Validating role of *Bin3* and its mammalian ortholog *MEPCE* as a tumor suppressor that antagonizes the function of oncoprotein YAP/Yki.

A thesis submitted towards partial fulfillment of

BS-MS Dual Degree program

by

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Index

Contents **Page No.**

Certificate

This is to certify that this dissertation entitled “Validating role of Bin3 and its mammalian ortholog MEPCE as tumor suppressor that antagonizes the function of oncoprotein YAP/Yki.” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Ruchi Wasnik, IISER Pune under the supervision of Prof. L. S. Shashidhara, Professor and Chair, Biology, IISER Pune during the academic year 2016-2017.

Prof. L.S. Shahsidhara Date: 14.3.17

Declaration

I hereby declare that the matter embodied in the report entitled “Validating role of *Bin3* and its mammalian ortholog *MEPCE* as tumor suppressor that antagonizes the function of oncoprotein YAP/Yki.” are the results of the investigations carried out by me at the Department of Biology, Indian Institute of Science Education and Research (IISER), Pune, under the supervision of Prof. L. S. Shashidhara, Biology, IISER, Pune and the same has not been submitted elsewhere for any other degree.

Ruchi Wasnik Date: 20.3.2017

Abstract

Tumor growth and metastasis could be an outcome of activation of oncogenes and inhibition of tumor suppressors. We identified *Bin3* and *Hexim* as potential tumor suppressors under *Yki* overexpression background from a genome-wide RNAi screening using *Drosophila*. Hippo pathway and its downstream effector Yki has implicated in organ size control and tumorigenesis. Previous work done in the lab suggested that large tumor growth is a result of increased activity of *Cdk9* when *Bin3* is downregulated in the *Yki* overexpression background. This also results in nuclear localization of pMad-Yki complex which might cause upregulation of Yki target genes resulting in proliferation. We investigated neoplastic nature of $Hexim^{RNAi}$ +Yki^{OEx} tumors in this study. We performed genetic rescue experiment to confirm the involvement of *Cdk9* where *Cdk9* knockdown rescues the tumorous phenotype. We also suggest a non specific function of *Cdk9* as a regulator of tumorigenesis. However, neither *Cdk9* overexpression and/or *Bin3*/*Hexim* knockdown lead to tumor formation suggesting that *Yki* overexpression acts as a driver for the tumorigenesis. Further, we extended this study using Human orthologs of potential tumor suppressor genes to understand their prognostic value in different cancers. *In silico* analyses performed on the patient's RNA-Seq data revealed potential tumor suppressor activity of *MEPCE* and *HEXIM1* in an aggressive form of lung cancer and bladder cancer respectively.

List of Figures

Figure **Page No.**

List of Tables

Table **Page No.**

Abbreviations

JNK: c-Jun N-terminal kinases,

EGFR: Epidermal Growth Factor Receptor,

SOCS: Suppressor of Cytokine Signaling,

GFP: Green Fluorescent Protein,

DPP: Decapentaplegic,

SMAD/ Mad: Mothers Against DPP

RUNX: Runt Related Transcription Factor,

TEAD: TEA Domain Transcription Factor,

LATS: Large Tumor Suppressor Kinase,

PABP: Poly (A) Binding Protein,

Ago2: Argonaute RISC Catalytic Component 2,

LARP: La-Related Protein,

Cdk9: Cyclin Dependent Kinase 9,

RNAPII: RNA polymerase II,

7SK-snRNP: 7SK-Small nuclear Ribonucleoprotein,

BRCA: Breast Cancer Type 1 Susceptibility Protein,

MMP: Matrix Metallopeptidase ,

DE-Cad: *Drosophila* E-cadherin,

PTEN: Phosphatase And Tensin Homolog,

CTGF: Connective Tissue Growth Factor,

AREG: Amphiregulin,

Her2: Receptor tyrosine-protein kinase erbB2

Acknowledgements

I would like to take this opportunity to thank my mentor Prof. L. S. Shashidhara for his constant valuable and encouraging guidance. I would also like to thank Shital and J.P Bhaiya, Madhumita, Sanket and other members of LSS lab for their valuable inputs during the course of the thesis project. I would like to thank Dr. Girish Ratnaparkhi, my TAC member for his constant support, encouragement and guidance. I would also like to thank the past and current members of the screening team who have managed to maintain an encouraging and fun atmosphere in the lab and helped me with stocks now and then.

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I would like to specially thank my brother and Akash for keeping me sane during my difficult times and also my friends Khushboo, Naveen, Agrim, Lakshmi, Rajarajeswari and Smita for making my life at IISER Pune enjoyable and exciting.

And lastly, I would like to dedicate this work to my mother…

Introduction

Crosstalk between various processes is required to maintain cellular homeostasis and further, regulation in organ size and development. Cancer cells possess characteristic chronic proliferation which could be the result of disruption of homeostasis. Formation of tumors from normal cells is a multistep phenomenon during which characteristic 'hallmark' traits are acquired. In 2011, Hanahan and Weinberg described eight 'hallmarks' to describe the complexity of cancer (Figure 1a). These include- previously known hallmarks such as replicative immortality, resisting cell death, sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, inducing angiogenesis, etc., and newly recognized hallmarks such as deregulating cellular energetics and avoiding immune destruction (Hanahan and Weinberg, 2011). Also, the interplay between these hallmarks leads to tumor formation and its sustainability. Hence the understanding of these hallmarks and complex interaction between them will have an effect on cancer treatment.

Accumulation of mutations in the genome is one of the factors responsible for tumor progression, but their functional relevance is unknown in many of the cases. Recently studies have shown that genetic and epigenetic mutations might cooperate with known oncogenic activity resulting in tumor and disease progression (Stratton, 2011). Such phenomenon is known as cooperative tumorigenesis/ oncogenesis. Other studies have investigated the importance of cooperative tumorigenesis where both activation of oncogenic pathway and loss of function in the tumor suppressor pathway required for tumor progression. A study by Doggett *et al*. 2011 in *Drosophila* model implicated that the activity of Ras in scribbled mutant background produced neoplastic growth. Also, oncoprotein Ras and loss of tumor suppressor scribbled act together to produce growth and invasion by the activity of JNK pathway (Wu et al., 2010).

Other pathways such as Hippo and EGFR have also been shown to affect the tumor growth by cooperative tumorigenesis. Study on *bantam* target *SOCS36E* showed that EGFR overexpression or *SOCS36E* knockdown do not lead to neoplastic overgrowth in wing imaginal discs. But, in the case of the $SOCS36E^{RNAi}$ transgene in EGFR overexpression background, the overgrowth showed neoplastic characters (Herranz et

al., 2012). Thus, *bantam* target *SOCS36E* limits the activity of EGFR pathway and thereby maintains homeostasis.

These studies investigated crosstalk between various processes by the use of diverse genetic techniques available in *Drosophila* model system. Use of TARGET system (temporal and regional gene expression targeting system) in *Drosophila* model of 'Epithelial transformation'(described in Materials and methods) has helped to find cooperation between various genes required for tumor progression (McGuire et al. 2003; Herranz et al. 2012).

We initiated a genome-wide RNAi screening in *Drosophila* using this model to identify novel tumor suppressor genes. Hyperplasia in wing imaginal discs was induced by overexpression of Yorkie (Yki^{OEx}); *Yki* is a downstream effector of Hippo pathway which acts as a transcriptional cofactor and regulates transcription of various genes important in growth and proliferation. And using RNAi lines (KK library); knockdown was made for one gene at a time. Crosses were set using Yki^{OEx} +*GFP* females and males from RNAi background. Later, the third instar giant larvae were screened for *GFP* positive wing imaginal discs (Figure 1b)

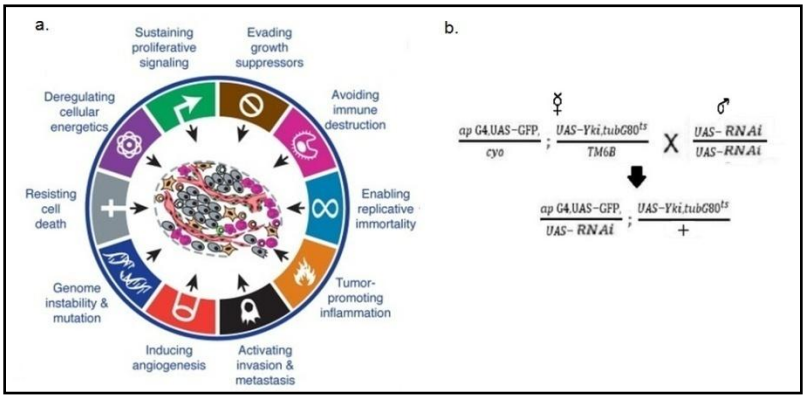


Figure 1; Hallmarks of Cancer and Crossing Scheme: a. Illustration for the eight hallmarks important for the characteristics of neoplastic tumors (Modified from: Hanahan & Weinberg 2011). **b.** The image depicts the genotype of the cross between virgin females with *GFP* and *Yki* overexpression and males with RNAi transgene. Above crossing scheme is followed to screen *GFP* positive giant larvae having *Yki* overexpression and RNAi knockdown.

Different studies have indicated the potential role of many of the positive hits obtained from the *Yki* screen in cancer and its progression. To mention, a few, well-known tumor suppressor- *p53* and *PTEN* were also found to be positive from the screen. Novel genes such as *Su(dx), Tsf1*, *Bin3*, and *LIPG* were identified as positives. Among these genes, the role of Bicoid Interacting Protein (Bin3) in transcriptional and translational regulation has studied in *Drosophila*. Also, previous work done in the lab suggests that loss of *Bin3* produces neoplastic growth in *Yki* overexpression background. Thus *Bin3* could act as a Potential Tumor Suppressor (PTS) in *Yki* mediated tumorigenesis. As *Bin3* and *Yki* affect the transcriptional activity in the cells, it was interesting to investigate if *Bin3* and *Yki* have some role to play in cooperative tumorgenesis.

Hippo signaling in cancers

Hippo signaling pathway was first discovered in *Drosophila* for its role in growth regulation and further studied in mammals and human cancers. The role of Hippo pathway is well conserved and is involved in the maintenance of organ size and cell fate. In *Drosophila*, Hippo signaling pathway contains over 35 different proteins. It has a core kinase cassette consisting of Hippo (HPO), Salvador (SAV), Mob as a tumor suppressor (MATS) and Warts (WTS). Regulation of tissue growth occurs by kinase activity of Wts via inhibition of downstream effector Yorkie(Yki) (Figure. 2a and 2b) (Harvey et al. 2013; Luo 2010). The activity of Yki changes depending on its phosphorylation status mediated by Wts. Wts inhibits the activity of Yki by phosphorylation at Ser127 residue which promotes association between Yki and 14-3-3. This complex causes cytoplasmic retention and further ubiquitin-mediated degradation of Yki. The similar regulatory process occurs for Yes-Associated Protein (YAP), the mammalian ortholog of Yki by LATS1 or LATS2 dependent phosphorylation at Ser168 residue (Oh and Irvine, 2009).

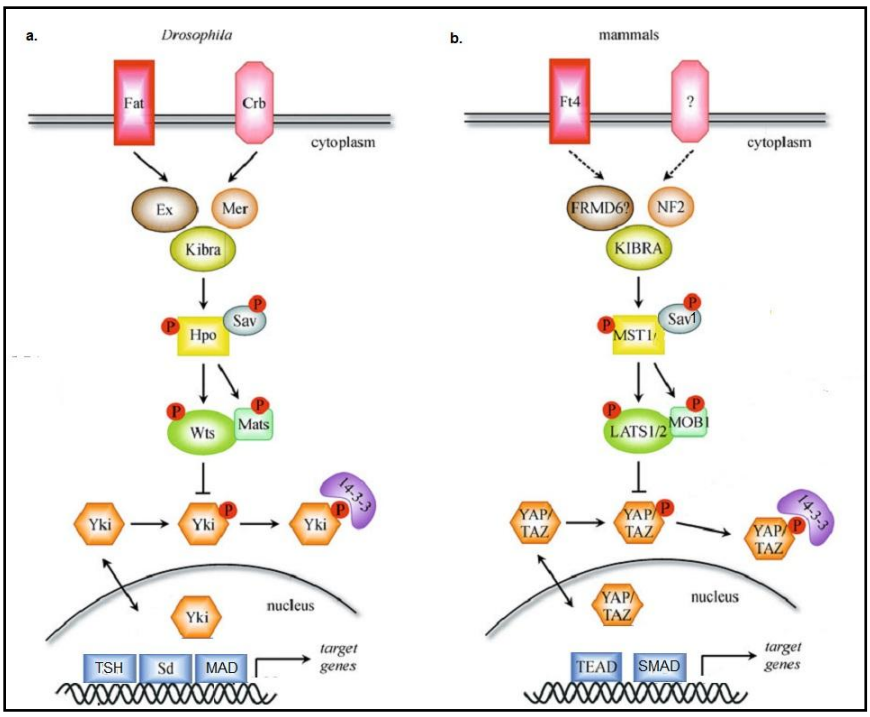


Figure 2; Hippo Signaling Pathway in *Drosophila* and mammals: Positive regulation is indicated by pointed arrows whereas blocked arrows indicate negative regulation. **a.** Extracellular signaling activates upstream membrane-associated proteins (Expanded: Ex, Merlin: Mer, Kibra). These proteins then activate core kinase cassette by phosphorylation of Hpo and Sav. Activation of Wts occurs by its phosphorylation which in turn phosphorylates Yki and facilitates its association with 14-3-3. Unphosphorylated form of Yki localizes to the nucleus and acts as a transcriptional cofactor for Scalloped: Sd, Mother for DPP: Mad, Teashirt: Tsh, etc. This association is important for regulation of target gene expression. **b.** Hippo signaling pathway is well conserved in mammals. The same color of the protein indicates homologous proteins. Upstream signaling has not yet understood properly and is indicated by dashed arrows (Modified from : Luo 2010).

Cell proliferation and apoptosis play an integral role in cancers, and Hippo pathway mediates regulation of these processes by the activity of Yki/YAP. When Hippo signaling is off, Yki/YAP associates itself with various transcription factors such as sd/TEADs, Mad/SMADs, RUNX2, etc. in the nucleus to promote tissue growth (Harvey et al. 2013; Luo 2010). Evidence suggest that mutations in different components of

Hippo pathway (e.g. Salvadore, Warts) lead to overgrowth of tissue (Justice et al., 1995). Similarly, *Yki*/*YAP* overexpression also results in tissue overgrowth (Zhao et al., 2007). Thus active Hippo signaling suppresses tissue growth and thereby plays an important role in tumorigenesis.

Role of *Bin3* and *Hexim* in development

Bicoid-interacting protein3 (*Bin3*) first identified in customized two-hybrid screen in *yeast* (Zhu & Hanes 2000). Studies, in the beginning, predicted based on protein sequence that the S-adenosyl methionine (AdoMet) motif of Bin3 acts as methyl-group donor similar to those of protein methyltransferases. Later, BCDIN3, the human ortholog of Bin3 in human cells had shown to act on small nuclear RNAs (e.g. 7SK, U6) (Jeronimo et al. 2007). It acts on 5' γ-phosphate of 7SK-snRNA and caps it with the methyl group. Thus human ortholog of *Bin3* is also called Methylphosphate Capping Enzyme (*MEPCE*).

As the name of the protein suggests, it was believed to interact with bicoid. *In vivo* studies revealed the essential role of *Bin3* in the early development of *Drosophila* and zebrafish. In the anterior compartment of *Drosophila* embryo, bicoid interacts with 3'-UTR of caudal mRNA and represses its translation with the help of complex formation between Hexim, Larp1, PABP and Ago2 proteins and 7SK-snRNA. This complex formation occurs due to methylation of γ-phosphate of 7SK by Bin3 (Singh et al., 2011). Also, MEPCE in mammals not only enhance the stability of 7SK-snRNA by protecting it from exonucleolytic degradation but also facilitates the formation of repressor complex (7SK-snRNP) for Positive Transcription Elongation Factor-b (P-TEFb). P-TEFb contains two proteins, Cdk9 kinase, and CyclinT. Studies suggest that active Cdk9 is required for RNAPII dependent transcription elongation (Xue et al., 2010). Thus 7SK-snRNP plays an important role in transcription regulation (Figure 3).

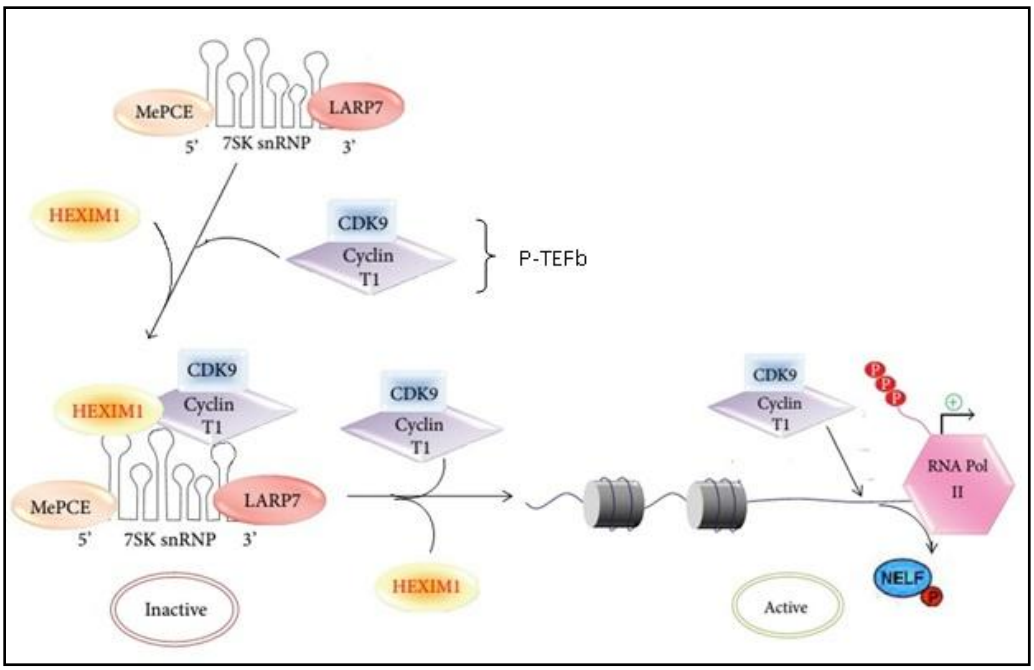


Figure 3; Transcription regulation by 7SK-snRNP: Binding of HEXIM1 to 7SK snRNP facilitates the interaction between P-TEFb and HEXIM1 and forms a repressor complex. Thus P-TEFb is no longer available for removing Negative elongation factor (NELF) from transcription paused site. Transcriptional elongation by RNAPII is activated once P-TEFb is freely available (Modified from: Chen et al. 2014).

Hexamethylene Bisacetamide Inducible (Hexim) belongs to one of the components of the 7SK-snRNP complex. Association between Hexim and 7SK-snRNA brings about conformational changes in Hexim dimer which sequester P-TEFb complex (Dey et al., 2007). Hence binding of Hexim to 7SK-snRNA is required for sequestering P-TEFb and thereby represses transcription elongation by RNAPII. Studies suggest that knockdown of *Hexim* in *Drosophila* leads to embryonic lethality and loss of expression in specific tissues causes severe developmental defects (Nguyen et al., 2012). Also, a recent study has proposed that *Hexim* regulates Hedgehog signaling by the activity of P-TEFb to give appropriate wing patterning (Nguyen et al., 2016). Apart from its role in development HEXIM1 in mammals lead to inhibition of Breast cancer tissues by modulating the transcriptional activity of ERα. It was also shown to inhibit *HIF1α* expression, important for angiogenesis in cancers by P-TEFb independent manner

(Chen et al., 2014). Thus *HEXIM1* has potential tumor suppressor role. But its role in the context of Hippo signaling has not been studied in detail.

This study focuses on the role of *Bin3*/*MEPCE*, and *Hexim*/*HEXIM1*or *HEXIM2* (functional homolog of *HEXIM1*) as potential tumor suppressors in *Yki*/*YAP*-mediated tumorigenesis. We divided this study into two parts based on two different approaches. The first approach involves the study of genetic interactions between candidate genes involved in the proposed pathway (described in Hypothesis) using *Drosophila* as a model and the second approach is that of *In Silico* analyses with the use of various databases (TCGA, METBRIC) to understand the clinical relevance of candidate genes.

Understanding Clinical relevance

Understanding of clinical heterogeneity present in cancer patients is very important to understand prognosis of cancer. Use of molecular profiling and clinical datasets allow identification of clinical heterogeneity and thereby play an important role in therapeutic drug development (Bedard et al., 2013). Thus, it is interesting to find if change in expression of genes has any role to play in the generation of such heterogeneity. So to understand if the candidate genes involved in the pathway have any prognostic value in cancer patients, we used RNA expression data and clinical data of cancer patients to perform various *In Silico* Analyses.

The second part of this study deals with RNA-Seq data obtained from The Cancer Genome Atlas (TCGA) Research Network: http://cancergenome.nih.gov/ in 2015 and METABRIC data. Since the hypothesis has drawn on the basis of Epithelial Transformation Model in *Drosophila*, we restricted our focus of analysis on carcinomas (i.e. cancers of epithelial origin). Here, we included Carcinoma of Breast, Bladder, Cervical, Kidney and lung. In this study, we started by analyzing expression patterns of candidate genes in normal tissue compared to cancer tissues.

American Joint Committee on Cancer (AJCC) assigns TNM (Primary tumor, T; regional lymph node, N; Distant metastasis, M) classification to tumors on the basis of various

pathological and clinical parameters (American Joint Committee on Cancer, 2009). This helps in understanding the progressive extent of the disease. Stages of cancer vary based on TNM classification and succession of stages serves as the best proxy for progression of cancer in patients. Thus, we performed Stage wise analysis to see the expression of candidate genes over the progression of cancer.

Poor prognosis of breast cancer patients likely associated with genetic markers such as *BRCA1, BRCA2, EGFR, MMP9* etc. Prediction models allowed us to understand that genes responsible for cell cycle, invasion and metastasis, angiogenesis are upregulated and associated with poor prognosis (van ’t Veer et al., 2002). But, to establish tumor suppressor activity of a particular gene, it is important to know the prognosis status due to its upregulation or downregulation. Here, we have studied the survival of patients using METABRIC data available for breast cancer for all the potential tumor suppressors and oncogene *YAP1*. Probabilistic estimate of survival using KM plots allowed us to understand if the low expression of PTSs correlates to poor prognosis or not.

Hypothesis

This study investigates the hypothesis (Figure 4) that the large tumor growth resulted from the increased activity of *Cdk9*; wherein *Bin3* is downregulated in the background of *Yki* overexpression. Knockdown of *Bin3* causes destabilization of 7SK-snRNA and thus Cdk9 is released which otherwise interact with Hexim on 7SK-snRNP and forms inactive complex (Xue et al., 2010; Peterlin et al., 2012). Availability of Cdk9 in the nucleus can phosphorylate linker domain of p-Mad protein in the presence of an agonist signal (Alarcón et al., 2010). This event allows the formation of a pMad-Yki complex which causes upregulation of target genes such as *bantam* mi-RNA responsible for proliferation (Parrish et al. 2010; Herranz et al. 2012).

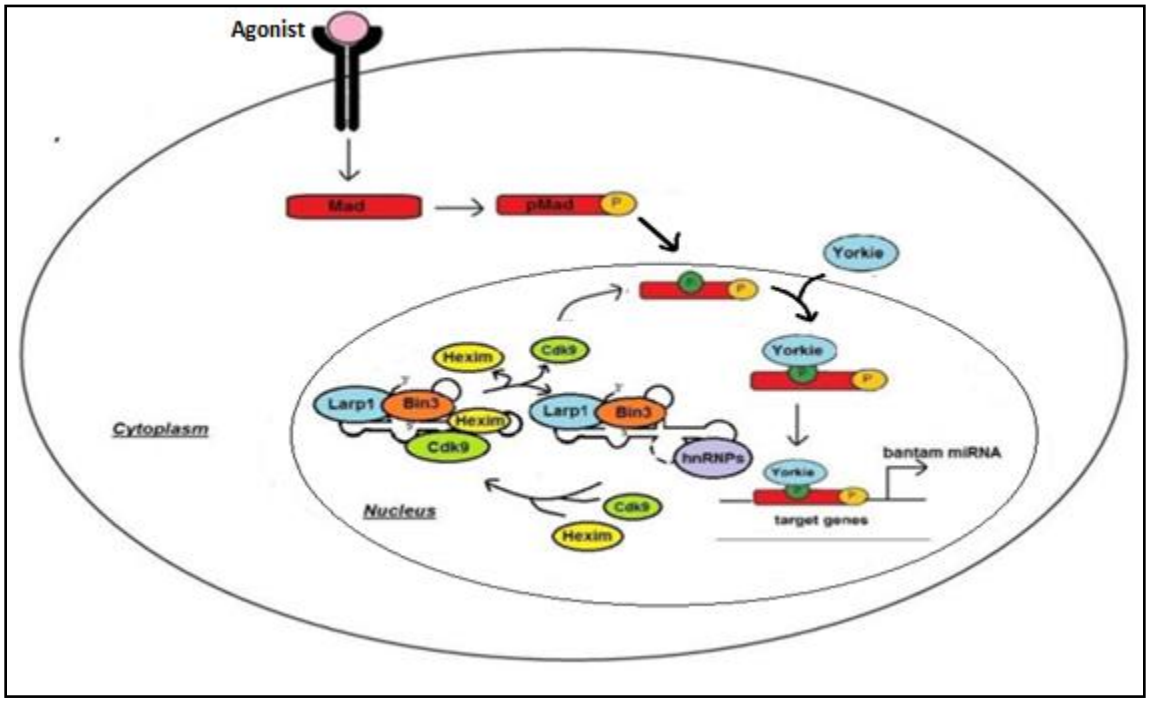


Figure 4; Proposed Hypothesis to investigate tumorigenesis: If *Bin3* is present it sequesters Cdk9 by forming a stable complex with Hexim and Larp1. In its absence, Cdk9 phosphorylates p-Mad protein. This event allows binding of Yki to the phospho p-Mad and affects target genes such as bantam miRNA, a well known pro-proliferative signal (Modified from: Thesis-Pravallika G.,2016). The similar pathway can be proposed for mammalian system using orthologs of above proteins since the genes are functionally conserved.

Aims and Objectives

Part A

Aim: To understand role of components of 7SKsnRNP complex in regulation of Yki mediated tumorigenesis in Drosophila.

Objectives:

1. To test if other proteins of 7SKsnRNP complex could act as potential tumor suppressor using VDRC and TRiP RNAi stocks.
2. Characterizing neoplastic nature of tumors by immunostaining against PH3, DE-cad and MMP1.
3. To test if downregulation of *Cdk9* rescues the neoplastic overgrowth.
4. To test if Bin3 and Cdk9 activity is Yki dependent.
5. To understand different genetic interactions important for *Yki* mediated tumorigenesis.

Part B

Aim: Understand the clinically important role of YAP and other potential tumor suppressor genes in human carcinomas using cancer datasets.

Objectives:

1. Check if stagewise progression of cancer has any effect on expression profiles of oncogene *YAP1* or PTSs *MEPCE*, *HEXIM*1/2.
2. Check if expression changes in *MEPCE*, *HEXIM*1/2 have an effect on *YAP1* targets in aggressive tumors.
3. Check the prognosis of cancer by survival analysis.

Materials and Methods

Drosophila Strains Used

The following stocks obtained from Prof. Stephen Cohen's lab:

1. $\frac{ap\ G4,\ UAS\text{-}GFP}{cyo}\ ;\ \frac{tub\ G80^{ts}}{TM6B}$
2. $\frac{ap\ G4,\ UAS\text{-}GFP}{cyo}\ ;\ \frac{UAS\text{-}Yki,\ tub\ G80^{ts}}{TM6B}$

All the RNAi stocks obtained from Vienna *Drosophila* RNAi Center (VDRC), and TRiP stocks obtained from Bloomington Stock Center. Other UAS lines such as UAS-*Bin3*; BL#20290, UAS-*Hexim*; BL#21200 and UAS-*Cdk9*; BL#12221 obtained from Bloomington Stock Center. Fly Facility at IISER, Pune provided double balancer stocks.

Genotypes for various abbreviations used for indicating parents and progeny:

Table 1: List of genotypes used in the study and their abbreviations

Sr.No	Abbreviation	Genotype
1.	*apG4*	*ap GAL4*
2.	*apG4> GFP*	$\frac{ap\ G4,\ UAS\text{-}GFP}{cyo}\ ;\ \frac{tub\ G80^{ts}}{TM6B}$
3.	*apG4> Yki*^{OEx}*+GFP*	$\frac{ap\ G4,\ UAS\text{-}GFP}{UAS\text{-}GFP}\ ;\ \frac{UAS\text{-}Yki,\ tub\ G80^{ts}}{+}$
4.	*apG4>* m*EGFR*^{OEx}*+GFP*	$\frac{ap\ G4,\ UAS\text{-}GFP}{UAS\text{-}GFP}\ ;\ \frac{UAS\text{-}mEGFR,\ tub\ G80^{ts}}{+}$
5.	*apG4> Bin3*^{RNAi}*+GFP*	$\frac{ap\ G4,\ UAS\text{-}GFP}{UAS\text{-}Bin3^{RNAi}}\ ;\ \frac{tub\ G80^{ts}}{+}$
6.	*apG4> Hexim*^{RNAi}*+GFP*	$\frac{ap\ G4,\ UAS\text{-}GFP}{UAS\text{-}Hexim^{RNAi}}\ ;\ \frac{tub\ G80^{ts}}{+}$

7.	*apG4> Bin3^{RNAi}+Yki^{OEx}+GFP*	*ap G4, UAS-GFP / UAS-Bin3^{RNAi} ; UAS-Yki, tub G80^{ts} / +*
8.	*apG4> Hexim^{RNAi}+Yki^{OEx}+GFP*	*ap G4, UAS-GFP / UAS-Hexim^{RNAi} ; UAS-Yki, tub G80^{ts} / +*
9.	*apG4> Cdk9^{RNAi}+Yki^{OEx}+GFP*	*ap G4, UAS-GFP / UAS-Cdk9^{RNAi} ; UAS-Yki, tub G80^{ts} / +*
10.	*apG4> Larp^{RNAi}+Yki^{OEx}+GFP*	*ap G4, UAS-GFP / UAS-Larp^{RNAi} ; UAS-Yki, tub G80^{ts} / +*
11.	*apG4> Cdk9^{OEx}+Yki^{OEx}+GFP*	*ap G4, UAS-GFP / UAS-Cdk9 ; UAS-Yki, tub G80^{ts} / +*
12.	*apG4> PTEN^{RNAi}+Yki^{OEx}+GFP*	*ap G4, UAS-GFP / UAS-PTEN^{RNAi} ; UAS-Yki, tub G80^{ts} / +*
13.	*apG4>PTEN^{RNAi}+ mEGFR^{OEX}+ GFP*	*ap G4, UAS-GFP / UAS-PTEN^{RNAi} ; UAS-mEGFR, tub G80^{ts} / +*
14.	*apG4>Bin3^{RNAi}+Yki^{OEx}+Cdk9^{RNAi} + GFP*	*ap G4, UAS-GFP, UAS-Cdk9^{RNAi} / UAS-Bin3^{RNAi} ; UAS-Yki, tub G80^{ts} / +*
15.	*apG4>Hexim^{RNAi}+Yki^{OEx}+Cdk9^{RNAi}+GFP*	*ap G4, UAS-GFP, UAS-Cdk9^{RNAi} / UAS-Hexim^{RNAi} ; UAS-Yki, tub G80^{ts} / +*
16.	*apG4>PTEN^{RNAi}+mEGFR^{OEX}+ Cdk9^{RNAi} + GFP*	*ap G4, UAS-GFP, UAS-Cdk9^{RNAi} / UAS-PTEN^{RNAi} ; UAS-mEGFR , tub G80^{ts} / +*
17.	*apG4> Bin3^{RNAi}+ Cdk9^{OEx} +GFP*	*ap G4, UAS-GFP, UAS-Cdk9 / UAS-Bin3^{RNAi} ; tub G80^{ts} / +*
18.	*apG4>Hexim^{RNAi}+Cdk9^{OE} +GFP*	*ap G4, UAS-GFP, UAS-Cdk9 / UAS-Hexim^{RNAi} ; tub G80^{ts} / +*

Spatial and Temporal Regulation of Expression

A TARGET system (Figure 5) using *GAL4, GAL80*^{ts} (ts-temperature sensitive) drivers under specific enhancers in *Drosophila* was used to regulate gene expression in a spatiotemporal manner. *GAL80*^{ts} was under control of *tubulin* enhancer, and *GAL4* was under *apterous* enhancer. Since apterous is present in the dorsal compartment of wing imaginal discs, the expression restricted to wing disc area. All the crosses initially kept at 18°C wherein *GAL80*^{ts} is active and inhibits the activity of *GAL4* driver and thereby all the downstream genes. At the late first instar larval stage the progeny was transferred to 28°C for activation of *GAL4*.

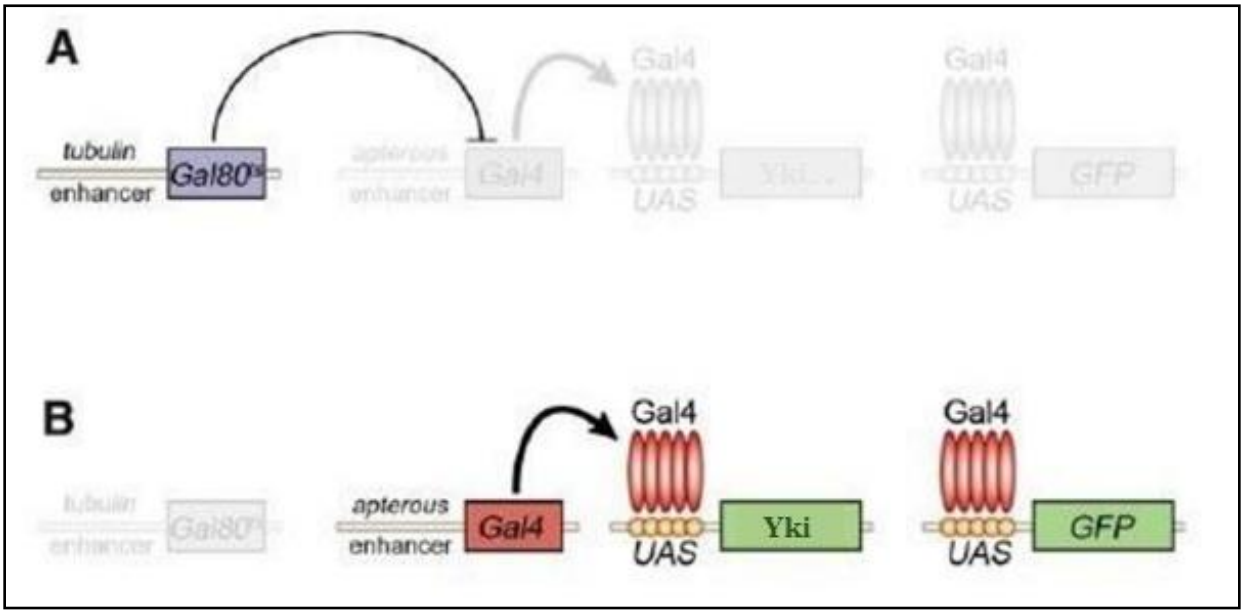


Figure 5; Spatiotemporal regulation of genes: At 18°C, *GAL80*^{ts} is active; represses *GAL4* activity and thus, UAS transgenes do not express (A). At 28°C, *GAL80*^{ts} is inactive, allowing apterous-*GAL4* to drive UAS-*Yki* or UAS-*EGFR* and UAS-*GFP* in the dorsal compartment of the wing imaginal disc (B). (Adapted from: Herranz et al., 2012)

Scoring and Selection

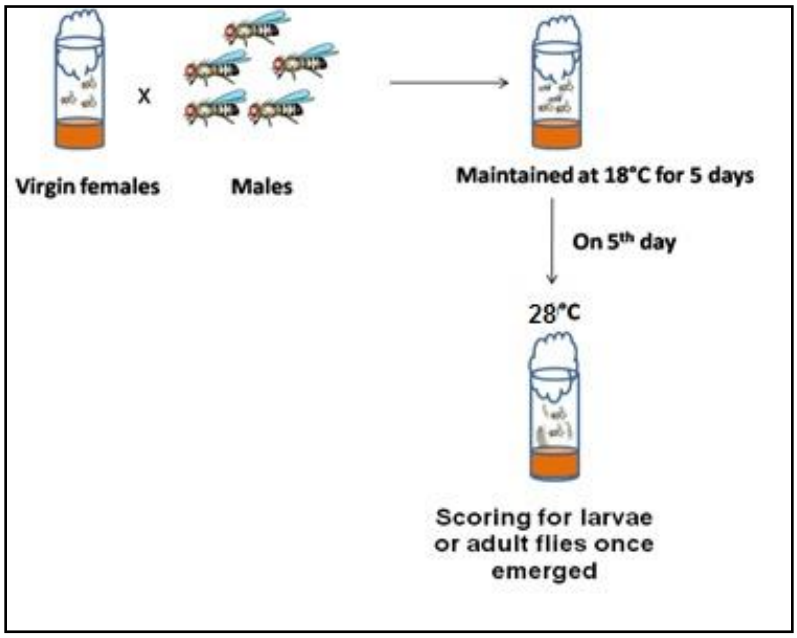
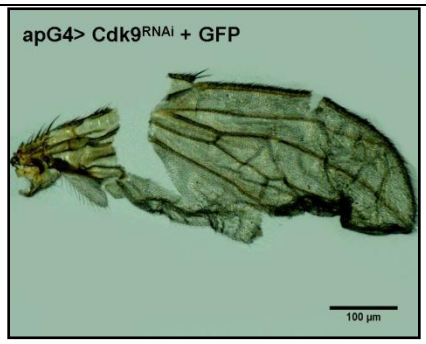
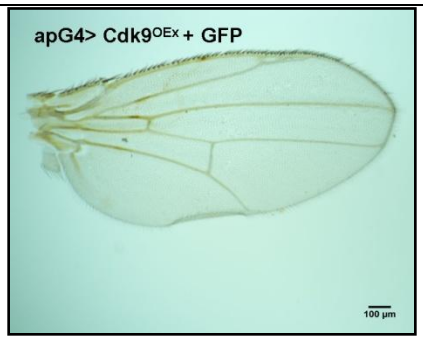
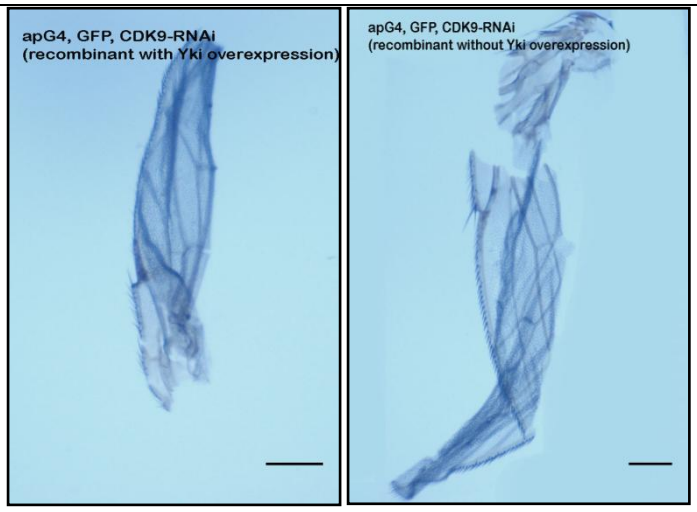


Figure 6; Graphical representation of crossing scheme followed: Virgin females of right genotype crossed with males of right genotype. Induction was done on the fifth day if required; otherwise, crosses maintained at 18°C.

The progeny scored for *GFP* positive giant larvae in the case of overgrowth. Otherwise, the adult flies were selected depending on defects in wing blade. The following table shows the wing phenotypes chosen to select flies for a given transgene:

Table 2: Defects in Wing blades chosen for selection of various genotypes

Sr. No.	Genotype	Phenotype
1.	*Bin3*^{RNAi}	

2.	*Hexim*^{RNAi}	
3.	*Cdk9*^{RNAi}	
4.	UAS-*Cdk9*	
5.	Recombined *Cdk9*^{RNAi} at 2nd chromosome (Scale: 50µm)	

Immunostaining

All the antibodies used here obtained from Developmental Studies Hybridoma Bank (DSHB), which include, anti-DECAD (1:100) and anti-MMP1 (1:50). The anti-pH3 (1:1000) antibody was a generous gift from Dr. Mayurika Lahiri's lab. (Parentheses indicate dilution factors)

Goat anti-rat (Alexa 568) and Goat anti-mouse (Alexa 633) are the secondary antibodies used with the dilution factor of 1:1000 unless otherwise mentioned. Images of wing imaginal discs from confocal microscopy were processed using Fiji and Adobe Photoshop 7.0.

Stock Preparations

The RNAi Stocks used in genetic schemes obtained from VDRC. All the RNAi inserts were on the 2nd chromosome at the same location. Thus we made recombinant stocks. Procedure 1 and 2 indicates the genetic scheme for the stock preparation of mentioned genotypes. UAS-*Cdk9* transgene was also on 2nd chromosome. Procedure 2 followed no induction since adult flies with an expression of UAS-*Yki* and UAS-RNAi transgene together might produce lethality.

1. To create $\frac{ap\ G4,\ UAS\text{-}GFP,\ UAS\text{-}Cdk9^{RNAi}}{cyo}\ ;\ \frac{UAS\text{-}Yki,\ tub\ G80^{ts}}{TM6B}$ stock

Procedure 1:

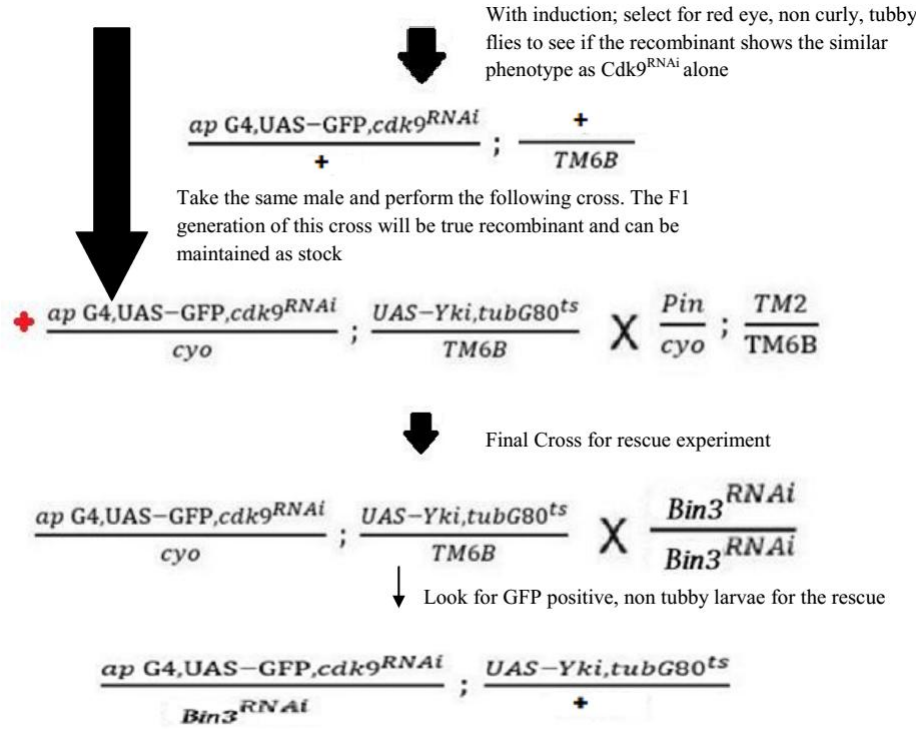
$$\frac{ap\ G4,UAS-GFP}{cyo}\ ;\ \frac{UAS-Yki,tubG80^{ts}}{TM6B}\ X\ \frac{cdk9^{RNAi}}{cdk9^{RNAi}}$$

⬇ Without induction; select for red eyed, non curly, non-tubby virgin flies

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

⬇ With Induction; select for red eye, GFP positive, Curly, Tubby, wing defects (shown in results) male

$$\frac{ap\ G4,UAS-GFP,cdk9^{RNAi}}{cyo}\ ;\ \frac{UAS-Yki,tubG80^{ts}}{TM6B}\ X\ \mathbf{w1118}$$



(Red star indicates that the same male has used in both the crosses)

Similarly, following stocks were prepared except the step of reconfirmation by w1118

$\frac{ap\ G4,\ UAS\text{-}GFP,\ UAS\text{-}Bin3^{RNAi}}{cyo}$; $\frac{tub\ G80^{ts}}{TM6B}$ $\frac{ap\ G4,\ UAS\text{-}GFP,\ UAS\text{-}Hexim^{RNAi}}{cyo}$; $\frac{tub\ G80^{ts}}{TM6B}$ nd

$\frac{ap\ G4,\ UAS\text{-}GFP,\ UAS\text{-}Cdk9}{cyo}$; $\frac{tub\ G80^{ts}}{TM6B}$

To create $\frac{ap\ G4,\ UAS\text{-}GFP,\ UAS\text{-}RNAi}{cyo}\ ;\ \frac{UAS\text{-}Yki,\ tub\ G80^{ts}}{TM6B}$ stock

Procedure 2:

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

↓

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

↓ Induction at 29°C, Select for GFP positive, Non ebony, Non curly, Non Tubby, Pin Bristles and bristles on haltare

$$\frac{ap\ G4,UAS-GFP,\ RNAi}{Pin}\ ;\ \frac{UAS-Yki,tubG80^{ts}}{TM2}\ \dashrightarrow (1)$$

Maintain this as a stock

$$\frac{ap\ G4,UAS-GFP,\ RNAi}{cyo}\ ;\ \frac{tubG80^{ts}}{TM6B}\ \dashrightarrow (2)$$

Cross (1) and (2) and maintain without induction

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

↓ Without induction, Select for Non ebony, Non curly, Pin Bristles, WT haltare and Tubby

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

↓ Without induction, Select for Non ebony, curly, Non Pin Bristles, WT haltare and Tubby to maintain stock

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

Similarly, the following stock prepared:

$$\frac{ap\ G4,\ UAS\text{-}GFP,\ UAS\text{-}Cdk9}{cyo}\ ;\ \frac{UAS\text{-}Yki,\ tub\ G80^{ts}}{TM6B}$$

In Silico Analyses

1. Stage wise analysis:

   Stage of tumor at the time of diagnosis provides a clinical parameter for progressiveness of cancer. The following table indicates the broad categorization for different stages used in TCGA clinical data:

   Table 3: Characterization of Stages in various cancer types.

Stages	**Characteristics**
Stage I	Primary small sized tumors
Stage II	Large tumors, LN negative, no metastasis
Stage III	Large tumors, LN positive, no metastasis
Stage IV	Large tumors, LN positive, Metastatic

   We plotted the expression profile of oncogene *YAP1* and Potential tumor suppressors, *MEPCE*, *HEXIM*1 and *HEXIM*2 using Box-Whiskers in MS-Excel for various stages. And a significant difference (p-value ≤ 0.05) between two distributions found by using Kolmogorov-Smirnov (KS) test in XL-Stat software. KS test is a non-parametric test. Thus it allows the identification of differences between two unknown distributions. Also, the difference between the median values of the distribution can be analyzed, even for small sample size.

2. Expression of *YAP1* targets in aggressive tumors

   Here we defined the aggressiveness of the tumor by three clinical parameters i.e. large tumors, LN-positive and late stage. The expression of bona fide *YAP1* targets, *AREG* and *CTGF*, was observed in normal tissue, in cancer tissue, in aggressive tumors, and aggressive tumors with high or low expression levels of *MEPCE*, *HEXIM*1, and *HEXIM*2. We observed the differences using Box-Whisker plot drawn in MS-Excel. And a significant difference (p-value ≤ 0.05) between two distributions found by using Kolmogorov-Smirnov test in XL-Stat software.

3. Kaplan-Meier Plots:

Here we drew KM plots by using METABRIC data for Breast cancer patients made available to us by Dr. T.S. Sridhar's lab. Survival analysis tool in XL-Stat software was used to produce KM curves, and Wilcoxon test gave the estimate of probability to find a significant difference between the trend lines. The software also predicted the number of patients at risk at a given point of time. First, we considered timed data of Disease-Free Survival below 60 months (5 Years) as this is the approximate time window to look at relapse of the disease. Next, the status of the patient considered as 1 if the patient survived disease-free and 0 if the disease reoccur. The expression of PTS genes divided into high, medium and low groups and *YAP1* into high and low groups depending on cutoffs as explained in Figure 7. Timed data, status, and expression grouping then fed into the KM plot analysis tool to generate survival curves for high, medium and low gene expression for 1. all patients 2. in patient cohort with particular PAM50 subtype and 3. in patient cohort where *YAP1* has high expression.

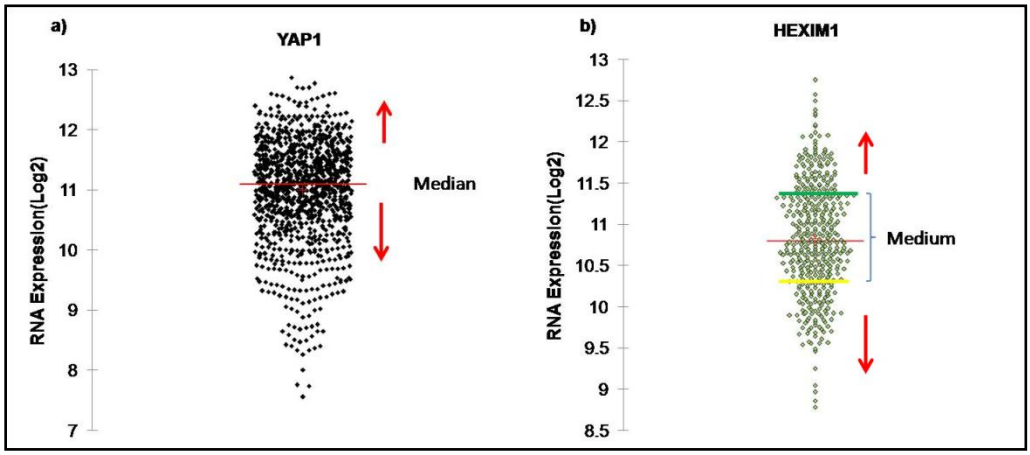


Figure 7; Cutoff values for dividing expression into high low and medium groups: Upward arrow indicates high expression whereas downward arrow indicates low expression of genes. Red line indicated the median value of the profile. For *YAP1*, median represents the cutoff for high and low. However, any other PTS gene expression considered as high for values ≥3rd quartile whereas low for values <1st quartile. 2nd and 3rd quartile represent medium level of expression.

Results- Part A

1. *Hexim* and *Larp* as Potential Tumor Suppressor Gene

Previous work done in the lab suggested *Bin3* as a potential tumor suppressor since the loss of *Bin3* in *Yki* overexpression background resulted in neoplastic overgrowth (Thesis-Pravallika G.,2016). Here, we tested other proteins belonging to the 7SK-snRNP complex for their potential role as a tumor suppressor. Control panel (Figure 8a: Control) show that overexpression of *Yki* alone causes minor growth of wing imaginal discs whereas knockdown of *PTEN* in *Yki* overexpression background causes large growth. $PTEN^{RNAi}$ in the background of Yki^{OEx} was used as a positive control due to its known activity as a tumor suppressor (Song et al., 2012). We observed that knockdown of *Bin3* and *Hexim* with KK-RNAi lines produce significantly large tumors (Figure 8a: KK, 8b) whereas TRiP line for $Hexim^{RNAi}$ does not produce an overgrowth. However, the TRiP line for $Larp^{RNAi}$ showed a significant overgrowth (Figure 8a: TRiP, 8c). Phenotypes for both the lines of $Cdk9^{RNAi}$ are similar to that of Yki^{OEx} alone. These results indicate that *Hexim* and Larp also can be potential tumor suppressors.

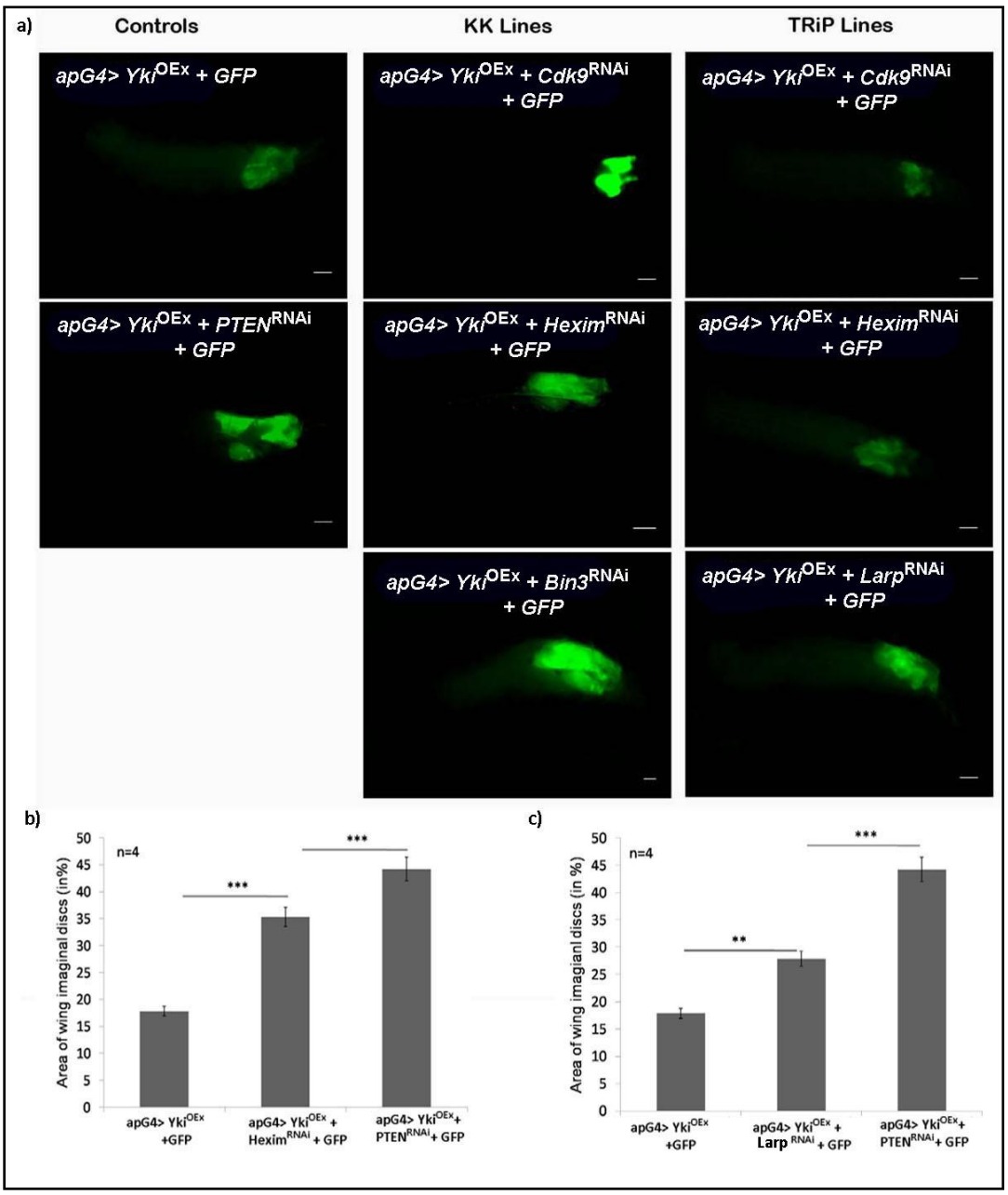


Figure 8; *Hexim* and Larp as potential tumor suppressor: a) *GFP*-positive 3rd instar larvae with the genotype indicated at the top. Tissue overgrowth observed in case of *Hexim* knockdown and Larp knockdown in *Yki* Overexpression background. 60% of the larvae showed similar phenotype (Scale: 50µm) **b)** Bar graph indicating a significant increase in the area of wing imaginal disc on *Hexim*(KK) knockdown. p≤0.05. **c)** Bar graph indicating a significant increase in the area of wing imaginal disc on *Larp* (TRiP) knockdown. Notice that *Bin3*^{RNAi} produces overgrowth more than half the larval length. p≤0.05 Unpaired two-tailed Student's *t*-test used to estimate P-value.

2. Characterization of tumors with *Hexim* Knockdown and *Yki* overexpression

A) Actively dividing cells in *Yki*^{OEx} + *Hexim*^{RNAi} tumors

To visualize the mitotically active cells, we performed PH3 (phospho-Histone 3) staining (Figure 9) on wing imaginal discs. Confocal micrographs of IHC performed against PH3 showed elevated PH3 levels in *Yki*^{OEx} + *Hexim*^{RNAi} tumors (Figure 9c). However, less PH3 staining observed in wing discs with only *Hexim* knockdown (Figure 9a) or only *Yki* overexpression (Figure 9b). Thus, overgrowth could be a result of the increased mitotic activity of cells in tumorous wing discs.

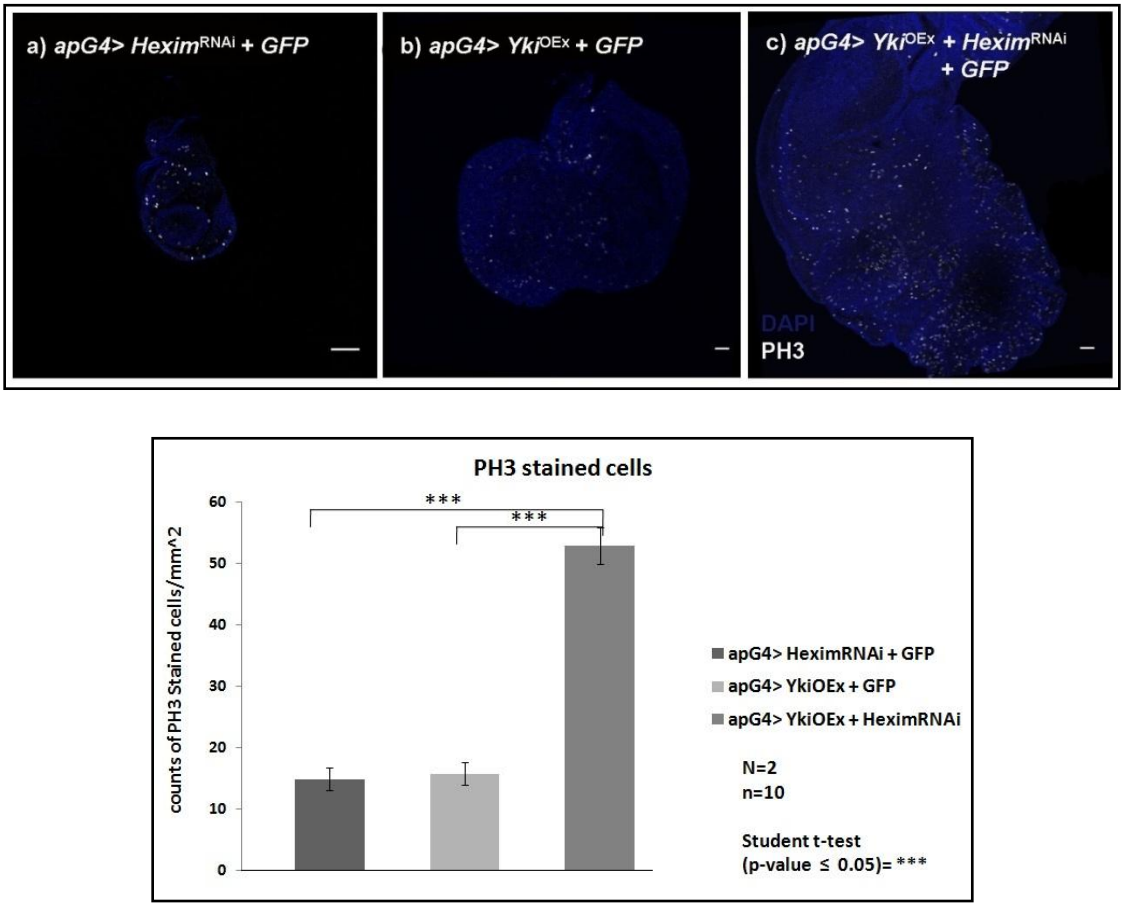


Figure 9; Mitotically active cells in *Yki*^{OEx} + *Hexim*^{RNAi} tumors: DAPI (stained in blue) and PH3 (stained in white) observed from above confocal micrographs of 3rd instar larval wing imaginal discs. Note the increased number of white puncta indicating mitotically active cells in tumorous wing disc (d). (Scale: 20μm) Unpaired two-tailed Student's *t*-test used to estimate P-value (p≤0.05).

B) Loss of Epithelial cell polarity in *Yki*^{OEx} + *Hexim*^{RNAi} tumors

Either loss or mislocalization of E-cadherin arises if the cell polarity gets compromised. To investigate the loss of epithelial cell polarity, we performed DE-cad staining. Figure 10 shows the confocal micrographs obtained by performing immunohistochemistry(IHC) against DE-Cad on 3rd instar larval wing imaginal discs. Results obtained here suggest that DE-cad localized to an apical region in wing discs with wild-type background (Figure 10a), *Yki*^{OEx} (Figure 10b) and *Hexim*^{RNAi} (Figure 10c). However, we observed mislocalization of DE-Cad in *Yki*^{OEx} + *Hexim*^{RNAi} tumors (Figure 10d) suggesting a loss of epithelial cell polarity in these tumors.

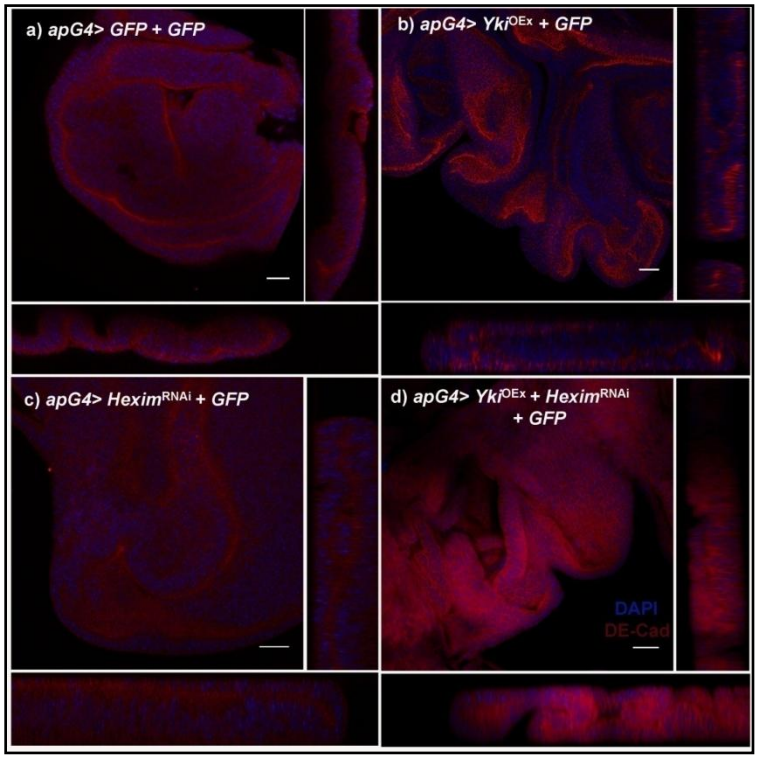


Figure 10; Loss of epithelial cell polarity caused by downregulation of *Hexim* in *Yki* overexpression background: DAPI (stained in blue) and DE-cad (stained in red) observed from above confocal micrographs of 3rd instar larval wing imaginal discs. Orthogonal optical sections depict the localization of DE-Cad. Note, mislocalization of DE-cad in *Yki*^{OEx} + *Hexim*^{RNAi} tumors (d). (Scale=20μm)

C) Epithelial-Mesenchymal transition of *Yki*^{OEx} + *Hexim*^{RNAi} tumors

Epithelial to mesenchymal transition is a well-known characteristic of tumor progression. Metalloproteases are released out of the cell to modify extracellular matrix. MMP1 is one of the metalloproteases which acts as a marker for EMT (Figure 11). Confocal micrographs of IHC performed against MMP1 showed the elevated levels of MMP1 in tumors with upregulation of *Yki* and downregulation of *Hexim* (Figure 11d, 11d') However, we observed, less amount of MMP1 in only *Hexim* knockdown (Figure 11a,11a') or only *Yki* overexpression (Figure 11b,11b').

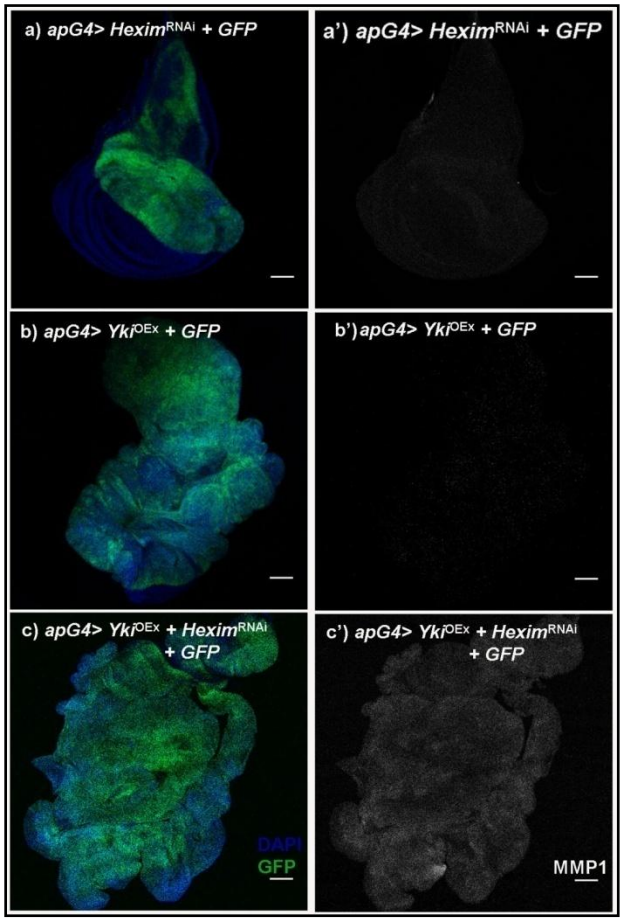


Figure 11; Tumors with *Yki*^{OEx} + *Hexim*^{RNAi} show elevated EMT marker: *GFP* (Green) marks the dorsal compartment of wing imaginal disc stained with DAPI (Blue) of 3rd instar larvae. Confocal micrographs shown here clearly depict the elevated levels of MMP1 (white) in tumors. Also, note the overgrowth of dorsal compartment due to *Yki*^{OEx} and downregulation of *Hexim* in *Yki*^{OEx} background. (Scale=20μm)

3. *Cdk9* knockdown genetically rescues the *Yki*^{OEx} + *Hexim*^{RNAi} and *Yki*^{OEx} + *Bin3*^{RNAi} tumors

According to the hypothesis, the neoplastic overgrowth observed for *Yki*^{OEx} + *Hexim*^{RNAi} (characterized in the previous section) and *Yki*^{OEx} + *Bin3*^{RNAi} (Thesis-Pravallika G., 2016) occurred due to increased activity of *Cdk9*. Thus, downregulation of *Cdk9* in these tumors might lead to the rescue of the tumorous phenotype. To investigate the same, we prepared a stable stock with recombinant *Cdk9*^{RNAi} at 2nd chromosome along with *Yki* overexpression at 3rd chromosome (described in Materials and Methods) since all RNAi inserts of KK library found at the same location. In this process, we made sure that *Yki*^{OEx} has no effect on adult wing phenotype upon downregulation of *Cdk9* (Table 1, 5.Recombined *Cdk9*^{RNAi} at 2nd chromosome). Hence, one can test the involvement of *Cdk9* in tumorigenesis. Here, we observed that downregulation of *Cdk9* indeed led to the rescue of tumorous growth observed when either *Hexim* or *Bin3* knocked down in *Yki* overexpression background (Figure 12c, 12f).

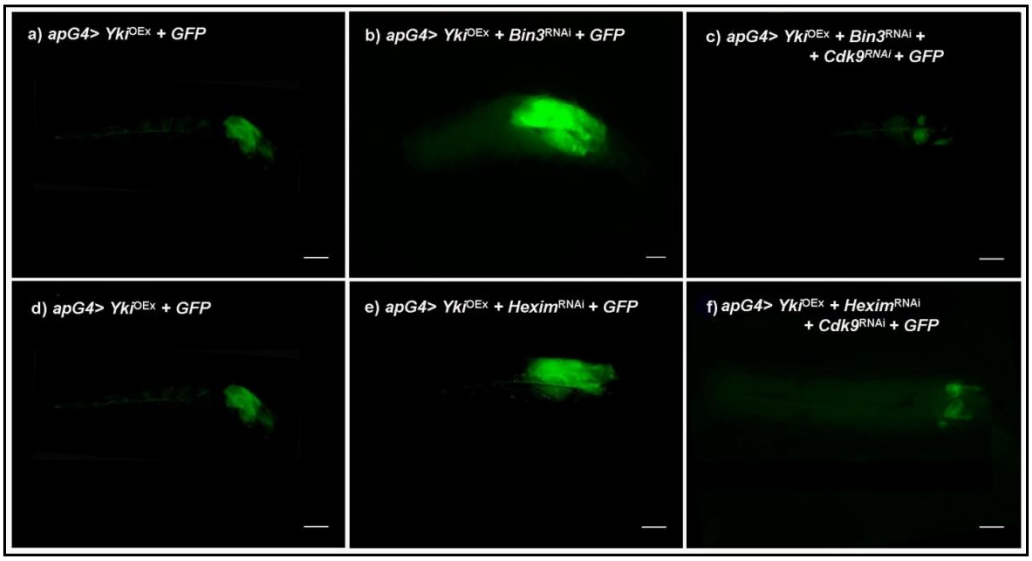


Figure 12; Downregulation of *Cdk9* suppresses the formation of *Yki*^{OEx} + *Hexim*^{RNAi} and *Yki*^{OEx} + *Bin3*^{RNAi} tumors: Image shows the non-tubby 3rd instar larvae scored for *GFP* positive wing discs. Interestingly, the morphology of wing discs in both the genetic rescue experiments matched with wild type but not with *Yki*^{OEx} alone. (Scale: 50µm)

The rescue observed due to downregulation of *Cdk9* not only restored the loss of apical polarity (Figure 13a, 13b) but also showed the levels of MMP1 similar to that of *Yki*^{OEx} (Figure 13a'' and 13b''). Moreover, the morphology of wing discs observed seemed to be similar to wild type. These results suggest that *Cdk9* mediate the tumorigenic effects caused by loss of *Bin3* and *Hexim* in *Yki* overexpression background.

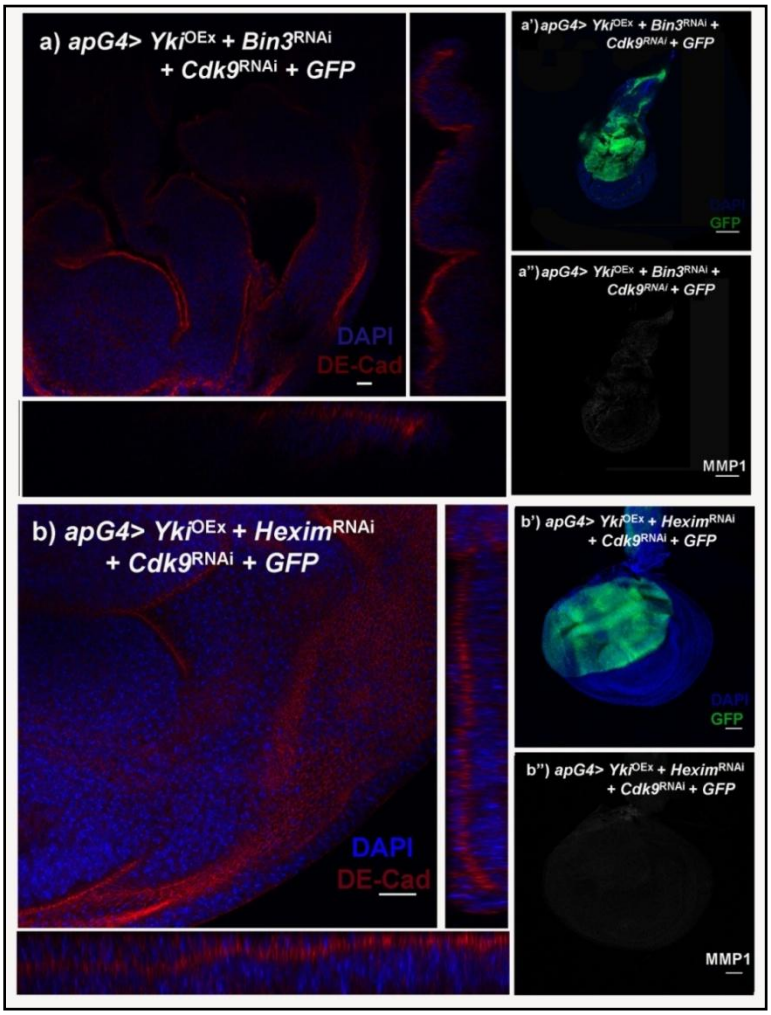


Figure 13; *Cdk9* knockdown in tumors with *Yki*^{OEx} + *Hexim*^{RNAi} restores the loss of cell polarity and EMT marker: Imaginal wing discs with DAPI (Blue), GFP (Green), DE-cad (Red) and MMP1 (White) of 3rd instar larvae depicted in the confocal micrographs. The optical cross-section in XZ plane illustrates the apical localization in rescued wing discs. (Scale=20μm)

4. Upregulation of *Yki* is important for tumorigenesis

Previous work done in the lab suggested that either downregulation of *Bin3* or upregulation of *Cdk9* in *Yki* overexpression background produces neoplastic tumors. We observed that downregulation of *Hexim* in *Yki* overexpression background led to neoplastic tumor formation (described in earlier results). And according to the hypothesis, *Bin3*, *Hexim* and *Cdk9* are the interactors in the 7SKsnRNP complex (Peterlin et al., 2012) which could regulate *Yki* mediated target gene expression. So, to understand if the interaction of *Bin3* or *Hexim* with *Cdk9* has a critical role to play in tumorigenesis, we wanted to downregulate *Bin3* or *Hexim* in *Cdk9* overexpression background. To do so, we prepared a recombinant stock of $Cdk9^{OEx}$ at 2nd chromosome without Yki^{OEx} (described in Materials and Methods). The non-tubby 3rd instar GFP positive larvae scored after crossing RNAi stock with a prepared stock of $Cdk9^{OEx}$ showed the wing discs similar to WT (Figure 14e, 14f). Therefore, knockdown of *Bin3* or *Hexim* along with overexpression of *Cdk9* is not sufficient to cause a tumor. Thus, *Yki* is the critical driver for tumorigenesis.

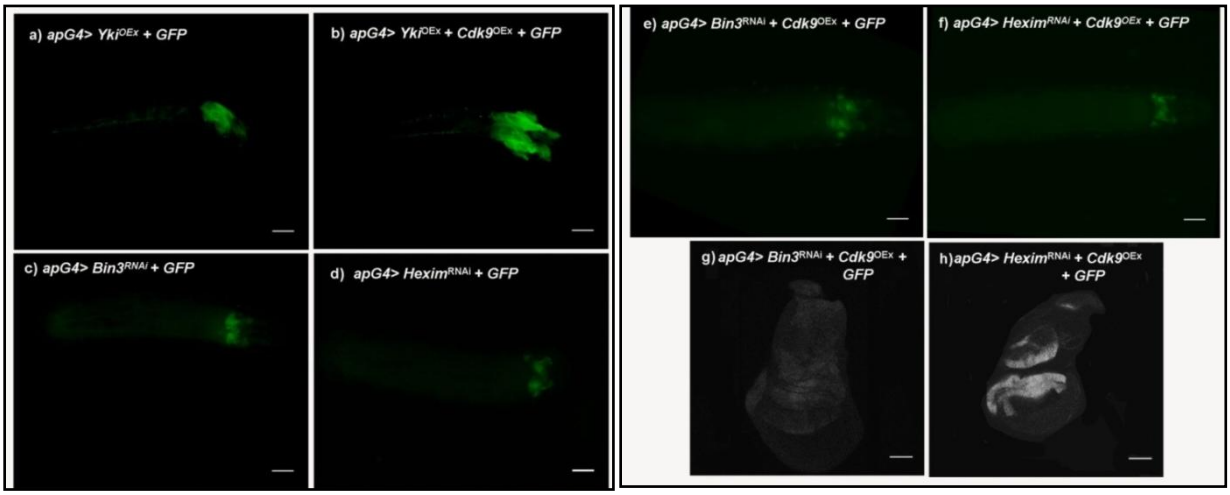


Figure 14; *Yki* overexpression is required for tumorigenesis: Non-tubby 3rd instar GFP positive larvae indicating wing discs with genotype mentioned at the top (Scale: 50µm). *Bin3* or *Hexim* knockdown in the $Cdk9^{OEx}$ background did not affect the morphology of wing discs observed from the micrograph (scale: 20 µm).

5. Non Specific role of *Cdk9* in tumorigenesis

As a part of the screen, we were also interested in looking at EGFR (Epidermal Growth Factor Receptor) pathway mediated tumorigenesis. Here also, *PTEN* knockdown led to tumor formation in *EGFR* overexpression background thereby serving as a positive control for *EGFR* screening (Figure 15b). According to the hypothesis, active *Cdk9* is important for *Yki* mediated tumorigenesis. But, the role of *Cdk9* has been established as a kinase important for removal of transcriptional pause and promoting productive elongation (see Figure 2). Thus, we wanted to test the extent of specificity of *Cdk9* function and whether *Cdk9* knockdown also has an effect on other tumorigenic pathways. To understand such role of *Cdk9* as a key regulator in tumorigenesis we prepared a stock of recombinant *Cdk9*^{RNAi} at 2nd chromosome with m*EGFR* (membraneEGFR) overexpression background (described in Materials and Methods). Interestingly enough, downregulation of *Cdk9* in m*EGFR*^{OEx} + *PTEN*^{RNAi} tumor led to the rescue of overgrowth (Figure 15c) highlighting the non specific role of *Cdk9* in tumorigenesis.

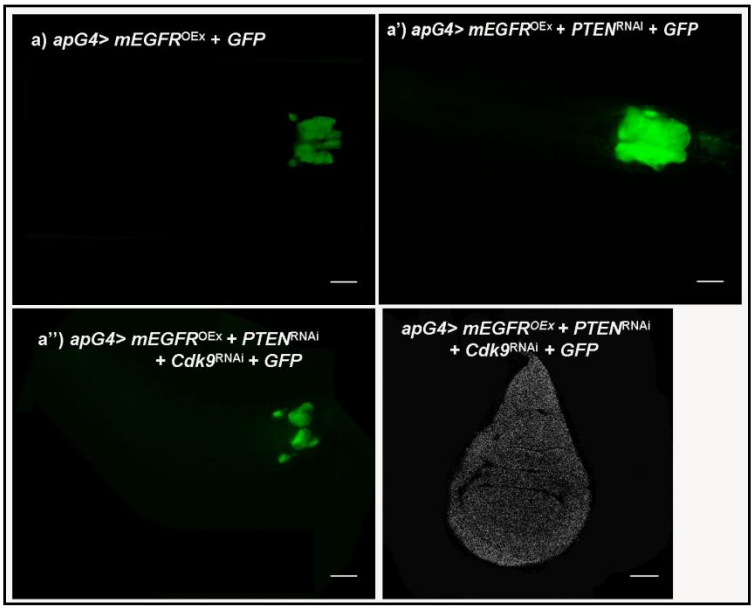
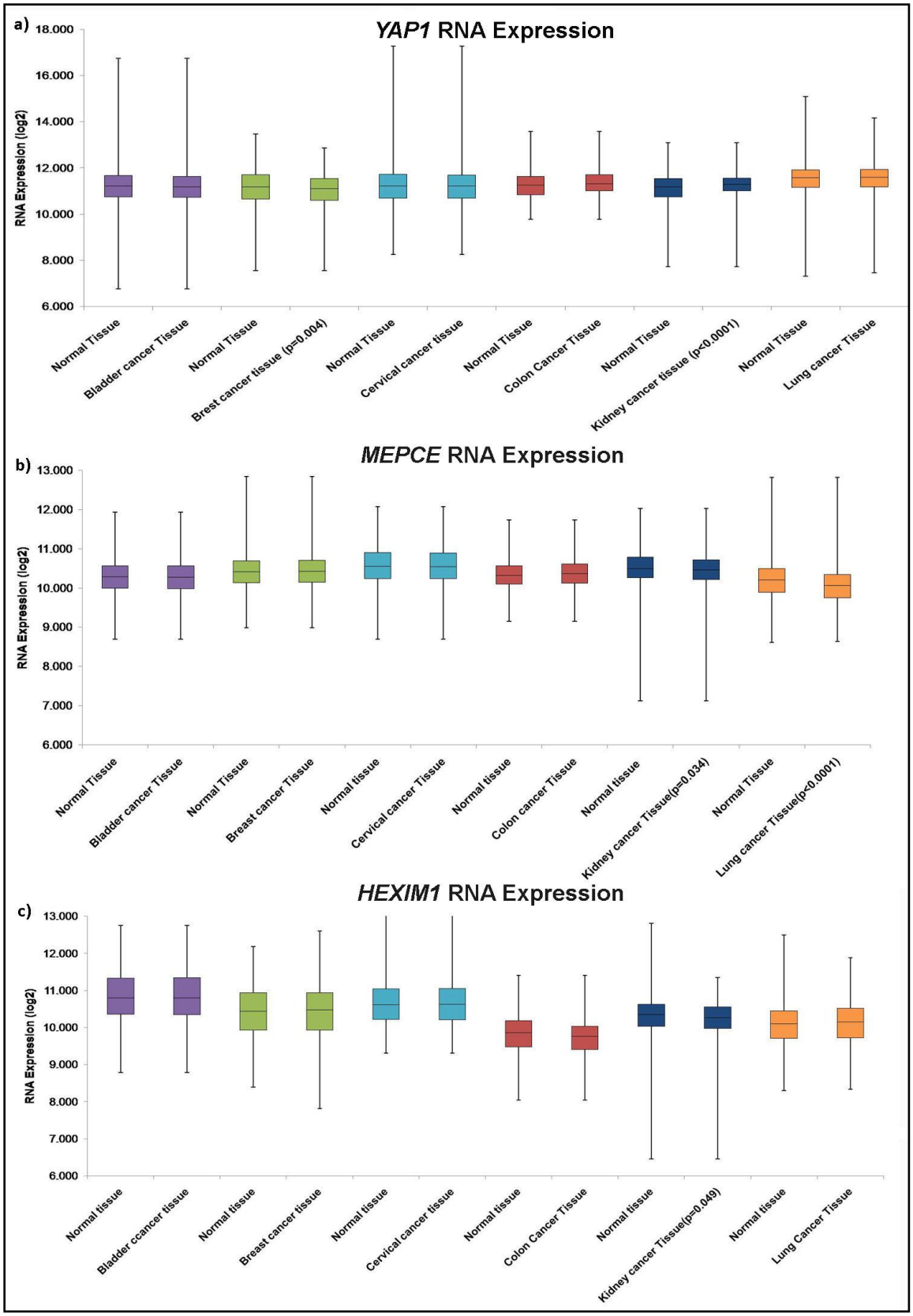


Figure 15; Downregulation of *Cdk9* rescues the mEGFR^{OEx} + PTEN^{RNAi} tumors: non-tubby GFP positive 3rd instar larvae with m*EGFR*^{OEx} produce slight overgrowth similar to that of *Yki*^{OEx} (Scale: 50µm). Note the morphology of wing discs from the micrograph (Scale: 20µm) rescued by *Cdk9*^{RNAi} similar to that of WT.

Results- Part B

1. Expression profiling of candidate genes at various stages of cancer

From the wet lab data, we established the role of *Hexim* and *Bin3* as potential tumor suppressor genes whereas *Cdk9* and *Yki* as potential oncogenes. Further to analyze the function of these candidate genes in cancer patients, we used TCGA RNA-seq data. We analyzed the expression profiles of human orthologs of these genes. Among the six cancers investigated here, a significant increase in *YAP1* expression occurs in only Kidney cancer whereas, in Breast cancers, the expression decreases significantly compared to respective normal tissue expression (Figure 16a). We observed, decrease in expression of *MEPCE* and *HEXIM1* in Kidney cancer whereas only *MEPCE* expression decreased in Lung cancer (Figure 16b, 16c). These profiles helped to understand overall differences of expression in normal vs. cancer condition. Further, to check if expression profiles differ according to the stage of cancer, we analyzed the distribution of patients at various stages (described in Materials and Methods). Analysis revealed that *YAP1* expression gradually increases over successive stages whereas *HEXIM1* expression goes down in Bladder cancer (Figure 16d, 16e). There were no significant changes in expression profiles in other cancers for any of the candidate genes except the one case where levels of *HEXIM2,* structural and functional homolog of *HEXIM1*, seem to increase from stage 3 to stage 4 (data not shown). Results obtained here indicate that the *YAP1* expression increases whereas *HEXIM1* expression decreases over the progression of Bladder cancer.


a)
YAP1 RNA Expression
RNA Expression (log2)
18.000
16.000
14.000
12.000
10.000
8.000
6.000
Normal Tissue
Bladder cancer Tissue
Normal Tissue
Brest cancer tissue (p=0.004)
Normal Tissue
Cervical cancer tissue
Normal Tissue
Colon Cancer Tissue
Normal Tissue
Kidney cancer tissue (p<0.0001)
Normal Tissue
Lung cancer Tissue
b)
MEPCE RNA Expression
RNA Expression (log2)
13.000
12.000
11.000
10.000
9.000
8.000
7.000
6.000
Normal Tissue
Bladder cancer Tissue
Normal Tissue
Breast cancer Tissue
Normal tissue
Cervical cancer Tissue
Normal tissue
Colon cancer Tissue
Normal tissue
Kidney cancer Tissue(p=0.034)
Normal Tissue
Lung cancer Tissue(p<0.0001)
c)
HEXIM1 RNA Expression
RNA Expression (log2)
13.000
12.000
11.000
10.000
9.000
8.000
7.000
6.000
Normal tissue
Bladder ccancer tissue
Normal tissue
Breast cancer tissue
Normal tissue
Cervical cancer tissue
Normal tissue
Colon Cancer Tissue
Normal tissue
Kidney cancer Tissue(p=0.049)
Normal tissue
Lung Cancer Tissue

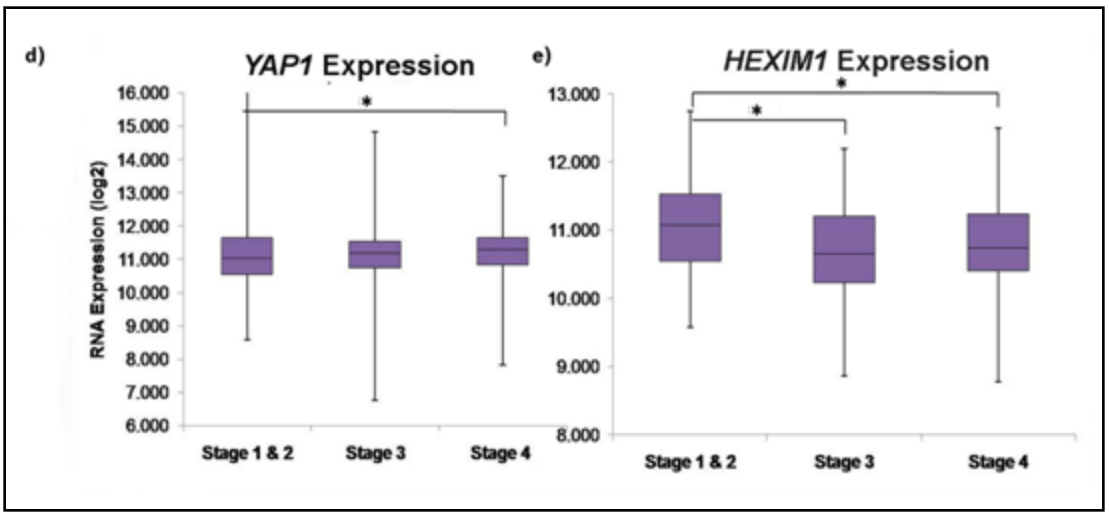


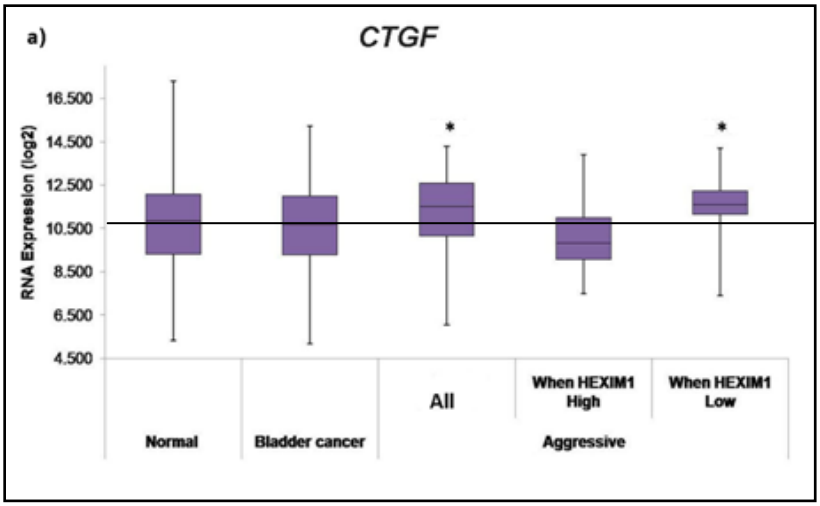
Figure 16; Upregulation of *YAP1* and downregulation of *HEXIM*1 expression in successive stages of Bladder cancer: RNA-Expression of *YAP1*, *MEPCE* and *HEXIM*1 across various cancer types in comparison with RNA-Expression in normal tissue indicated in Box-whisker plots. Significant differences indicated by p-value in parentheses (a, b, c). Stage 1&2 form population of early stage cancer patients whereas, Stage 3 and Stage 4 considered as late stages. The differences in two distributions analyzed by using KS-test in XL-Stat software (*=p≤0.05).

2. Effect of *MEPCE* and *HEXIM*1 expression on *YAP1* targets in aggressive tumors

In parallel to the hypothesis proposed here, we wanted to look at bona fide *YAP1* target expression in normal vs. aggressive tumors (Described in Materials and Method) in populations with high and low expression levels of *MEPCE* or *HEXIM1/2* (PTS). According to estimate, the expression of *YAP1* targets should decrease if expression levels of *MEPCE* or *HEXIM1/2* become high or vice versa. To check this, we produced box-whisker plots of *YAP1* target gene expression.

In the literature, CTGF and AREG found as bona fide YAP1 targets to study YAP1 activity (Liang et al., 2014; Zhao et al., 2008). These genes have also been shown to regulate YAP driven oncogenesis. Thus, we used *CTGF* and *AREG* expression as a

proxy for YAP1 activity and checked their expression levels when either *MEPCE* or *HEXIM1* levels changed in the aggressive form of cancer. The population of patients with aggressive tumor helps in creating a cohort which could correlate to a malignant form of cancer. In the expression plot of *CTGF*, we observed significant upregulation in the cohort of aggressive tumors of Bladder cancer and also in the cohort with low expression of *HEXIM1* (Figure 17a). A similar analysis done on Lung cancer patients revealed upregulation in *AREG* in aggressive tumors as well as in the cohort with low *MEPCE* expression in aggressive tumor (Figure 17b). Hence, low expression levels of *MEPCE* and *HEXIM1* in an aggressive form of lung cancer and bladder cancer respectively increase the expression of *YAP1* targets. However, analysis done for other cancer types does not show any significant differences in the expression profile of *YAP1* targets.



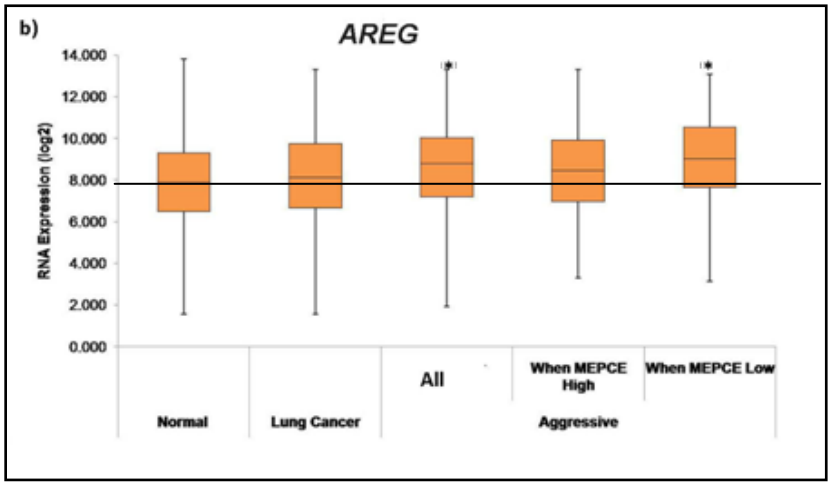


Figure 17; Expression of *YAP1* targets increase in the aggressive form of cancer: Purple indicates Bladder cancer whereas orange indicates lung cancer data. All the distributions of expression compared with normal tissue expression of *YAP1* targets. The black line indicates the median of normal expression profile. No differences observed in the whole cancer population compared to normal. But in an aggressive form of cancer and as well as in cohort with low levels of PTS, *CTGF* and *AREG* expression increased respectively.

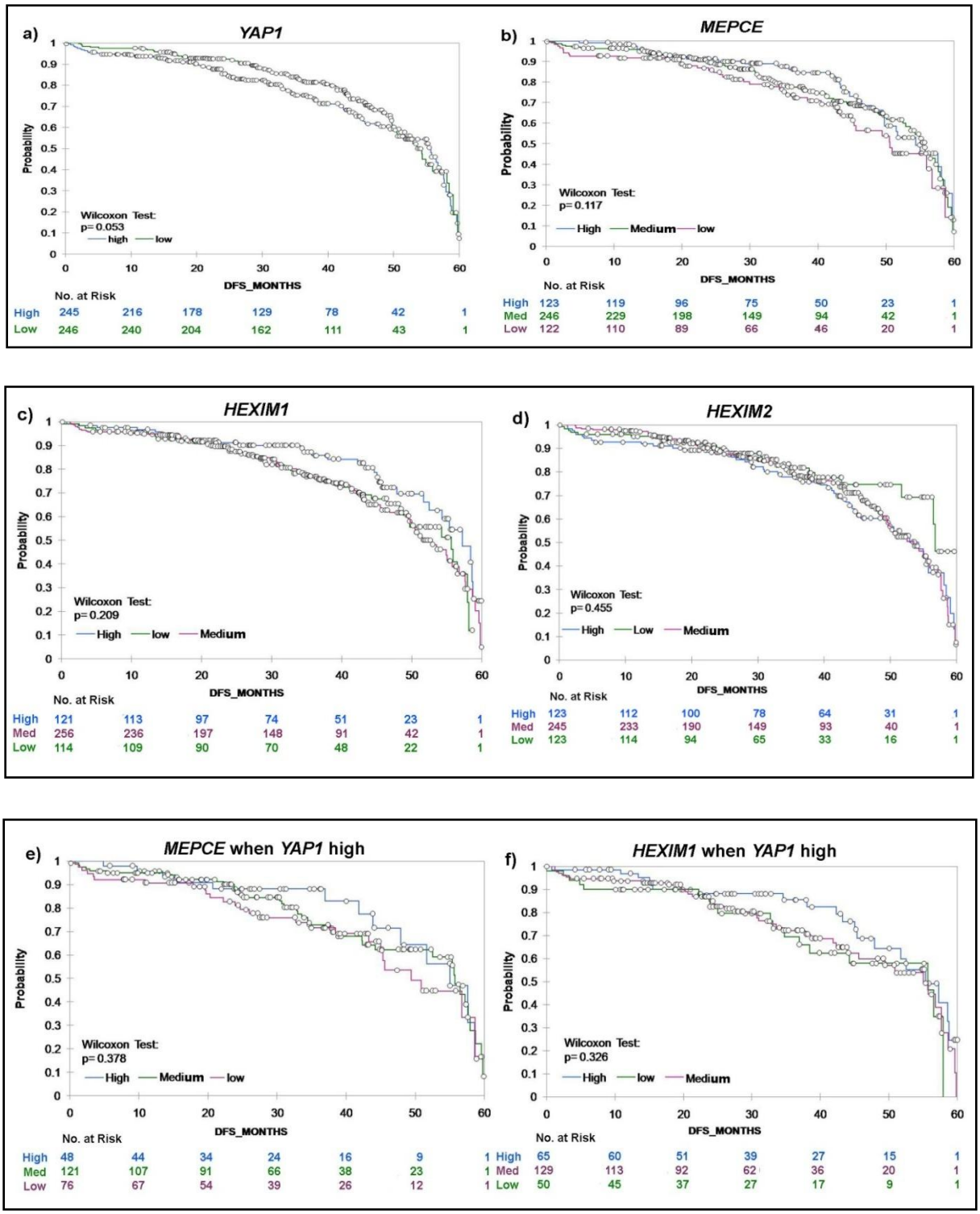
3. Prognosis of Breast cancer patients

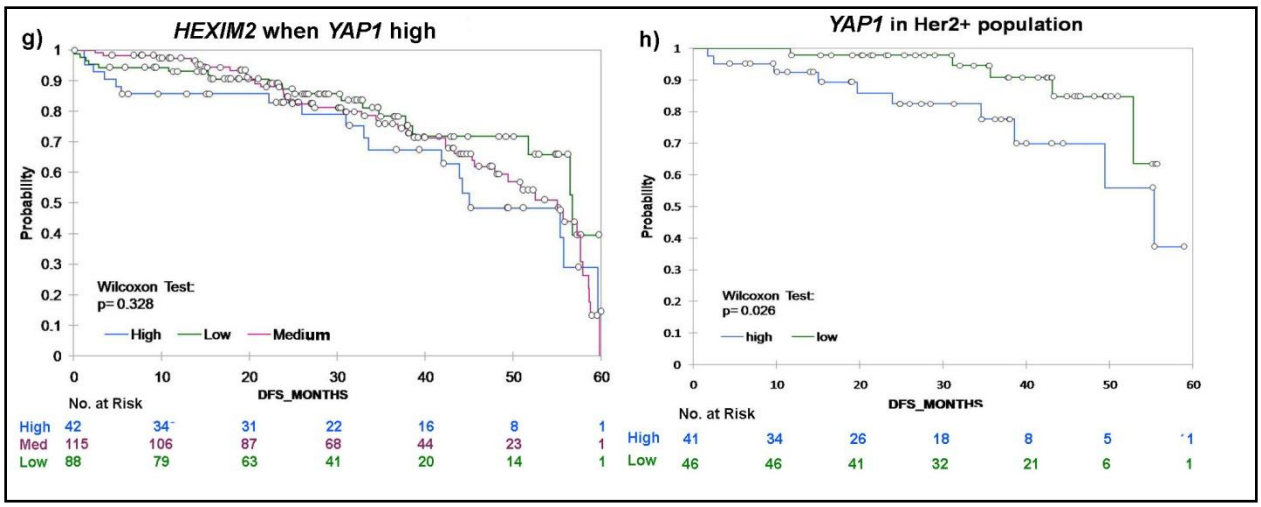
Disease-free survival (DFS) of patients plays an important role in understanding prognosis. To estimate DFS in Breast cancer patients, we used METABRIC follow-up data. We wanted to investigate how a change in expression of *MEPCE*, *HEXIM1/2*, and *YAP1* correlates to better or poor prognosis of patients. Using KM-Plot analysis in XL-Stat, we drew survival curves as shown in Figure 15. Graphs also indicate the number of patients at risk due to high, medium or low expression levels of given genes in given conditions.

The increase in *YAP1* expression leads to marginally significant (p=0.053) correlation to poor prognosis in Breast cancer patients (Figure 18a). However, no significant results observed for any PTS genes (Figure 18 b-d). We obtained similar results even in *YAP1* high conditions for all the PTS genes (Figure 18 e-g). Studies suggested that there are various subtypes of Breast cancer depending on Prediction Analysis of Microarray 50 (PAM50) classification which includes LuminalA, LuminalB, Her2 enriched, Basal type and Normal-like tumors (Bastien et al., 2012). From the analysis, we found that high expression levels of *YAP1* expression in Her2 enriched population correlates with poor prognosis (Figure 18h).

Also, in the very aggressive form of breast cancer known as basal type, low levels of *HEXIM1* correlated to the poor prognosis of patients under *YAP1* high conditions (Figure 18i). These results, suggest that *YAP1* high expression could be a driver for poor prognosis in Breast cancer patients and in particular for Her2 enriched patients.

And *HEXIM1* could act as a tumor suppressor in the very aggressive form of Breast cancer, but only in a population with high *YAP1* expression levels.





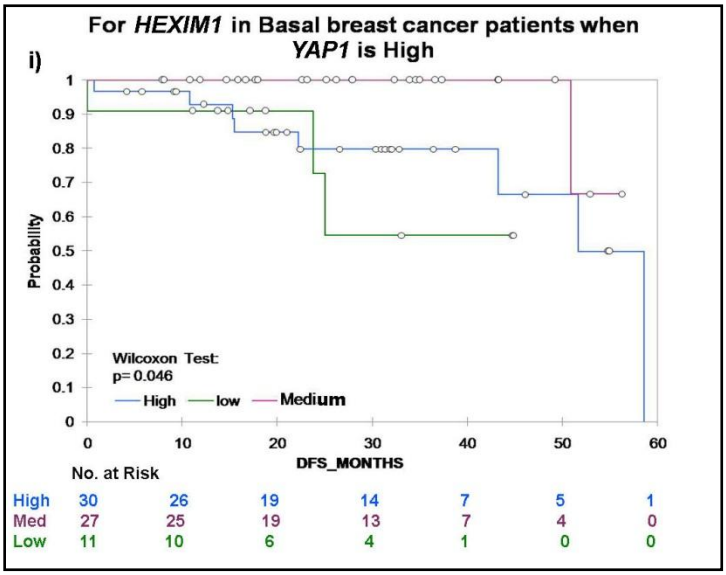


Figure 18; Disease Free Survival of Breast cancer patients: KM plots depict the survival curves for high, medium or low expression levels of genes indicated at the top with specified conditions. The number of patients at risk bears a color of trend line representing that population. To find the significant differences between the curves, we considered Wilcoxon Test with p≤ 0.05.

Discussion

Growth control and regulation of organ size was extensively studied in developmental biology using *Drosophila* as a model system. *Drosophila* provides a genetic background which can be tweaked easily to study a particular context. And because many of the genes conserved well from *Drosophila* to human, these studies can easily be extrapolated to mammals. So, to study a complex genetic disease such as cancer, this model provides a uniform genetic background as well as microenvironment which could approximate to human cancer scenarios. Thus, we established a genome-wide genetic screen to identify potential tumor suppressors in the epithelial context in three different genetic backgrounds which include *Yorkie* overexpression, *EGFR* overexpression and *Socs36E* knockdown in EGFR overexpression. The objectives of the study conducted here are based on the previous work from the lab wherein *Bin3* has picked as a potential tumor suppressor from the *Yki* screen and further studied to validate its role in *Drosophila* by proposing a hypothesis (explained in Figure 4). This study has been an extension to elucidate the role of *Bin3* and its interacting complex (7SKsnRNP) further, in *Yki* mediated tumorigenesis. From this study, we also developed an understanding of human orthologs of the genes involved in the pathway for their potential roles in different cancers.

We observed that other components of the 7SKsnRNP complex such as *Hexim* and Larp also act as potential tumor suppressors. Moving forward with *Hexim* study, we observed, *Hexim* knockdown in *Yki* overexpression background produced neoplastic tumors. These tumors characterized by the loss of apical cell polarity and the potential to undergo EMT when compared to either *Hexim* knockdown or *Yki* over expression alone. The neoplastic overgrowth in tumors could be because of rapid proliferation since the cells are undergoing active mitosis. Thus, along with *Bin3*, *Hexim* could also be a tumor suppressor in *Yki* mediated tumorigenesis.

To understand the involvement of *Cdk9* in this pathway, we performed epistatic genetic experiment wherein downregulation of *Cdk9* led to the rescue of tumors formed by downregulation of *Bin3* or *Hexim* in *Yki* overexpression background. The rescue observed for these tumors also restored neoplastic characters. These results indicate

that *Yki* mediated tumorigenesis requires *Cdk9*. To perform such experiments, we created a stable stock of recombinant *Cdk9*^{RNAi} at 2nd chromosome and larvae of right genotype in the experiment contained four different UAS-transgenes. Thus, the result we observed could be because of the dilution of *GAL4*. To rule out this fact, we need to cross this stable stock with random RNAi transgenes of *Yki* screen positives to see whether tumors get rescued. But, it is also possible that knockdown of *Cdk9* is solely responsible factor for the rescue since Cdk9 controls the transcriptional activity of RNAPII. This possibility is based on the experiments where we downregulated *Cdk9* in *PTEN* knockdown with *mEGFR* overexpression background. *Cdk9* rescued *mEGFR*^{OEx} + *PTEN*^{RNAi} tumors suggesting a global role of *Cdk9* as a regulator for tumorigenesis. The observed results could suggest the known activity of *Cdk9* as regulating the target gene expressions at the transcriptional level.

As *Cdk9* overexpression whereas *Bin3* or *Hexim* knockdown in *Yki* overexpression background gave neoplastic tumors, it was interesting to understand if *Bin3* or *Hexim* knockdown and *Cdk9* overexpression together could drive tumorigenesis. But, the results suggest that knockdown of *Bin3* or *Hexim* in *Cdk9* overexpression background is not enough to cause a tumor. Thus, *Yki* overexpression plays an important role as a driver for tumorigenesis. Also, this result suggests that *Bin3* and *Hexim* activity is *Yki* dependent though the mediator, *Cdk9* has nonspecific role to play in tumorigenesis.

It has observed previously that p-Mad and Yki together localized to the nucleus in *Yki*^{OEx} + *Bin3*^{RNAi} tumors. Therefore, it could bring about the transcription of Dpp target genes (Thesis-Pravallika G.,2016). To understand the role of *Bin3* and *Hexim* as a key regulator of p-Mad-Yki target activity, it would be interesting to see the activity of Dpp target gene such as *bantam* getting upregulated in *Yki*^{OEx} + *Bin3*^{RNAi} or *Yki*^{OEx} + *Hexim*^{RNAi} tumors. Thus, we can use a *bantam*-LacZ or *bantam*-sponge to see the expression changes of *bantam* in tumorous wing discs.

Moving on to *In silico* analysis, here we made use of cancer patients' RNA expression data and clinical data to understand the role of human orthologs of the candidate genes. *HEXIM1* expression decreased whereas *YAP1* expression increased over successive stages of bladder cancer. In the aggressive form of bladder cancer increased

expression of *YAP1* target gene, *CTGF* was observed for low *HEXIM1* expression cohort, suggesting that the low levels of *HEXIM1* lead to the expression of *YAP1* targets as proposed in the hypothesis. Also, low expression levels of *HEXIM1* lead to the poor prognosis of Basal breast cancer patients in high *YAP1* expressing population. Thus, in parallel to the wet lab data, the analysis done here suggested the role of *HEXIM1* as a potential tumor suppressor in bladder cancer and breast cancer. But, the role of *HEXIM1* turns out to be very specific for progressive or aggressive tumors where malignancy is a marked characteristic. It is also important to perform such analysis on protein expression of the candidate genes to come to the conclusion of their role.

From the literature, we already know the oncogenic activity of *YAP1* and the analysis also revealed the similar results. In bladder cancer, *YAP1* expression increased in successive stages suggesting its role in the progression of cancer. But, the expression profiles observed for various cancers revealed that *YAP1* expression goes significantly down in breast cancer patients. However, high expression levels of *YAP1* lead to poor prognosis in Her2 enriched population suggesting the context-specific role of *YAP1* in breast cancer. But to conclude the role of *YAP1*, phosphorylation status of the protein plays an important role. Thus, it is important to understand the change in protein or phospho-protein levels of *YAP1*.

Similar to *HEXIM1*, *MEPCE* also correlated with increased expression of *YAP1* target, AREG in an aggressive form of lung cancer. If we compare the expression profiles of patients bearing aggressive form of tumors with normal tissue, the *CTGF* and *AREG* expression increases in bladder and lung cancer respectively. Similarly, a significant increase in expression of *CTGF* and *AREG* observed for the patient cohort with low levels of *HEXIM1* or *MEPCE* but not with their high levels. Therefore, a patient cohort with low levels of *HEXIM1* or *MEPCE* in respective cancers could contribute to the increase in expression of *YAP1* targets in the population bearing aggressive tumor (3rd box plot in Figure 17).

Taken together, results from the current study suggest that *MEPCE* and *Hexim*/ *HEXIM1* could act as a tumor suppressor in a context-dependent manner. Also, the tumor suppressor activity of *Bin3* and *Hexim* was specific to *Yki* mediated tumorigenesis

since knockdown of these genes in EGFR overexpression background did not produce any tumor. But, the role of *Cdk9* could be global to regulate transcription of the genes. To further understand the genetic interactions between *Bin3*, *Hexim*, and *Cdk9* we intent to test if *Bin3*^{OEx} or *Hexim*^{OEx} could rescue *Yki*^{OEx} + *Cdk9*^{OEx} tumors for which we are in the process of preparing a recombinant stock of *Cdk9*^{OEx} at 2nd chromosome and Yk^{OEx} at 3rd chromosome. Also, it would be interesting to understand if two tumor suppressors *Bin3* and *Hexim* could interact genetically, for which *Bin3* and *Hexim* can be knocked down together.

The definitive goal of using *Drosophila* as an epithelial transformation model and *In silico* analyses of cancer patients is to recognize different markers in the specific context of cancer. Finally, as a future prospect of this entire study, we would like to know if the proposed pathway is conserved by using mammalian cell lines and mouse xenografts.

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