón et al. 2010) in the nucleus. pMad-Yorkie complex is localized to the nucleus and brings about the expression of Dpp target genes such as bantam mi-RNA; a gene well-known for its pro-proliferative signal (Teleman & Cohen 2000; Theisen et al. 2007; Moser & Campbell 2005; Kirkpatrick et al. 2001).

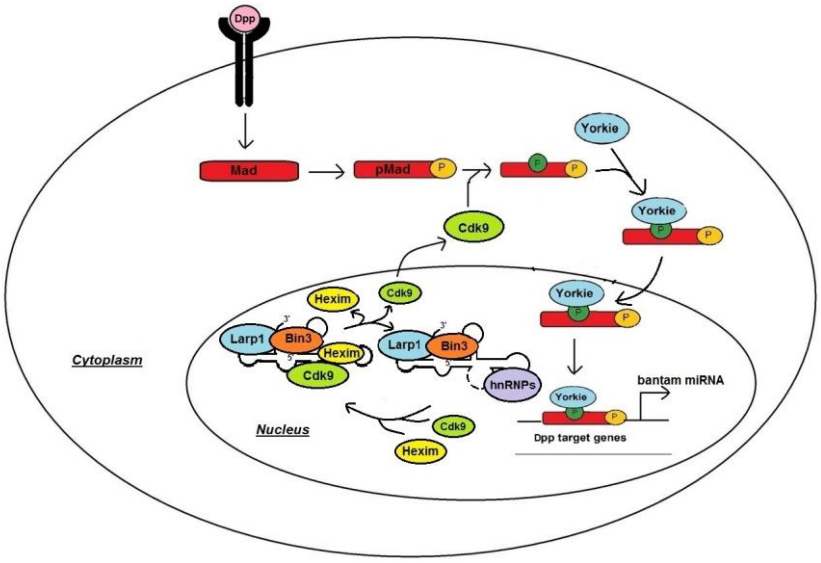


Figure 4: The proposed hypothesis to investigate tumorigenesis
As described previously, when Bin3 is not available 7SKsnRNA is not stabilized. This results in an increase in the levels of active Cdk9. Active Cdk9 phosphorylates pMad and promotes formation of Yorkie-pMad complex. Yorkie-pMad complex is localized to the nucleus and results in transcription of Dpp target genes. Of the activated genes is *bantam* miRNA which is well-known for its pro-proliferative signal.

Objectives

(A) Characterise the tumors, induced by Yki in the background of loss of *bin3*, by immunostainings that indicate loss of cell polarity, Epithelial-Mesenchymal Transtition (EMT), replicative immortality and evasion of cell death.

(B) Examine the effect of over-expression of Cdk9 and downregulation of Hexim on Yki-mediated tumor growth.

(C) Test if down regulation of Cdk9 rescues the tumor phenotypes observed when *bin3* is down regulated in the background of Yki upregulation.

Materials and Methods

Genotypes of various parents and their progeny

Table 3: List of genotypes examined and their abbreviation used

The genotypes enlisted in the table above indicate the both genotypes of parent lines and the progeny that was examined. (Note: UAS-Yki has been abbreviated as 'Yki' and 'Yki^{OE}' interchangeably, where 'Yki' has been used for figures and 'Yki^{OE}' has been used for text)

Abbreviation Used	Genotype Examined
ap >	*ap*-GAL4
ap > GFP	*ap*-GAL4, UAS-GFP / cyo; tubGAL80^{ts} / TM6B
ap > Yki	*ap*-GAL4, UAS-GFP / cyo; UAS-Yki, tubGAL80^{ts} / TM6B
ap > GFP + *bin3^{RNAi}*	*ap*-GAL4, UAS-GFP / UAS-*bin3^{RNAi}*; tubGAL80^{ts} / +
ap > Yki + *bin3^{RNAi}*	*ap*-GAL4, UAS-GFP / UAS-*bin3^{RNAi}*; UAS-Yki, tubGAL80^{ts} / +
ap > Yki + *PTEN^{RNAi}*	*ap*-GAL4, UAS-GFP / UAS-*PTEN^{RNAi}*; UAS-Yki, tubGAL80^{ts} / +
ap > Yki + *bin3^{RNAi}* + *cdk9^{RNAi}*	*ap*-GAL4, UAS-GFP, UAS-*cdk9^{RNAi}* / UAS-*bin3^{RNAi}*; UAS-Yki, tubGAL80^{ts} / +
ap > Yki + *hexim3^{RNAi}*	*ap*-GAL4, UAS-GFP / UAS-*hexim^{RNAi}*; UAS-Yki, tubGAL80^{ts} / +
ap > Yki + Cdk9^{OE}	ap-GAL4, UAS-GFP / UAS-Cdk9; UAS-Yki, tubGAL80^{ts} / +

Drosophila Strains

The following stocks were provided by Dr. Steve Cohen's lab.

Abbreviation Used	Genotype of Parent Line
ap > GFP	*ap*-GAL4, UAS-GFP / cyo; tubGAL80^{ts} / TM6B
ap > Yki	*ap*-GAL4, UAS-GFP / cyo; UAS-Yki, tubGAL80^{ts} / TM6B

All the RNAi stocks (total 334) were obtained from VDRC (Vienna Drosophila RNAi Center). The other UAS lines (UAS-cdk9; BL#12221) were obtained from Bloomington Stock center. The double balancer stocks were obtained from the IISER Pune stock center.

Temporal Regulation of Yorkie overexpression

A GAL4-GAL80 system was used to temporally regulate the expression of Yorkie. When the stocks or crosses were maintained at 18°C, GAL80 is active and represses GAL4 activity. At the developmental stage of interest, when embryos/larvae were shifted to 29°C, GAL80 would become inactive, thereby making GAL4 driver active. GAL4, in turn, transcribes genes/RNAi of interest downstream of UAS element. In the experiments described here, GAL4 was activated by shifting larvae to 29°C at late 2nd instar stage.

Screening and Confirmation of Positives

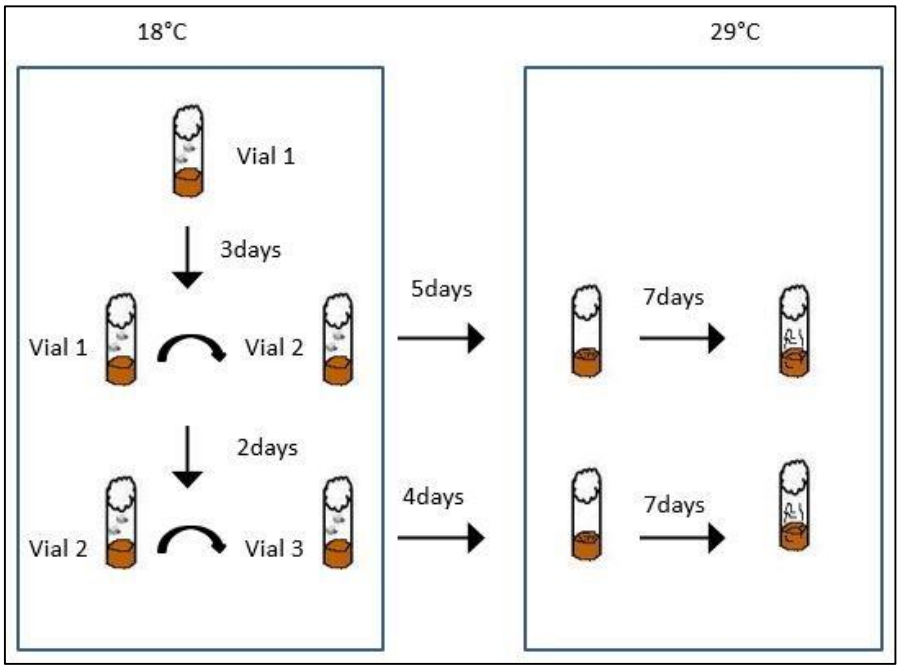


Figure 5: Screening and retesting of positives

The 'Vial 1' indicates the initial setup of cross. 'Vial 2' and 'Vial 3' indicate the technical replicates of the cross generated from 'Vial 1' after 3 days and 2 days of egg laying, respectively.

Since Yorkie overexpression is lethal for the larval development, the larvae were grown until they reach late 2nd instar stage and then Yorkie overexpression was induced after 5 days for vial 2 and after 4 days for vial 3. These crosses were scored for GFP positive giant larvae (indicating delay in development due to overgrowth) and overgrown wing imaginal discs. The positives were retested using biological replicates wherein a similar schedule is followed.

Measurement of Cell Size

Cell size was measured as described earlier (Singh et al. 2015). 10 cells were randomly chosen in a wing imaginal disc of a particular genotype wherein; the shape of cells is clearly visible after DE-cadherin staining. The analysis was done on randomly chosen 10 wing imaginal discs per genotype, each obtained from different set of dissections.

Immunostaining

The anti-pMad antibody (1:150) and anti-Yorkie antibody (1:200) are a generous gift from Ginés Morata. The anti-pH3 antibody (1:1000) was a generous gift from Dr.

Mayurika Lahiri. Other antibodies were obtained from DSHB (Developmental Studies Hybridoma Bank), which include, anti-DECAD (1:100) and anti-mmp1 (1:150). (Dilution factors are indicated in the parentheses.)

The secondary antibodies used include Goat anti-rat (Alexa 633), Goat anti-mouse (Alexa 568), Donkey anti-rabbit (Alexa 594) and Donkey anti-guniea pig (Alexa 633). All antibodies were used with the dilution factor 1:1000, unless otherwise mentioned.

Imaginal Disc Transplantations

Making Glass Capillaries

Glass capillaries were a gift from Dr. Aurnab Ghose. The capillaries were pulled using the P-97 Flaming / Brown Micropipette Puller. The capillaries were then broken approximately 1/4th starting from the tapered end at an angle of 45° in order make a needle.

The wing imaginal discs were dissected out from third instar larvae and cut into fragments using forceps. The fragments were introduced into the abdomen of virgin females of the strain w^{1118} using sharpened glass capillaries. The transplants were maintained at 29°C and scored only after 5-7 days.

Trypsin Cell Dissociation Assay

The wing imaginal discs were dissected out from third instar larva. The wing discs were immersed in 200µL of 2X Trypsin (25g/L solution of 10X Trypsin from Sigma Aldrich). Gentle swirling of glass cavity block was done to ensure that enzyme is always in contact with the wing imaginal discs during the experiment. The readings were taken until the wild type wing discs were completely dissociated.

Stock Preparation for rescue of *bin3^{RNAi}* phenotype by *cdk9^{RNAi}*

Both c*dk9^{RNAi}* and *bin3^{RNAi}* (VDRC RNAi-lines) are on 2nd chromosome. *cdk9^{RNAi}* was combined to create the stock: *ap* > UAS-GFP, *cdk9^{RNAi}* / cyo; UAS-Yki, tubG80^{ts} / TM6B. Genetics crosses towards making this stock are shown below. .

$$\frac{If}{cyo} ; \frac{TM3}{\text{Ser}} \quad X \quad \frac{cdk9^{RNAi}}{cdk9^{RNAi}}$$

Select for non-If, Non-TM3, cyo and serrate wings

$$\frac{cdk9^{RNAi}}{cyo} ; \frac{+}{\text{Ser}} \quad X \frac{ap\ \text{G4,UAS}-\text{GFP}}{cyo} ; \frac{UAS-Yki,tubG80^{ts}}{TM6B}$$

Select for flies with wing defects

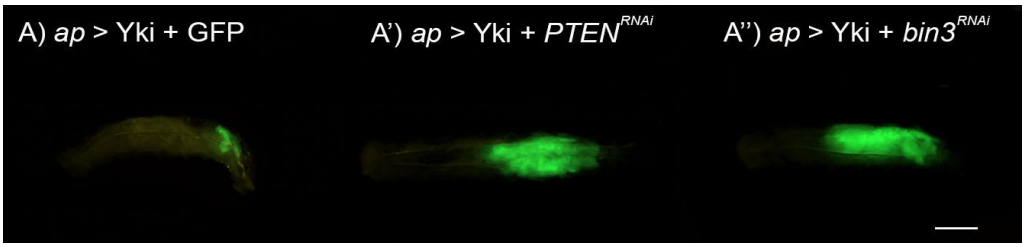
$$\frac{ap\ \text{G4,UAS}-\text{GFP}}{cdk9^{RNAi}} ; \frac{UAS-Yki,tubG80^{ts}}{Ser} X \frac{Pin}{cyo} ; \frac{TM2}{\text{TM6B}}$$

$$\frac{ap\ \text{G4,UAS}-\text{GFP},cdk9^{RNAi}}{cyo} ; \frac{UAS-Yki,tubG80^{ts}}{TM6B}$$

RESULTS

A) Bin3 as Potential Tumor Suppressor Gene:

Knock-down of a known tumor suppressor, PTEN (Keng et al. 2012) was used as a positive control to have a measure of increase in growth of wing imaginal disc when *bin3* is downregulated. Over-expression of Yki alone causes marginal increase in the size of wing discs (Fig. 6A), which is substantially increased when *PTEN* is down regulated (Fig. 6A'). *bin3* down regulation also caused substantial increase in wing disc size (Fig. 6A"), comparable to that of *PTEN*^{RNAi}.



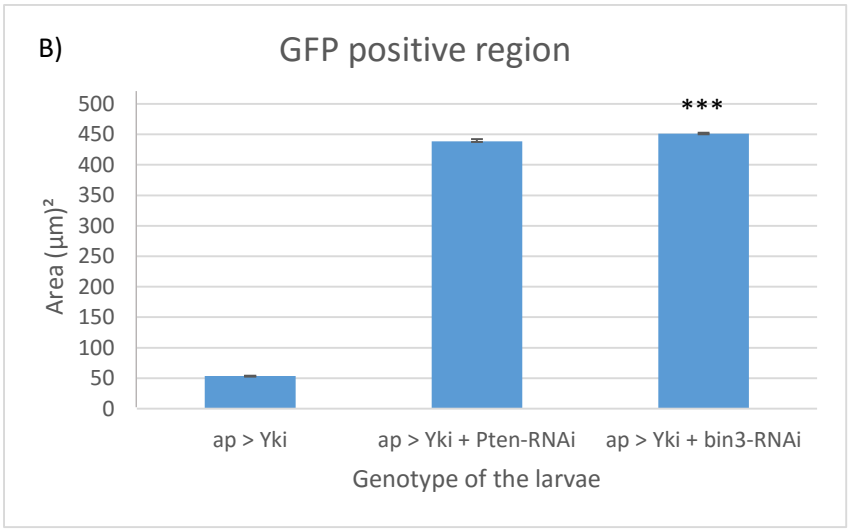


Figure 6: GFP positive 3rd instar larvae indicating tissue overgrowth. Genotypes of the larvae are shown on the images A)-A''). Note increased wing disc size (almost filling half of the larval body) when *PTEN* or *bin3* are down regulated in the background of Yki over-expression.
B) Bar Graph Indicating the change in area as a result of increase in tissue size, ***p=<0.001.

B) Characterizing the observed tumors:

a) *bin3* down regulation alone has no effect on wing disc growth:

Down regulation of *bin3* alone resulted in wing developmental defect (Fig. 7A), although there was no effect on wing disc size at larval stages (Fig. 8B).

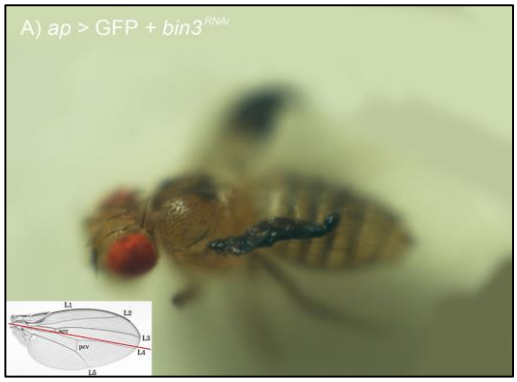


Figure 7: Down regulation of *bin3* affects wing development

Knock-down of *bin3* in the dorsal compartment results in defects in adult wings. The adult wings do not have a definitive boundary and vein formation as compared to the wild type wings (inset).

b) Loss of epithelial polarity and epithelial-mesenchymal transition in Yki^{OE} *bin3*^{RNAi} tumors:

Loss of DE-cadherin or misclocalised DE-cadherin indicates that epithelial organisation is compromised. Thus, to investigate loss of epithelial organisation, localisation of DE-cadherin was examined. DE-cadherin is apically localised in the wing imaginal discs of wild type (Fig. 8A), *bin3*^{RNAi} (Fig. 8A') and Yki^{OE} (Fig. 8A'') discs. However, we observed reduced DE-Cadherin expression and also its and mis-localization in *bin3*^{RNAi} Yki^{OE} wing discs (Fig. 8A'''). This suggests loss of polarity, albeit partial, in the tumors induced by *bin3*^{RNAi} Yki^{OE} .

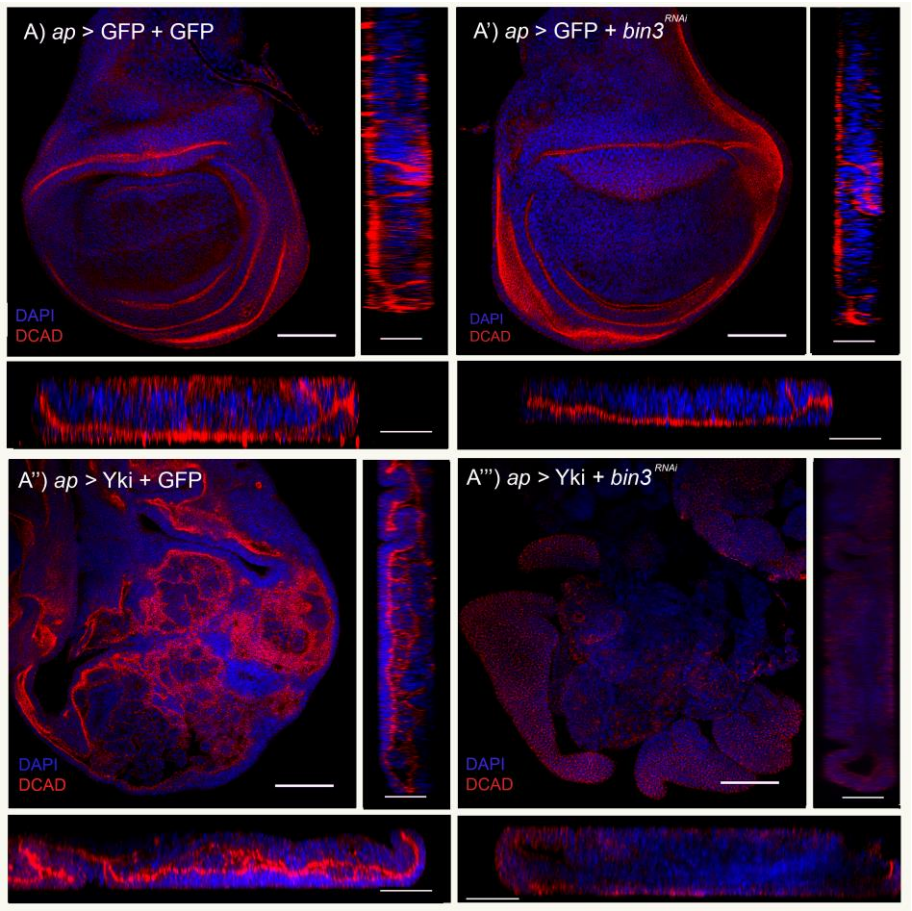


Figure 8: Loss of Cell Polarity in tumors caused by the down regulation of *bin3* and over-expression of Yki. Confocal micrographs of third instar wing imaginal discs stained for DNA (using DAPI; blue) and DE-cadherin (using antibodies; red). Optical cross-sections of the respective phenotypes indicate the localisation of DE-CAD. Note, reduced and mis-localized DE-CAD in $bin3^{RNAi}$ Yki^{OE} wing discs.

Another hallmark of cancer includes the invasive capability of the cells. During EMT the cells lose polarity and adhesive properties to acquire invasive capabilities. This process involves breakdown of basement membrane which is facilitated by proteases such as Mmp1 (Matrix Metalloprotease 1). Thus, to investigate the extent of EMT, the levels of Mmp1 was examined using immunostainings.

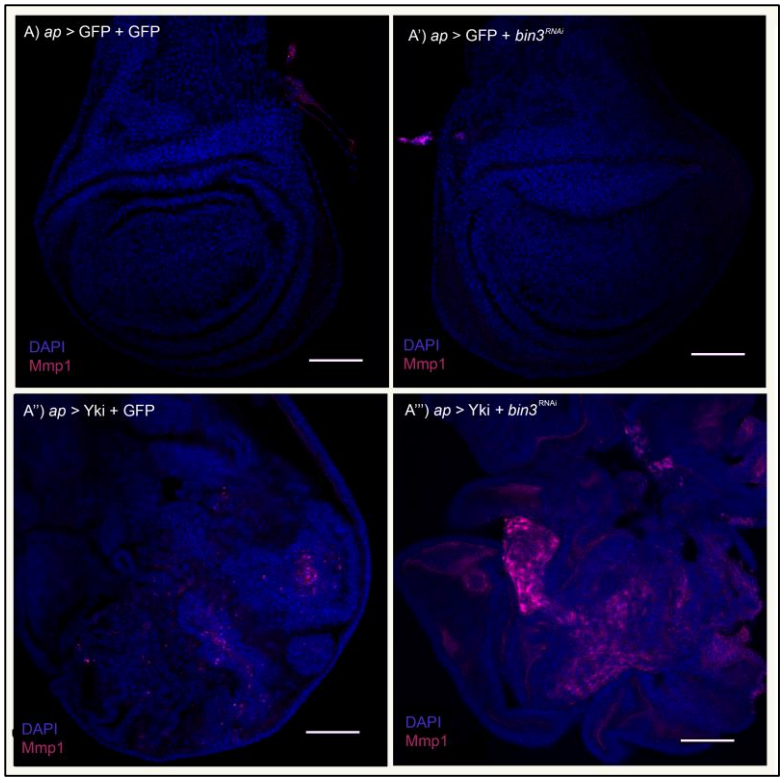


Figure 9: Overexpression of Mmp1 caused by the down regulation of *bin3* and over-expression of Yki
Confocal micrographs of third instar wing imaginal discs stained for DNA (using DAPI; blue) and Mmp1 (using antibodies; magenta). Note elevated levels of Mmp1 in $bin3^{RNAi}$ Yki^{OE} wing discs.

Mmp1 is absent in the wing imaginal discs of wild type (Fig. 9A), $bin3^{RNAi}$ (Fig. 9A') and Yki^{OE} wing discs (Fig. 9A''). However, we observed elevated levels of Mmp1 in $bin3^{RNAi}$ Yki^{OE} wing discs (Fig. 9A'''), suggesting that these cells are undergoing EMT.

c) <u>Increased Cell Size in Yki^{OE} $bin3^{RNAi}$ tumors</u>:

During normal growth, both cell size and cell number are tightly regulated. For example, any increase in cell number is compensated by decrease in cell size to keep the organ size intact. However, during the uncontrolled growth of tumors, particularly neoplastic tumors, cell size also increases substantially, along with cell number. Thus, change in cell size is another indicator of tumor formation. To investigate if there is a change in the size of cells, the area of cells was measured for wild type, Yki^{OE} and $bin3^{RNAi}$ Yki^{OE} wing imaginal discs (Figure 10).

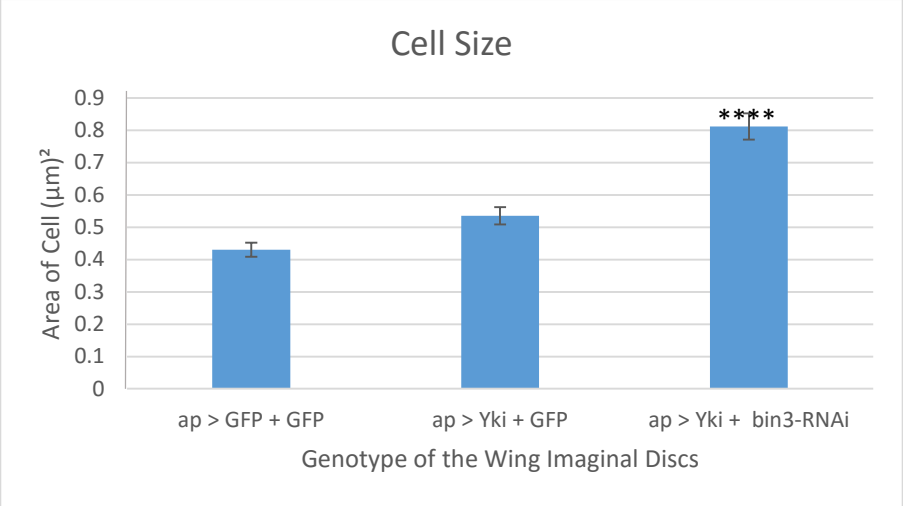


Figure 10: Increase in cell size caused by the down regulation of *bin3* and over-expression of Yki

Note significant increase in the size of cells in $bin3^{RNAi}$ Yki^{OE} wing imaginal discs. ****p=<0.0001

d) Cdk9^{OE} Yki^{OE} and *Hexim1*^{RNAi} Yki^{OE} wing disc overgrowth resemble *bin3*^{RNAi} Yki^{OE} tumors

According to the proposed hypothesis, down regulation of bin3 results in down regulation of *hexim* and thereby activation of Cdk9, which may cause increased cell proliferation. We tested this, by RNAi-mediated down regulation of *hexim* or by over-expressing Cdk9 in the background of Yki over-expression.

While down regulation of *hexim* or activation of Cdk9 alone did not cause tumor formation, in the background of Yki over-expression, they caused substantial increase in tumor size, comparable to $bin3^{RNAi}$ background (Fig. 11). This validated the hypothesis on which this work was initiated.

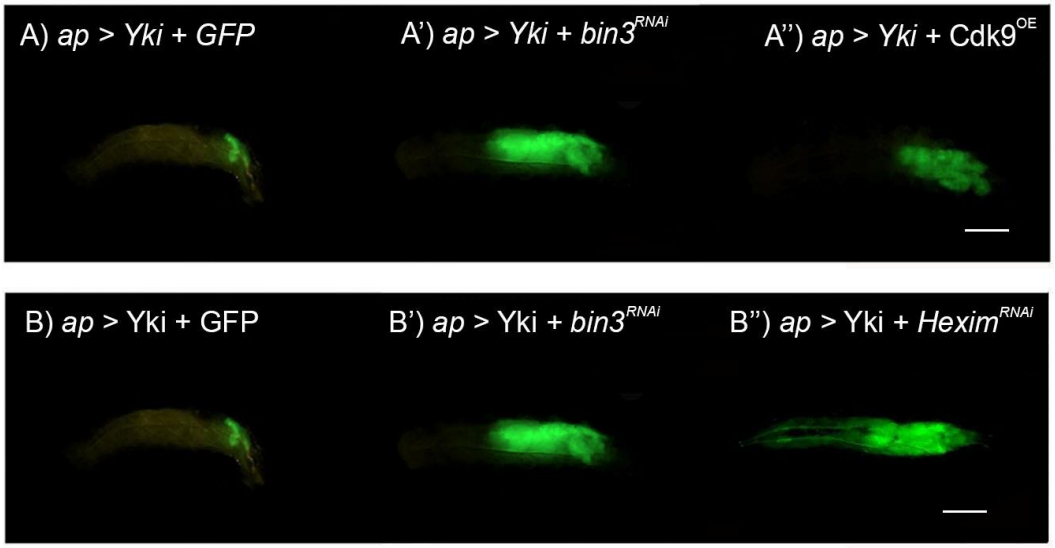
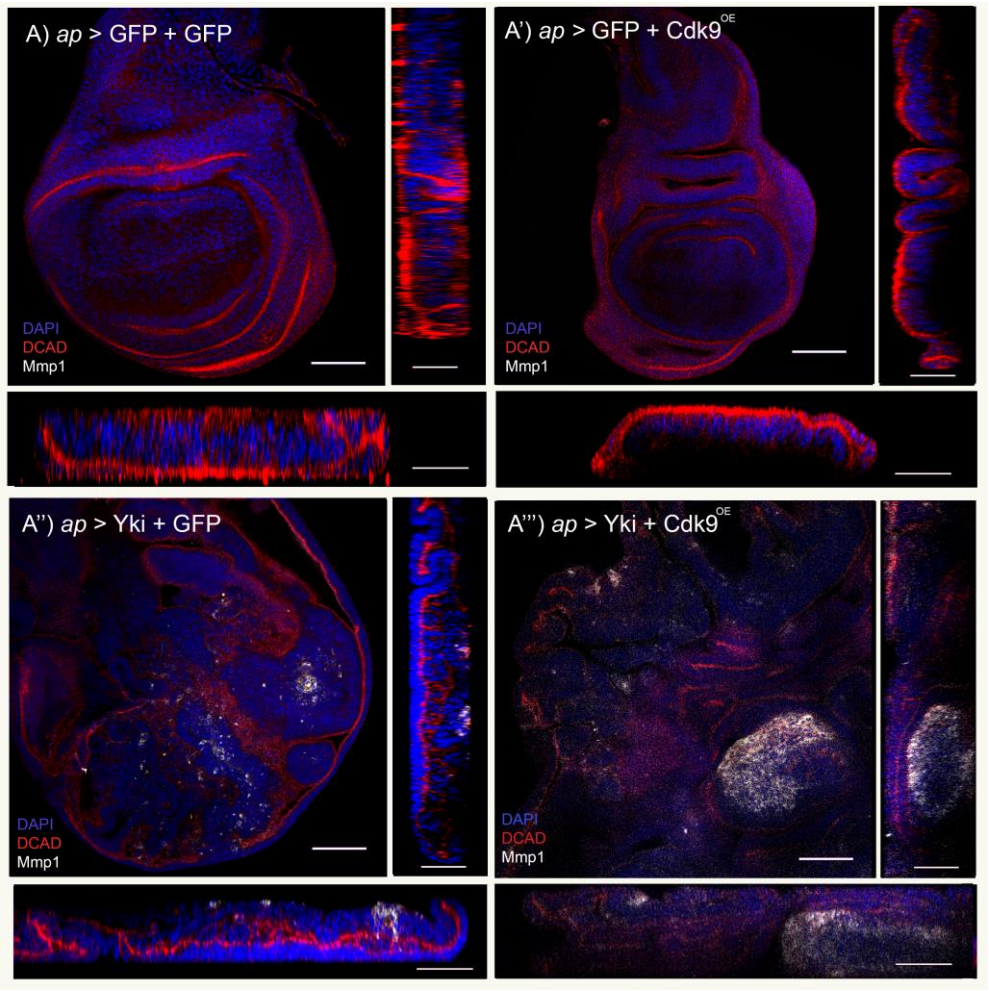


Figure 11: GFP positve 3rd instar larvae indicating tissue overgrowth. Genotypes of the larvae are shown on the images.

Yki^{OE} alone causes marginal increase in the size of wing discs (A, B), which is substantially increased when *bin3* is down regulated (A', B'). Cdk9^{OE} (A") and *hexim*^{RNAi} (B") also caused substantial increase in wing disc size comparable to that of *bin3*^{RNAi}.

We then investigated if the Cdk9^{OE} Yki^{OE} wing discs also lose polarity and undergo EMT, similar to that of *bin3*^{RNAi} Yki^{OE} tumors.

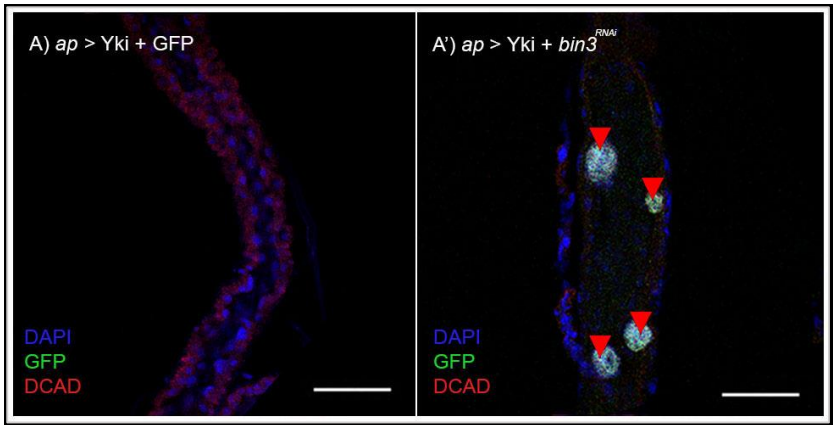
Indeed, we observed loss of DE-cadherin and also its mis-localization, suggesting partial loss of polarity in Cdk9^{OE} Yki^{OE} wing discs (Fig. 12A"'). We also observed increased Mmp1 levels in Cdk9^{OE} Yki^{OE} wing discs (Fig. 12A"'), suggesting that these cells too are undergoing EMT.



Fi gure 12: Loss of Cell Polarity and EMT in tumors caused by the combined overexpression of Cdk9 and Yki. Confocal micrographs of third instar wing imaginal discs stained for DNA (using DAPI; blue), D E-cadherin (using antibodies; red) and Mmp1 (using antibodies; grey). Optical cross-sections of the respective phenotypes indicate the localisation of DE-CAD. Note, reduced and mis-localized DE-CAD and also, elevated levels of Mmp1 in Cdk9^{OE} Yki^{OE} wing discs.

e) Confirmation of metastatic behaviour of *bin3*^{RNAi} Yki^{OE} tumors

Since, *bin3*^{RNAi} Yki^{OE} wing imaginal discs indicated elevated elevls of Mmp1, we investigated if they exhibit metastiatic behaviour. Closer observation of *bin3*^{RNAi} Yki^{OE} larvae revealed GFP positive cells in the larval gut, while Yki^{OE} larvae are devoid of any GFP positive cells in any place other than the wing disc itself. As ap-GAL4 driver is not expressed in the larval gut, it is likley that these cells are migratory (deatched from wing discs), thus suggesting their metastiatic ability.



B)

Area (in %) / Genotype	ap > Yki	ap > Yki + *bin3*^{RNAi}
Gut Cells	43	14
GFP Positive Cells	0	39

Figure 13: Presence of GFP positive cells in the midgut provides evidence of metastasis. Confocal micrographs ((A)-(A')) of larval gut stained for DNA (using DAPI; blue) and DE-cadherin (using antibodies; red). Note GFP positive cells (indicated by red arrowheads) in larval gut, suggesting metastases.
B) Area covered by wild type cells with respect to GFP positive cells within the gut.

f) Invasive capability of *bin3*^{RNAi} Yki^{OE} tumors

We then examined if *bin3*^{RNAi} Yki^{OE} tumors are invasive i.e. if they have ability to invade wildtype tissues by performing transplantation experiments. Fragments of wing imaginal discs were transplanted into adult flies of the strain w^{1118} as described in materials and methods. We transplanted tumorous wing discs from Yki^{OE} and *bin3*^{RNAi} Yki^{OE} larvae. We also used Yki^{OE} *SOCS36E*^{RNAi} as positive control. We observed GFP-postive tissues at locations far from the sites of original transplantation in *bin3*^{RNAi} Yki^{OE} tumors, to the same extent as Yki^{OE} *SOCS36E*^{RNAi} tumors. This suggests that *bin3*^{RNAi} Yki^{OE} tumors are neoplastic and matastatic with invasive capabilities.

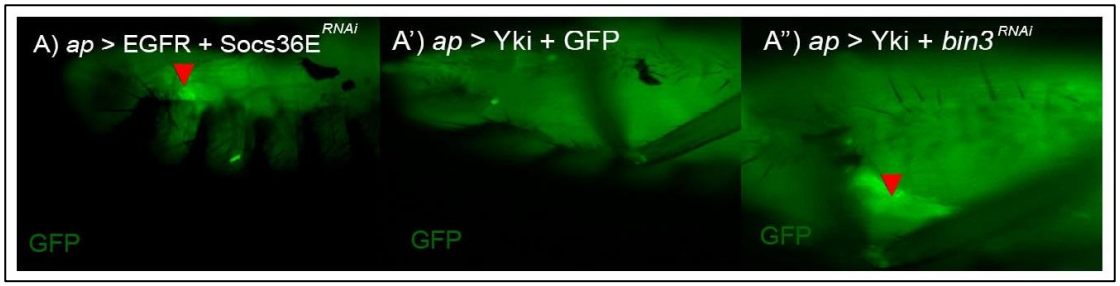


Figure 14: Growing GFP positive tissue fragments at locations far way from the site of injection indicate the invasive capability of tumors. Representative images of the abdominal regions of the w^{1118} virgin flies into which tumor fragments of variosu genotypes were transplanted. Transplants of *Socs36E*^{RNAi} Yki^{OE} disc fragments (A) were used as a positive control, as it has been shown previously that these transplants give rise to tumors (Herranz et al. 2012). Transplants of disc fragments of Yki^{OE} tumors did not give rise to tumors in the adult flies (A'). However, transplants of *bin3*^{RNAi} Yki^{OE} tumor fragments gave rise to tumors that grow significantly larger than tumor fragments of Yki^{OE}. Note the GFP-positive disc fragments that form tumors (indicated by red arrowheads).

Transplantations were also done for 5-day old and 8-day old tumors (data not shown). We did not observe any growth of the transplanted tissue. This indicates that the *bin3*^{RNAi} Yki^{OE} tumors are only hyperplastic until 9 days of tissue growth, and they attain invasive capability on or after 9th day of tumor growth.

g) Neoplastic nature of *bin3*^{RNAi} Yki^{OE} tumors

As described above, *bin3*^{RNAi} Yki^{OE} tumors are metastatic and have the ability to give rise to tumors in organs other than their organ of origin. Loss of cell-cell adhesions is one of the characteristics that enables this invasive capibility. We directly measured loss of cell adhesion by measuring how fast cells of these tumors can disassociate, compared to wildtype, when treated with a proteolytic enzyme, trypsin.

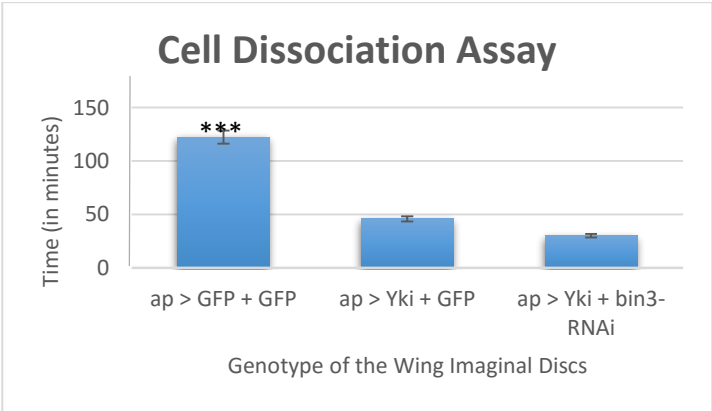
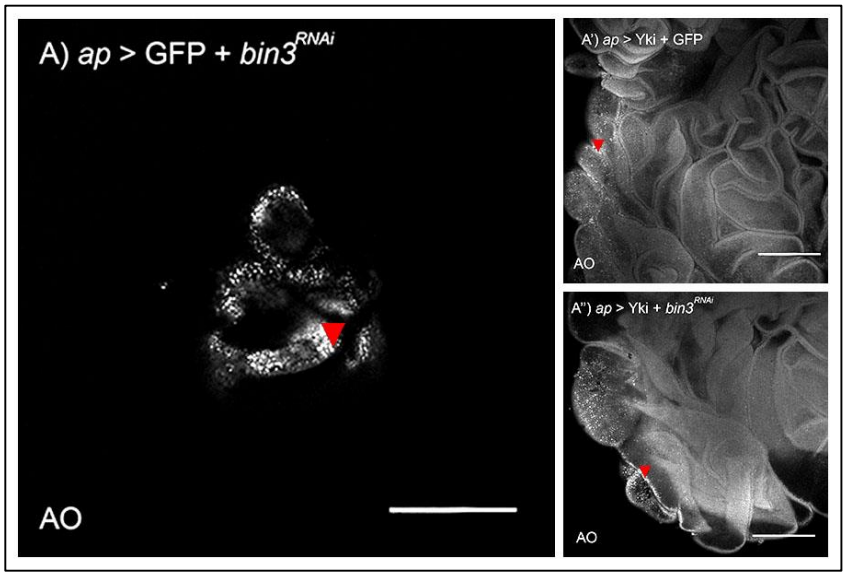


Figure 15: Rapid dissociation of cells indicates loss of cell adhesion in *bin3*^{RNAi} Yki^{OE} tumors.
Note the cells of *bin3*^{RNAi} *Yki*^{OE} tumors dissociate rapidly as compared to wild type discs and *Yki*^{OE} tumors. ***p=<0.001.

h) Attenuated apoptosis in *bin3*^{RNAi} Yki^{OE} tumors

We used acridine orange to perform a crude assay to investigate the capability of cells to undergo apoptosis. Apoptic cells have a very low pH value and acridine orange after interacting with apoptotic cells turns orange (wavelength; 630-590nm)



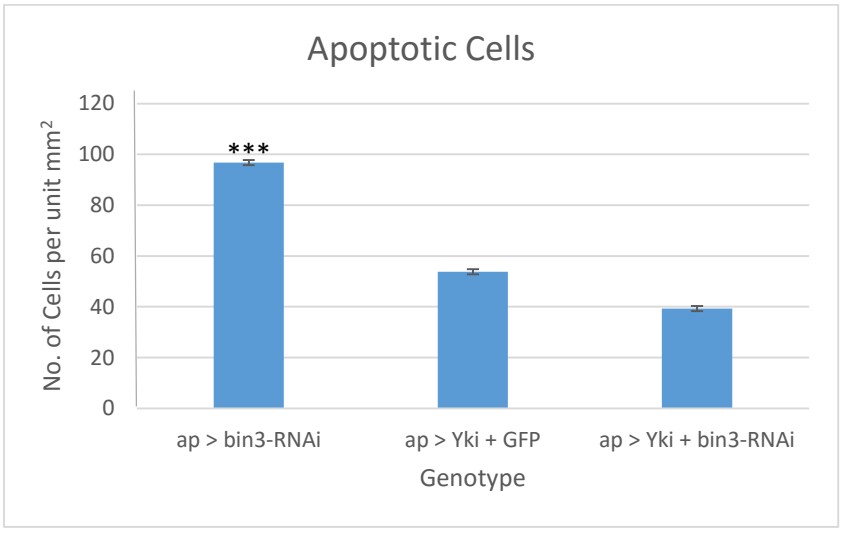


Figure 16: Attenuation of apoptosis in *bin3*^{RNAi} Yki^{OE} tumors.

A) – A'') Confocal micrographs of wing discs stained to visualise apoptotic cells (using Acridine orange; gray). The extent of apoptosis is higher in the *bin3*^{RNAi} wing imaginal discs (A) compared to, Yki^{OE} (A'). *bin3*^{RNAi} Yki^{OE} tumors too show low levels of apoptosis (A''). This suggests that although bin3 is required for cell survival, a combination of its loss of function and Yki over-expression leads to both cell proliferation and decreased apoptosis.

B) Bar graph indicating the number of apoptotic cells per unit area (mm^2), ***p=<0.001.

i) Increased mitosis *bin3*^{RNAi} Yki^{OE} tumors

Mitotically active cells can be visualized using antibodies against pH3 (phosphorylated Histone 3). Using this as a marker, we observed significant increase in mitosis in *bin3*^{RNAi} Yki^{OE} tumors (Fig. 17A''), when compared to Yki^{OE} wing discs (Fig. 17A').

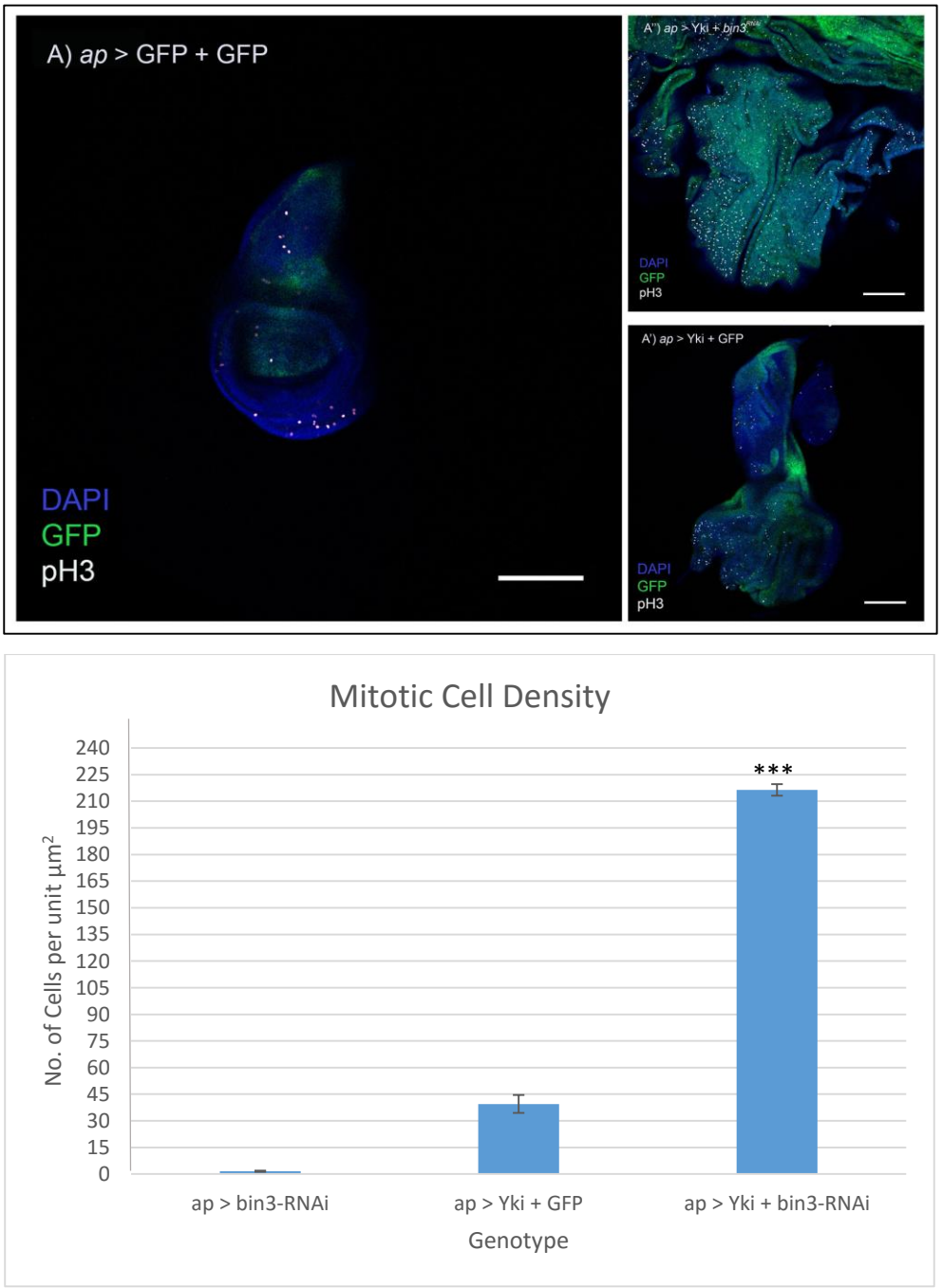


Figure 17: Increased mitosis in *bin*^{RNAi} Yki^{OE} tumors.
A) – A'') Confocal micrographs of wing imaginal discs stained for DNA (using DAPI; blue) and pH3 (using antibodies; gray). Dorsal compartment is indicated by GFP positive region. Note increased mitosis in *bin3*^{RNAi} Yki^{OE} tumors.
B) Bar Graph indicating the mitotic cells per unit area (µm^2), ***p=<0.001.

j) Genetic Rescue Experiments validate the involvement of Cdk9 in *bin3*^{RNAi} Yki^{OE} tumors

As per the hypothesis of this work, down regulation of *cdk9* in *bin3*^{RNAi} Yki^{OE} tumors should suppress the development of neoplastic tumors. For the rescue experiment, the male flies of the genotype *ap* > UAS-GFP, *cdk9*^{RNAi}; UAS-Yki, *tubG80*^{ts} were crossed with the virgin females of the *bin3*^{RNAi} stock. While scoring, non-tubby larvae or non-tubby flies which are GFP positive were considered. We observed strong rescue of tumor phenotype of *bin3*^{RNAi} Yki^{OE}, when *cdk9*^{RNAi} was introduced in this background (Fig. 18A").

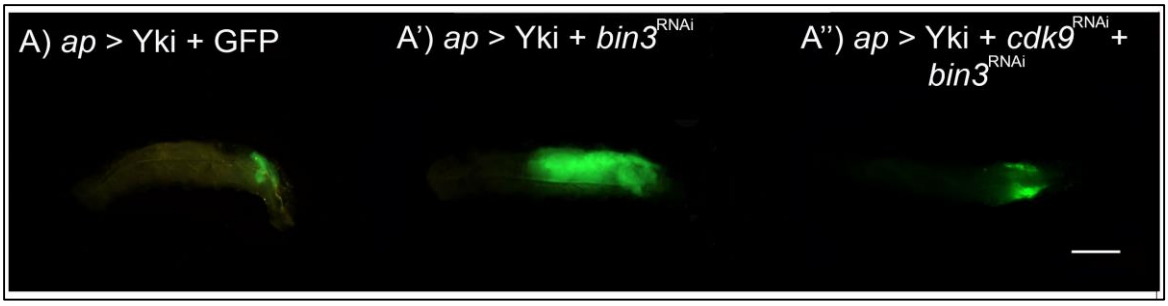


Figure 18: RNAi-mediated downregulation of *cdk9* suppresses *bin3*^{RNAi} Yki^{OE} tumor grwoth. Size of tumor in *cdk9*^{RNAi} *bin3*^{RNAi} Yki^{OE} larvae is smaller compared to *bin3*^{RNAi} Yki^{OE}. It is comparable to that of Yki^{OE} alone. Nevertheless, these larvae do not grow into adult flies.

k) Increased nuclear localisation of pMad in *bin3*^{RNAi} wing discs and in *bin3*^{RNAi} Yki^{OE} tumors

A possible mechanistic explanation for large metastatic *bin3*^{RNAi} Yki^{OE} tumors is increased nuclear localization of pMad. pMAD is normally localised to the cytoplasm and only when cell division is induced, it transiently localized to the nucleus. We examined the sub-cellular localization of pMAD in different genotypes.

pMad is mostly localised to the cytoplasm in wild type wing discs (Fig. 19A, B). However, pMad is mostly nuclearly localised in *bin3*^{RNAi} wing imaginal discs (Fig. 19A', B'). Interestingly, pMad is localised to the cytoplasm in Yki^{OE} wing discs as well (Fig. 19A'', B''). In *bin3*^{RNAi} Yki^{OE} tumors, pMad is mostly localised to the nucleus, although its overall levels are significantly reduced (Fig. 19B''').

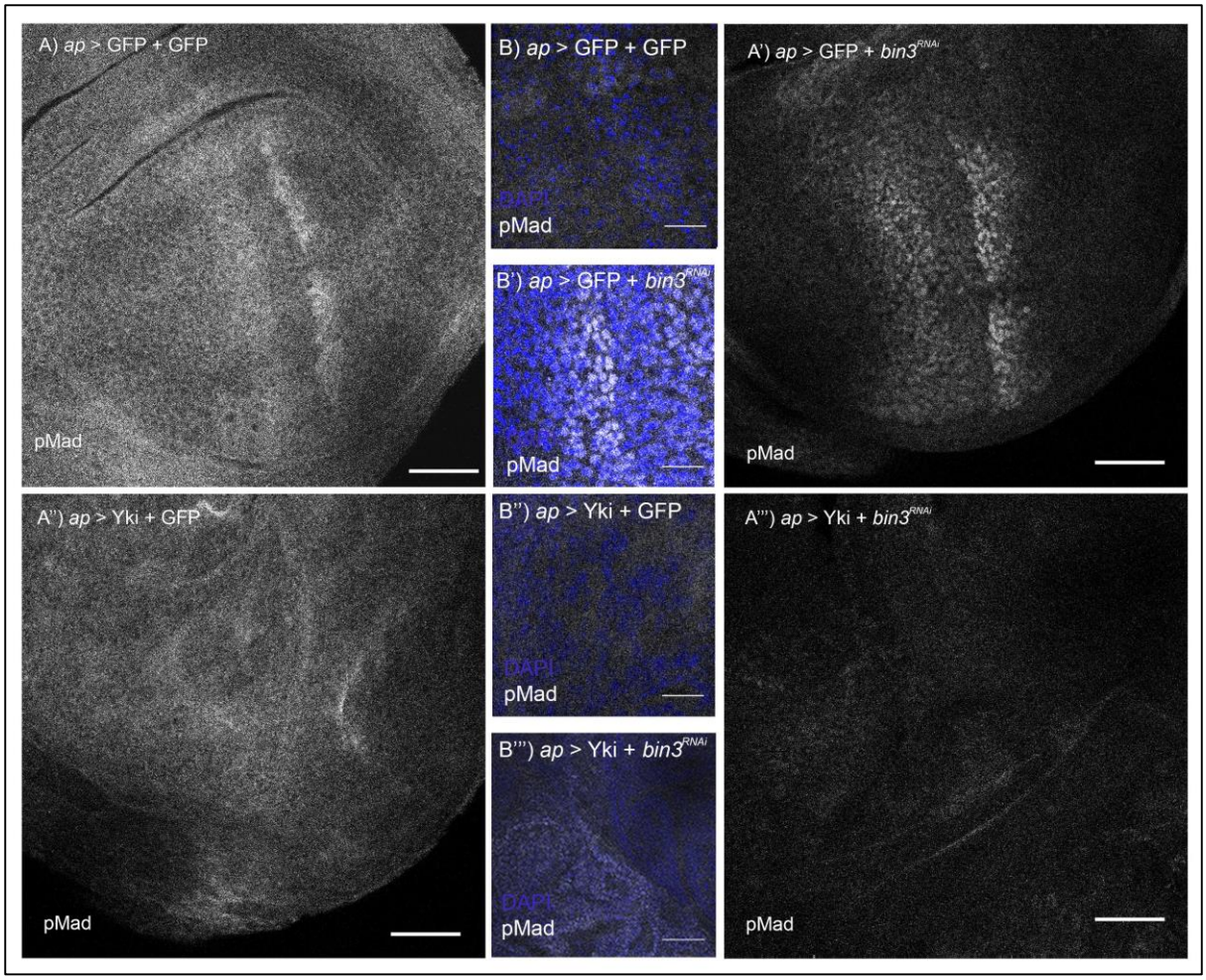


Figure 19: Increased nuclear localisation of pMad is observed when *bin3* is knocked down.
A)-A''') Confocal micrographs stained for DNA (using DAPI; blue) and pMad (using antibodies; gray). Note, increased nuclear localization of pMAD in *bin3*^{RNAi} and in *bin3*^{RNAi} Yki^{OE} discs. B) – B''') Zoomed in on dorsal compartment of wing imaginal discs to indicate the nuclear localisation of pMad.

I) Increased nuclear localisation of Yki in *bin3*^{RNAi} wing discs

Similar to pMAD, Yki too is normally localized in the cytoplasm and cell-proliferation signals recruit Yki to the nucleus. In our observations too, Yki is localised to the cytoplasm in wildtype wing imaginal discs (Fig. 20A, B). We observed increased nuclear localization of Yki in *bin3*^{RNAi} wing imaginal discs (Fig. 20A', B'). It is, therefore, likely that there would be increased nuclear localization of over-expressed Yki in *bin3*^{RNAi} background, compared to wildtype background.

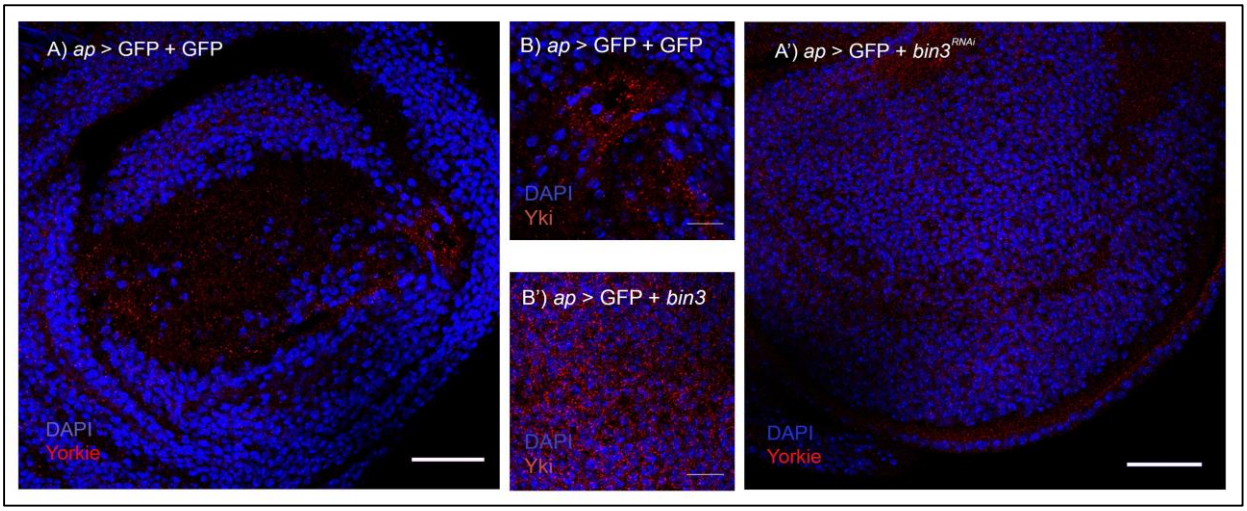


Figure 20: Increased nuclear localisation of Yki when *bin3* is knocked down.

A)-A''') Confocal micrographs of wing imaginal discs stained for DNA (using DAPI; blue) and Yorkie (using antibody; red). Note the increase in nuclear localisation of Yki in $bin3^{RNAi}$ wing discs. B) – B''') Zoomed in on dorsal compartment of wing imaginal discs to indicate the nuclear localisation of Yki.

In summary, results presented in this thesis report that down regulation of bin3 causes increased nuclear localization of both pMAD and Yki. The latter two, along with Cdk9, induce cell proliferation.

Discussion

Drosophila melanogaster has been used extensively to study and understand regulation of organ size, identify genes involved in growth control and their function. Considering the fact that many pathways controlling growth discovered in flies are highly conserved from flies to human, some of the growth regulators are likely to be classical tumor suppressors in the context of human cancer. Several studies have attempted to mimic complex environments of cancer, wherein they have reported and characterised genes implicated in cooperative tumorigenesis. But, what lacks is a study which aims at developing a comprehensive network involving all potential tumor suppressors in a given genetic background (such as over-expression of Yki). The objective of this study is derived from an ongoing genomic-scale screen, which aims at identifying all tumor suppressor genes that antagonise the functions of the oncoprotein Yorkie. In this screen, Bin3 was identified as potential negative regulator of Yki function in *Drosophila*. In the context of cancer, it is predicted to function as a tumor suppressor.

We have observed that the tumors caused by the knock down of *bin3* in the background of Yorkie overexpression are neoplastic in nature. These tumors lose polarity and rapidly undergo EMT, when compared to Yki overexpression alone or *bin3* knock-down alone. Furthermore, for these tumors, EMT manifests only post 9 days of induction suggesting a time line of events taking place during tumor progression, wherein the tumors are hyperplastic in nature until 9 days of tumor growth and attain metastatic properties on or after 9^{th} day of tumor growth.

These tumors are also highly mitotic in nature and evade apoptosis fulfilling two more hallmarks of cancer, which are ‘Enabling Replicative Immortality’ and ‘Resisting Cell Death’. Thus, the above results indicate that *bin3* knock down in the background Yki overexpression leads to cancer, suggesting that *bin3* is a potential tumor suppressor that antagonises the function of Yki as an oncoprotein.

Antibody staining for Yorkie and pMAD reveal a likelihood of new role for Bin3 in Yorkie and pMAD regulation. We observed increased nuclear localization of Yki and pMAD in wing discs wherein *bin3* is down regulated. However, further validation is required in order to investigate the role of Bin3 as a regulator of sub-cellular localization of Yorkie and pMAD. At the mechanistic level, Bin3 may antagonize Yki function, directly or indirectly, by retaining Yki, pMAD and Cdk9 in the cytoplasm.

When *bin3* is down regulated, these three may form a complex in the nucleus driving proliferation. However, *bin3* knock-down alone had no tumor phenotype, suggesting that amount of Yki is critical in this process. When Yki is over-expressed and at the same time *bin3* is down regulated, the levels of nuclear Yki (perhaps independently), nuclear pMAD and Cdk9 may cause the formation of neoplastic tumors.

Epistasis genetic experiments suggest that indeed increased levels of Cdk9 is the cause of formation of neoplastic tumors when *bin3* is down regulated in the background of Yki over-expression. It is important to note that in the rescue experiment, the larvae of right genotype had four UAS transgenes. It is possible that the amount of GAL4 activating the transgenes is diluted due to its distribution across four UAS insertions. This, might give rise to a false positive, wherein the rescue might be a result of the decreased activity of each gene. Thus, another control such as UAS-lacZ may have been used to investigate this effect.

In addition, several experiments are pending to understand precise role of *bin3* in Cdk9 localization. We also need to determine if over-expression of Cdk9 in $bin3^{RNAi}$ background mimics Yki^{OE} phenotype (Table 4).

Table 4: Table summarizing the results and experiments of future interest
The combination highlighted in bold, green and blue indicate the role of *bin3* as a tumor suppressor, the role of Cdk9 in promoting tumorigenesis when *bin3* is knocked down in Yki background and the unresolved role of Cdk9 in promoting growth when only *bin3* is knocked down, respectively. The combinations highlighted in Yellow indicate the effect of downregulation of Cdk9 on overexpression of Yorkie and downregulation of *bin3,* respectively.

Expression of Yorkie	Expression of bin3	Expression of Cdk9	Resultant Phenotype
Overexpressed	**Downregulated**	**NA**	**Tissue Overgrowth**
Overexpressed	**NA**	**Overexpressed**	**Tissue Overgrowth**
Overexpressed	**NA**	**Downregulated**	**Defects in adult wings**
NA	**Downregulated**	**Downregulated**	**GFP positive adult flies**
Overexpressed	**Downregulated**	**Downregulated**	**GFP positive pupae (adult GFP positive flies absent)**
NA	**Downregulated**	**Overexpressed**	**To be Done**

The definitive goal of using *Drosophila* as an epithelial transformation model is to identify various markers and causal agents that help understand the cancer milieu in humans. Hence, the validation of the proposed pathway in mammalian system using mammalian cell lines and mouse xenograft models is of future interest.

To conclude, our study reported here reveals a possible role for *bin3* as a tumor suppressor and also a pathway through which it antagonizes function of Yorkie as an oncoprotein.

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