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Diwas Srivastava

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# THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En BIOLOGIE - SANTE

École doctorale- Biologiques pour la Santé (CBS2)

Unité de recherche- Institut de Génétique Moléculaire de Montpellier

## Modulation of Hippo Pathway by Alternative Splicing

Présentée par Diwas SRIVASTAVA

Le 25 Juin 2019

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---

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## Abstract

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The Hippo pathway is a conserved pathway involved in tissue growth and tumor suppression. Studies have demonstrated its significance in the development of human cancers. This cascade controls the activity of the transcription co-activator Yorkie (Yki) in flies and Yes-associated protein (YAP) in mammals. Due to Alternative Splicing (AS), both Yki and YAP proteins exist as two isoforms containing one (Yki1/YAP1) or two (Yki2/YAP2) WW domains. Since WW domains are essential for interaction with specific partners, the alternative inclusion of this domain in Yki/YAP protein may remodel their interaction network and therefore their activity. The regulation and functional consequences of AS of *yki/YAP* *in vivo* are unknown.

In this Ph.D. project, we identified that depletion of splicing factor B52 in *Drosophila* lowers inclusion of the alternative exon in *yki* mRNAs and favors the expression of Yki1 isoform at the expense of the Yki2 isoform. B52 depletion in the wing reduces growth and Yki activity. We demonstrate that Yki1 isoform is an attenuated version of Yki protein that can compete with Yki2 isoform in the nucleus. To ascertain the role of *yki* AS *in vivo* and the importance of short isoform Yki1, we abrogated this splicing by using CRISPR/Cas9 technology and created flies that can express Yki2 isoform only. *yki*<sup>2only</sup> flies are viable but display a random phenotype of asymmetric wing size. This rise in “fluctuating asymmetry” that is the consequence of subtle deviation from normal development, suggests that AS of *yki* is crucial for the development robustness. Taking together, these results highlight a new layer of modulation of Hippo pathway *via* AS of *yki*.

Alternative inclusion of the second WW domain is a conserved feature between Yki and YAP. This further supports the idea that Yki1 and YAP1 isoforms have an important function *in vivo* and that AS of *yki/YAP* is a conserved mechanism of control of the Hippo pathway. This study opens up new perspectives for modulation of the Hippo pathway in cancer cells by altering *YAP* AS.

# Résumé

---

La voie Hippo est une voie conservée impliquée dans la croissance des tissus et la suppression de tumeurs. Des études ont démontré son implication dans le développement des cancers chez l'homme. Cette cascade contrôle l'activité du co-activateur transcriptionnel Yorkie (Yki) chez la drosophile et de la protéine YAP (Yes Associated Protein) chez les mammifères. En raison de l'épissage alternatif de leur transcrits, les protéines Yki et YAP existent sous deux isoformes contenant un domaine WW (Yki1/YAP1) ou deux (Yki2/YAP2). Puisque les domaines WW sont essentiels pour l'interaction avec des partenaires spécifiques, l'inclusion alternative de ce domaine dans la protéine Yki/YAP peut remodeler leur réseau d'interaction et donc leur activité. La régulation et les conséquences fonctionnelles de l'épissage alternatif de *yki / YAP in vivo* sont inconnues.

Dans le cadre de ce doctorat, nous avons constaté que la déplétion du facteur d'épissage B52 chez la drosophile réduit l'inclusion de l'exon alternatif dans l'ARNm de *yki* et favorise l'expression de l'isoforme Yki1 aux dépens de l'isoforme Yki2. La déplétion en B52 dans l'aile réduit la croissance et l'activité de Yki. Nous montrons que l'isoforme Yki1 est une version atténuée de la protéine Yki qui peut entrer en concurrence avec l'isoforme Yki2 dans le noyau. Pour déterminer le rôle de l'épissage alternatif de *yki in vivo* et l'importance de l'isoforme courte Yki1, nous avons abrogé cet épissage en utilisant la technologie CRISPR/Cas9 et avons créé des mouches capables d'exprimer uniquement l'isoforme Yki2. Ces mouches *yki<sup>2only</sup>* sont viables mais présentent un phénotype aléatoire d'ailes asymétriques. Cette augmentation de l'«asymétrie fluctuante», qui traduit une déviation par rapport au développement normal, suggère que l'épissage alternatif de *yki* est crucial pour la stabilité développementale. Ces résultats mettent en évidence un nouveau niveau de modulation de la voie Hippo *via* l'épissage alternatif de *yki*.

L'inclusion alternative du deuxième domaine WW est une caractéristique conservée entre Yki et YAP. Cela conforte l'idée que les isoformes Yki1 et YAP1 ont une fonction importante *in vivo* et que l'épissage alternatif de *yki/YAP* est un mécanisme conservé de contrôle de la voie Hippo. Cette étude ouvre de nouvelles perspectives pour la modulation de la voie Hippo dans les cellules cancéreuses en modifiant l'épissage alternatif de *YAP*.

## Short Introduction and Objectives :

---

Over the last decade, the Hippo pathway has emerged as a major player in tissue size control, regeneration and cancer progression. The pathway is, in particular, regulated by mechanical cues and allows to link growth of the cells with their environment. First discovered in *Drosophila*, this conserved signaling pathway regulates the activity of a transcription co-activator called Yorkie (Yki) in flies and YAP (Yes-Associated Protein) in human. The core of the Hippo pathway is composed by the kinase Hippo (MST1/2 in mammals) which activates the kinase Warts (LATS1/2 in mammals) that phosphorylates the transcription co-activator Yki in *Drosophila* and YAP in mammals. Phosphorylated Yki/YAP is sequestered in the cytoplasm through interactions with 14-3-3 proteins. Inactivation of the pathway leads to translocation of unphosphorylated Yki/YAP in the nucleus and activation of its target genes. Yki/YAP does not bind DNA directly but interacts with several transcription factors, mainly members of the TEAD family such as Scalloped (Sd) in flies and TEAD1–4 in mammals. Yki/YAP bound to Sd/TEAD recruits cofactors and chromatin remodeling complexes to activate transcription of its target genes, which promote cell proliferation and inhibit apoptosis. Overexpression of Yki/YAP, or inactivation of the Hippo pathway, induces tissue overgrowth both in *Drosophila* and mouse models. Studies of mouse models and clinical samples have now demonstrated the significance of Hippo pathway for the development of human cancers.

Our lab is interested in the mechanism of Alternative Splicing (AS), that allows producing multiple mRNAs from a single gene, by modulating inclusion or exclusion of alternative exons for example, therefore encoding different protein isoforms. By studying the function of the RNA binding protein B52 in *Drosophila*, a splicing factor of the SR protein family, the lab previously showed that overexpression of B52 increases cell growth, whereas B52 depletion reduces it. By the use of RNAseq data, they identified several AS events modulated by B52 level in genes involved in cell growth, including several genes linked to the Hippo pathway and especially *yki*.

They observed that depletion of the B52 splicing factor favors skipping of *yki* exon 3, which encodes one of the two WW domains of Yki protein. Thus, Yki exists as two isoforms containing one or two WW domain(s), respectively called Yki1 and Yki2. WW domains are



protein-protein interaction modules that bind proline-rich motifs (PPxY) present in several proteins of the pathway such as the kinase Warts, the cytoskeleton-associated protein Expanded (Ex) and the transcription co-activator Wbp2, as examples. Therefore, modulating the inclusion/exclusion of one WW domain could change Yki phosphorylation, localization or co-activator activity. The expression and function of the short Yki1 isoform has never been described. Remarkably a similar AS event exists in the human *YAP* homolog: AS of exon 4 gives rise to two isoforms, YAP1 and YAP2, which respectively contain one or two WW domain(s). Therefore, alternative inclusion of the second WW domain is a conserved feature between Yki and YAP. Nevertheless, the signaling differences among YAP splicing variants remain to be elucidated, as is the regulation of *YAP* AS.

The objectives of my Ph.D. were to investigate and reveal the role of B52-triggered *yki* AS and to determine if this AS constitutes a new level of modulation of Hippo pathway in *Drosophila*. Our results show that B52 depletion favors expression of the Yki1 isoform carrying a single WW domain, and reduces growth in part through modulation of *yki* AS. Compared to the canonical Yki2 isoform containing two WW domains, Yki1 has reduced transcriptional and growth-promoting activities, decreased binding to PPxY motifs-containing proteins, and an inability to bridge two proteins containing PPxY motifs. Nevertheless, Yki1 and Yki2 interact similarly with transcription factors and thus compete in vivo. Flies in which the *yki* AS has been abrogated, thereby expressing only the Yki2 isoform, exhibit increased fluctuating wing asymmetry, a signal of increased developmental noise. These results show that *yki* AS represents an additional layer of modulation of Yki activity that unexpectedly participates in buffering developmental noise, and provide the first experimental evidence that AS participates in developmental robustness.

I hereby introduce the process of splicing, key components that facilitate splicing and alternative splicing in general introduction. In a second part I cover in my introduction the Hippo pathway and its key components in *Drosophila* and mammals, starting from its discovery to most recently described key processes that regulate the pathway. The results obtained during in the thesis are presented in the form of pre manuscript followed by a discussion

# INTRODUCTION

## 1.0 INTRODUCTION

### 1.1 The Expansion of proteome: Single gene, multiple mRNA isoforms

Humans have approximately 21000 protein-coding genes while *Caenorhabditis elegans* with much simpler physiology has a genome of 19 000 protein-coding genes and at the same time *Oryza sativa Japonica*, commonly known as rice contains approximately 35,825 protein-coding genes. For decades researchers were puzzled by the fact that the complexity of the organism does not correlate to the number of protein-coding genes they contain. This question, however, was answered by the discovery of how common and abundant post-transcriptional modifications are.

Once messenger RNA (mRNA) starts to be transcribed from DNA, the primary transcript (pre-mRNA) is generated. To be successfully exported out of the nucleus, this pre-mRNA would undergo a series of processing events, such as 5' capping, splicing, transcription termination and 3' polyadenylation (Matlin, Clark, & Smith, 2005). During splicing, some regions of the pre-mRNA are removed (i.e., introns), and stretches of sequence that contain the necessary information for protein synthesis (i.e., exons) are ligated together. Alternative splicing is the phenomenon where different combinations of sequences could be included or excluded in the final transcript leading to generation of structurally and functionally different mRNA variants (Nilsen & Graveley, 2010). High frequencies of alternative splicing, post-translational modifications even leads to increased protein diversity (Wilhelm et al., 2014).

#### 1.1.1 Transcription: Where it all begins

In order to express the encoded information contained in the double-stranded molecule made of deoxyribonucleic acid (DNA), several key steps have to be completed. The genome contains this information in genes that code for proteins which in turn control nearly all functional aspects of a cell. Among the diversity of identified RNA species, messenger RNAs (mRNAs) are regarded as those that contain the necessary information for the synthesis of proteins.

During transcription, genes (stretches of DNA) are used as templates for the synthesis of complementary single-stranded RNA molecules (i.e., transcripts). In eukaryotic cells, based on the type of gene being targeted, such reaction can be catalyzed by three different enzymes RNA polymerases (I, II and III). RNA polymerase II is responsible for the synthesis of RNAs derived from the majority of genes, including those that encode for proteins. While, RNA

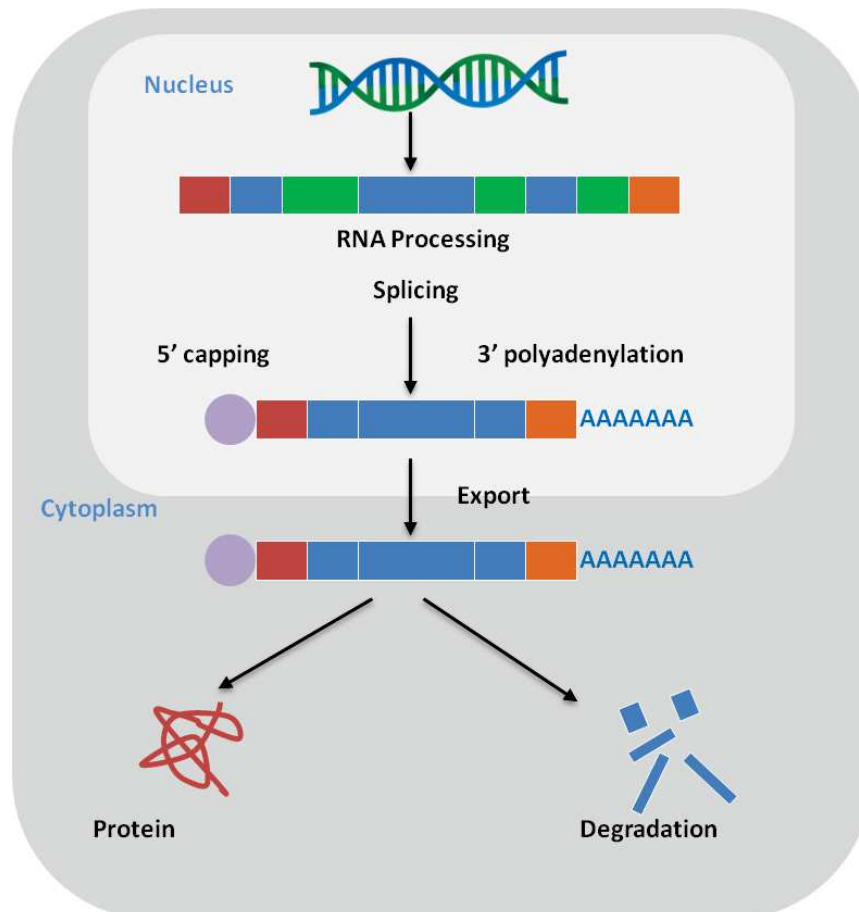
polymerase I and III are specifically involved in the transcription of ribosomal RNAs (rRNAs), transfer RNAs (tRNAs) and several small RNAs (Paule & White, 2000).

Transcription by RNA pol II starts with the binding of several transcription factors to a regulatory region located upstream of the gene, known as the promoter (Fuda, Ardehali, & Lis, 2009). The transcription factors enable the subsequent assembly of the polymerase and the formation of the transcription initiation complex. After the assembly steps and further conformational rearrangements, RNA pol II releases from the large complex and enters the elongation phase (Kwak & Lis, 2013). During elongation, RNA is synthesized from the transcription start site (TSS), and nucleotides are incorporated in a complimentary basis in the 5' to 3' direction. Eventually, the polymerase transcribes through the cleavage and polyadenylation signals that mark the end of the gene, and it is released from the DNA template (Kuehner, Pearson, & Moore, 2011)

### **1.1.2 A short overview of mRNA processing:**

All mRNA molecules undergo several modifications before they are exported to the cytosol, which includes the addition of a 5' cap, the polyadenylation of the 3' end and the removal of introns via splicing. The carboxyl-terminal domain (CTD) of the RNA polymerase II is involved in the cap formation by recruiting the capping enzymes (triphosphatase, guanylyltransferase and methyltransferase). During 5'capping a chemical group is added to the 5'end of the pre-mRNA. 5'cap is the addition of a modified guanine (5' guanine-N7 cap) nucleotide which is subsequently methylated, and it functions to prevent the novel transcript from degradation (Mandal et al., 2004). Reviewed by (Martinez-Rucobo et al., 2015).

Splicing is a much more complex reaction. During this process, introns are removed, and exons are ligated together. As a result, a mature mRNA product is obtained. Many proteins are involved in the splicing process, including small ribonucleoproteins (snRNPs), heterogeneous nuclear ribonucleoproteins (hnRNPs) and other additional proteins. (Matlin et al., 2005). To finalize the processing, the 3' end of the pre-mRNA is first cleaved at a specific site. Sites of cleavage are encoded in the DNA sequence of the gene, and for the vast majority it is between the highly conserved polyadenylation signal (PAS) AAUAAA and a downstream sequence element (DSE), usually U or GU-rich (Proudfoot, Furger, & Dye, 2002) and then polyAdenosine (polyA) tail is added, which is typically 200-250 nucleotides long in mammalian cells. (Figure1).



**Figure 1 Regulation of eukaryotic gene expression:**

mRNA expression starts with the nuclear transcription of genes. Following several processing steps, some of which occur co-transcriptionally, the transcription products are further transformed into mature mRNAs that can then be exported to the cytosol and localized to sub-cellular compartments. mRNA export is linked to strict quality control mechanisms unprocessed RNAs degraded. Once in the cytosol, mRNAs can be recognized by ribosomes and translated into proteins or will be degraded.

Initially, mRNA processing was thought to happen post-transcriptionally. However, in reality, transcription and processing are not consecutive, but simultaneous and interdependent (Neugebauer, 2002) and reviewed in (Bentley, 2014). The 3' poly(A) tail prevents the degradation by 3'-to-5' exoribonucleases, facilitates the transfer from the nucleus to the cytoplasm and promotes mRNA translation. This transfer, however, depends on the correct processing and successful detection of CBC (cap binding complex) which is bound to 5' cap of the pre-mRNA. Detection of CBC facilitates mRNAs transfer via nuclear pore (J. D. Lewis & Izaurralde, 1997).

### 1.1.3 Splicing Sites:

The process of splicing is complex, in eukaryotes it is carried by a large complex of proteins and RNAs and together they do the intricate job of removal of introns and stitching of exon together. This large complex is aptly termed as spliceosome, and it is also one of the most complicated machinery within the cell (Nilsen, 2003). Recognition of and removal of introns

by spliceosome is facilitated by the presence of specific sequence elements within the introns and at exon-Intron boundaries. The 5' and 3' splice sites of an intron are specific and very crucial recognition sequences that are recognized by the components of splicing machinery. These sequences extend a few nucleotides into the flanking exon.

There are two types of introns in metazoan pre-mRNA. They are classified as minor introns also known as U12 type or major class of introns also known as U2 type. The U2 and U12 introns differ by the presence of characteristic splice site sequence, and thus they are spliced by a different spliceosomal complex.

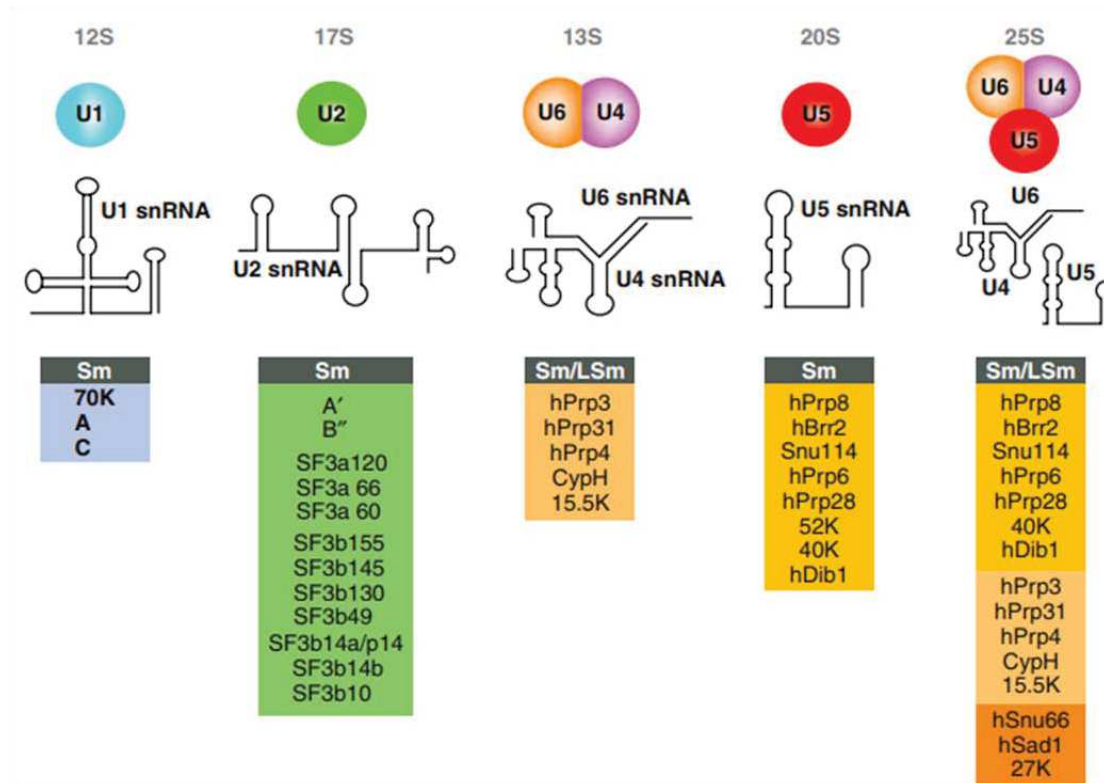
U2 type introns form the majority of the introns present in metazoans, and they contain the canonical GT-AG intron boundaries. It must be noted that very few U2 introns also contain GC-AG boundaries. However, GC-AG introns represent only 0.82% of U2-type introns in humans or 0.45% of U2 type in *Drosophila melanogaster*. Overall U2 type introns account for 99% of all the introns present in humans, *Drosophila melanogaster*.

Minor class or U12 type introns are so rare that they amount to mere 0.4% of total human introns (Sheth et al., 2006) while *Drosophila melanogaster* contains only 19 U12 type introns (C. F. Lin, Mount, Jarmoowski, & Makaowski, 2010).

In humans, the majority of the Introns are U2 type, in these introns, the 5' splice sites or the splice donor site is defined by a nine nucleotide (nt) consensus sequence, YAG/GURAGU (where Y is a pyrimidine, R is a purine. Even though the consensus exists, splice site sequences are degenerate. The 3' splice site is comprised of three elements, placed within around 40 nucleotides upstream the intron/exon junction. The 3' splice site itself has a consensus YAG/N, where Y is a pyrimidine (uracil or cytosine). The spliceosome also recognizes another sequence motif called Branch Point Sequence (BPS) which is located at 20-40 nt upstream of the 3' splice site. The BPS YNYURAY (where Y is C or U nucleotide) contains essential adenosine that is required for the first nucleophilic attack of the splicing reaction (Reed, 1996). BPS is followed by a polypyrimidine tract (PPT), a pyrimidine-rich motif which is a string of uracil bases is crucial for efficient BPS utilization and selection — reviewed in (Valadkhan, 2007a) (Figure 2). On the other hand, U12 type introns so not contain polypyrimidine tract upstream of 3' splice site. Reviewed in (Sheth et al., 2006).







**Figure 3 Protein composition and snRNA secondary structures of the major human spliceosomal snRNPs:**

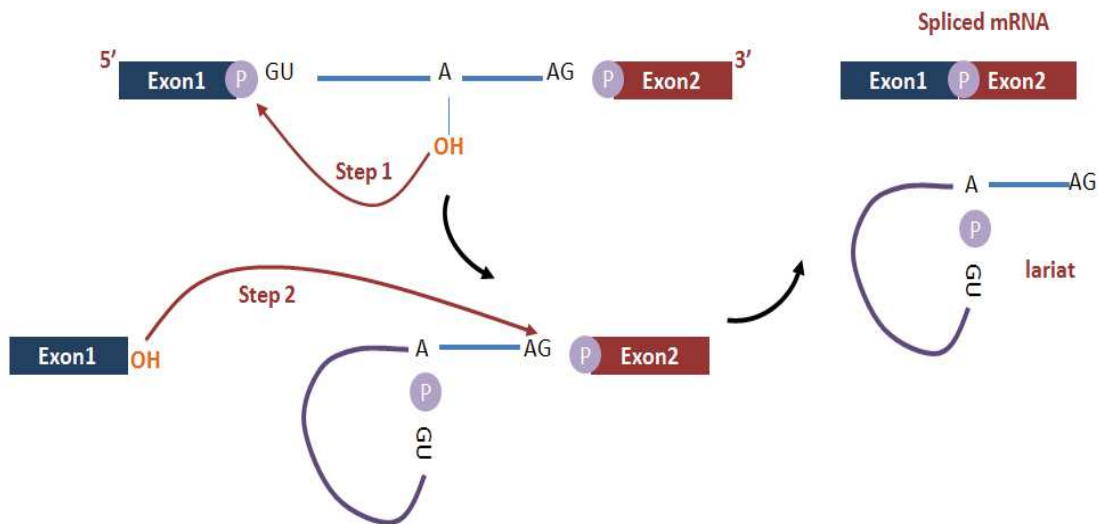
All seven Sm proteins (B/B', D3, D2, D1, E, F, and G) or LSm proteins (Lsm2-8) are indicated by "Sm" or "LSm" at the top of the boxes showing the proteins associated with each snRNP. The U4/U6.U5 tri-snRNP contains two sets of Sm proteins and one set of LSm proteins Adapted from (Will & Lührmann, 2011)

Such snRNPs form the core of the spliceosome and are directly involved in recognition of splice sites and branch-point sequences, as well as the catalysis of the splicing reaction. (Will & Lührmann, 2011). The minor spliceosome is functionally analogous to the major spliceosome but differs in the use of snRNAs (minor snRNAs are U11, U12, U4atac/U6atac, and U5). There are many RNA binding proteins (RBPs) in the dynamic structure of the spliceosome that helps in splicing (Matlin & Moore, 2010).

### 1.1.5 Two key steps of splicing:

Pre-mRNA splicing is achieved by two consecutive trans-esterification reactions which are based on nucleophilic attacks between RNA nucleotides. In the very first reaction, 5' exon is cleaved from the intron through a nucleophilic attack of the 2' hydroxyl group of an adenosine nucleotide in the branch point sequence on the phosphate group of the GU dinucleotide at the 5' splice site, resulting in the formation of a lariat intermediate. In the second step, the free 3' hydroxyl group at 5'ss makes the second nucleophilic attack on the phosphodiester bond at 3'ss (3' exon-intron junction) (Figure 4). This results in ligation of the two exons and release of excised intron as a lariat. Reviewed in (Valadkhan & Jaladat, 2010) and (Will & Lührmann, 2011).





**Figure 4 The two steps of splicing catalysis:**

During the first step, the 5'ss and branch adenosine are joined by a 2'-5' nucleophilic attack, leading to the formation of a lariat structure and a free upstream exon. The second step consists of the 3'-5' nucleophilic bond between the free exon and the 3'ss. Adapted from (Scotti & Swanson, 2016)

### 1.1.6 Assembly of Spliceosome and catalysis of splicing reaction:

During the course of the splicing reaction and before the actual intron can be removed, the active catalytic site of the spliceosome needs to be created, an event that requires many changes in its composition and conformation. Since spliceosome is both highly dynamic and flexible, it assembles and disassembles tediously to facilitate each splicing event. It undergoes both radical structural and compositional changes at every step of its assembly. The assembly of spliceosome has been studied extensively in vitro. Based on biochemical methods, six different complexes can be distinguished: the E, A, B, Bact, B\*, and C complex. Reviewed in (Shi, 2017).

RNA helicases from the DExD/H-box family (composed of the DEAD-box, DEAH-box, and Ski2-like helicases) facilitate the extensive structural and compositional remodeling of spliceosome assembly and transition to various steps (Cordin, Hahn, & Beggs, 2012). Cyclophilins, a subfamily of peptidyl-prolyl *cis-trans* isomerases (PPIases), facilitate conformational changes within the spliceosome. Reviewed in (Thapar, 2015). Hereby, the major spliceosome catalyzes the splicing in the following steps (Figure 5).

**Step1:** The crucial first step in the assembly of the major spliceosome is the association of U1 snRNP with the 5'ss and U2 snRNP with 3' ss via formation of protein dimer. This results in the formation of the commitment complex; also referred to as "E complex."

**Step2:** U2 snRNP then recognizes branch point sequence followed by interaction with U1 snRNP leading to the formation of pre-spliceosome. This complex made by the interaction between U1 snRNP and U2 snRNP spans across the exons and brings together the 5' and 3' splice sites and BPS in close vicinity. Thus this pre-spliceosome Complex A is also referred to as “Exon definition complex.”

**Step3:** U4/U6.U5 is a large pre-assembled tri-snRNP spliceosomal complex. It contains U5 snRNA, extensively base-paired U4/U6 snRNAs in addition to over 30 proteins. Recruitment of this tri-snRNA to complex A results into formation of Complex B.

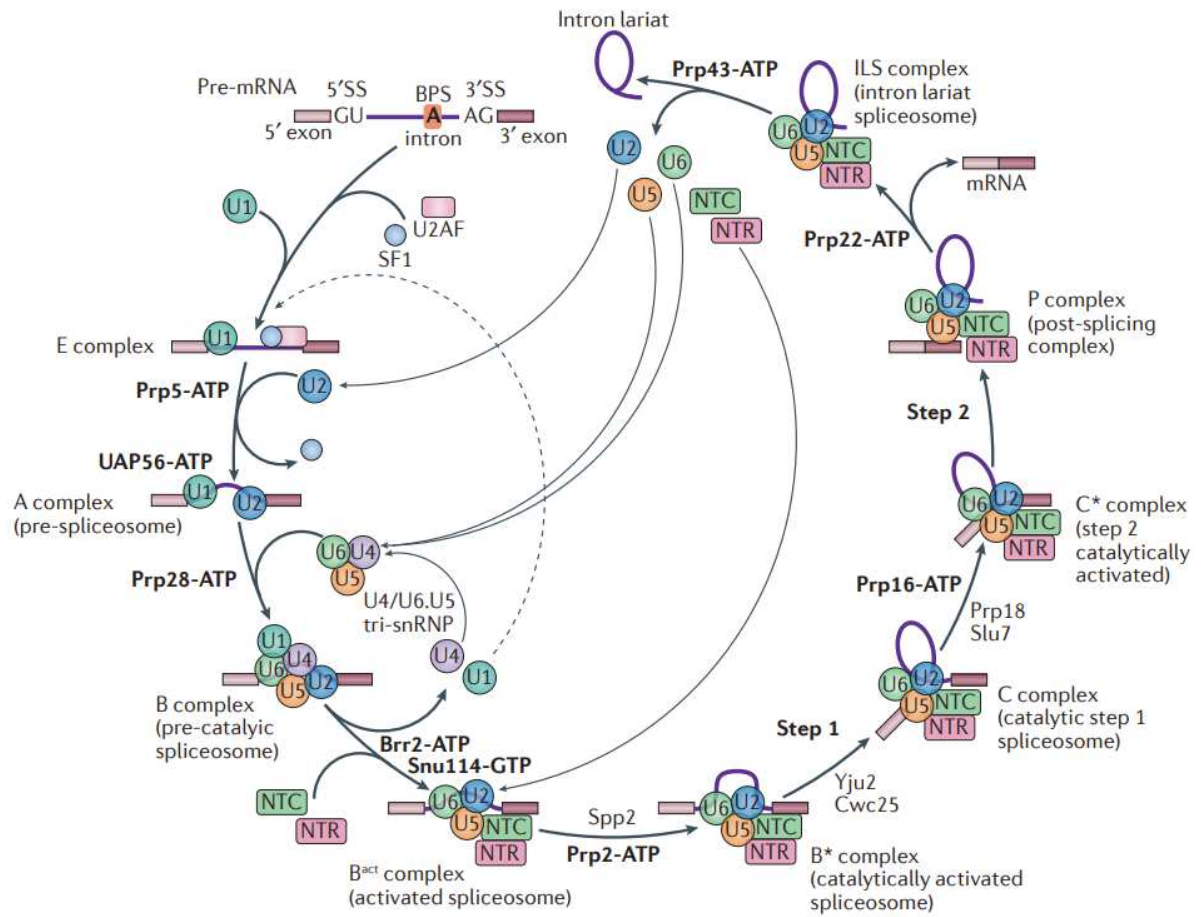
**Step4:** The complex B undergoes a series of conformational and compositional rearrangements resulting in a catalytically active complex called complex B<sub>act</sub> or complex B\*. Several remodeling of protein and RNA-RNA interactions facilitate the formation of U2-U6 snRNA structure that brings the 5' splice site and BPS in close proximity and forms the catalytic core. The activation of complex B leads to unwinding of U4 and U6 snRNA and expulsion of U4 and U1 from the complex.

**Step5:** The activated B complex (B<sub>act</sub>) catalyzes the first step of splicing. This leads to the formation of complex C, which contains Intron-lariat intermediate.

**Step6:** Complex C catalyzes the second step of splicing after it undergoes further conformational rearrangements.

**Step7:** After this second transesterification reaction, the ligated exons and a lariat intron are released. The intron lariat structure is degraded. This is followed by the release of U2, U5 and U6 snRNP and which are recycled for further rounds of splicing.

However, the mRNA is just not yet free in the nucleus. A complex of proteins is then deposited to the new exon junction (thus termed as exon junction complex, EJC). This marks the completion of splicing and allows the mRNP particle to travel to the cytoplasm. Reviewed in (Black, 2003) (Valadkhan, 2007b) (Wahl et al., 2009) (Will & Lührmann, 2011).



**Figure 5 Splicing assembly:** Schematic representing the key steps of assembly and activation of the yeast spliceosome and the complete splicing-reaction cycle. Adapted from (Shi, 2017).

## 1.2 Alternative Splicing: *Harbinger of complexity!*

Constitutive splicing refers to the type of splicing in which introns are systematically removed, and the exons are ligated together to generate a final mRNA. However, splicing is also regulated *alternatively*, i.e., particular exon might be entirely or partially spliced out, or introns might be retained, or the splicing machinery could make the choice of the different 5' or 3' splice sites. Such processing of pre mRNA transcript which results in the inclusion of different part of a transcript into the final mRNA product is called Alternative splicing (AS). It leads to the formation of alternative mRNA products from a given gene locus. According to (Q. Pan, Shai, Lee, Frey, & Blencowe, 2008) (E. T. Wang et al., 2008), almost 95% of multiexonic genes in humans are regulated by produce several RNAs by alternative splicing. Interestingly, proteins resulting from splicing variants often have distinct molecular functions. AS can result in the generation of isoforms of proteins with different biological function, structure, localization and interaction capabilities (Keren, Lev-Maor, & Ast, 2010) (Nilsen & Graveley, 2010). For example, the two variants of *survivin* have opposite functions: one has pro-apoptotic while the other has anti-apoptotic properties (Végran et al., 2007). It has been estimated that on average, each gene generates around ten mRNA isoforms (Z. Hu et al., 2015). Around 40% of *Drosophila* genes contain one or more alternative exon (Q. Pan et al., 2008)

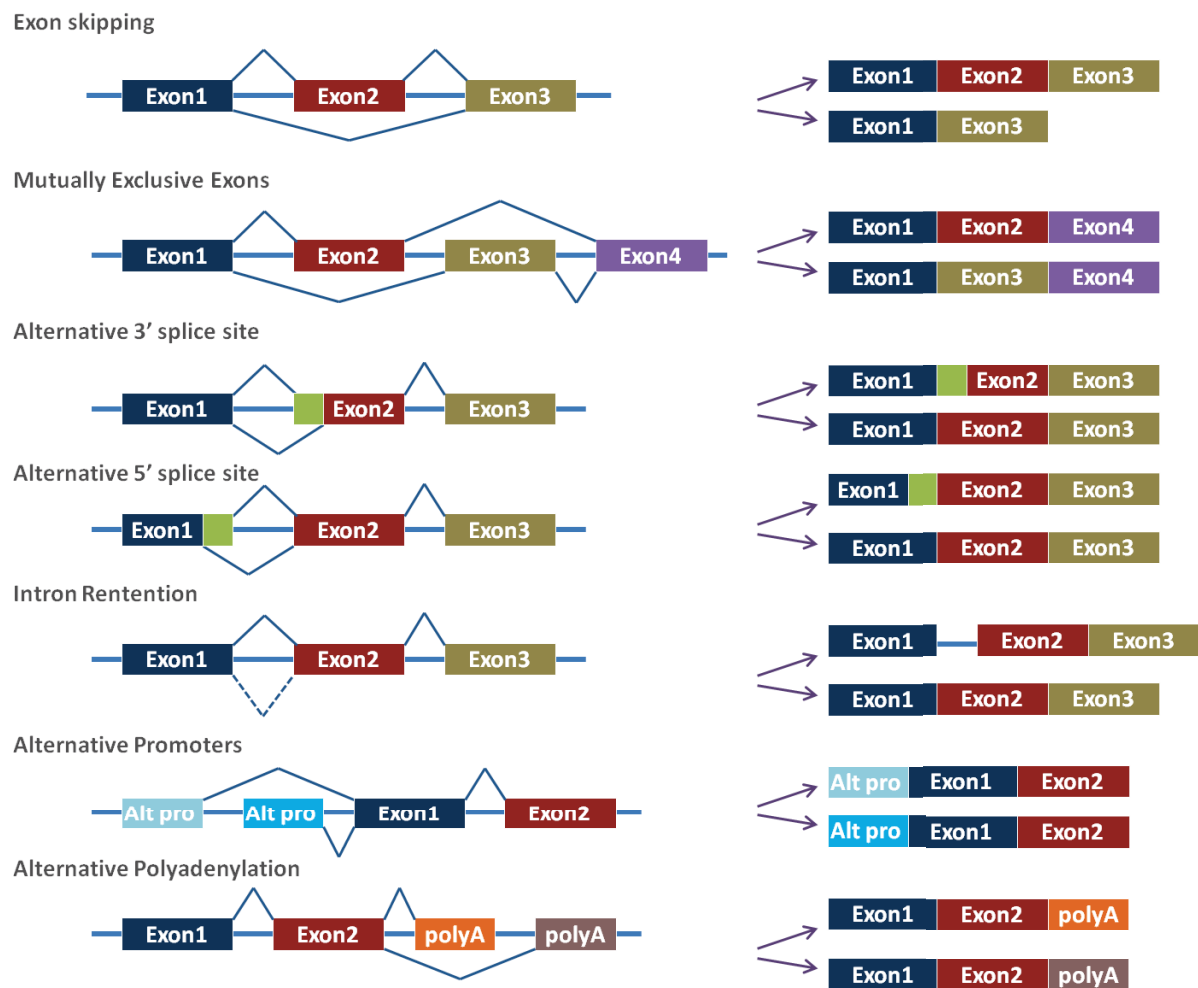
Alternative Splicing plays a crucial role in the expansion of the coding capacity of eukaryotic genomes by giving rise to several structurally and functionally different protein isoforms from a single gene locus. Thus, it fills the gap between the total number of protein-coding genes (< 20,000) (Ezkurdia et al., 2014) compared to the overall number of proteins (> 100,000) and also imparts to the complexity of the organism (Nilsen & Graveley, 2010).. In unicellular cells, alternative splicing is absent or very rare, and one gene provides one protein product (Ast, 2004).

One of the extreme examples of such expansion of protein-coding capacity via alternative splicing is *dscam* gene in *Drosophila*. It can produce a whopping 38,016 different mRNA isoforms via alternative splicing in four different regions of its pre-mRNA (Black, 2000) (Graveley, 2001)(Schmucker et al., 2000).

### 1.2.1 Types of Alternative Splicing:

In eukaryotes, alternative splicing can result via employing one of the following mechanisms: exon skipping or inclusion, choice of alternative 3' or 5' splices site selection, intron retention, the inclusion of mutually exclusive exon and alternative polyadenylation (J. Chen

& Weiss, 2015). The most common type of alternative splicing is a cassette type alternative exon, i.e., Exon skipping in vertebrates and invertebrates. While in lower metazoans Intron retention is more frequent (Figure 6). Which splicing event takes place is often determined by the contributions and activity of the different splicing activators or repressors in the tissue or during a developmental stage.



**Figure 6 Seven types of alternative splicing:** (From Top to bottom) Seven types of alternative splicing. Exon skipping, Mutually exclusive exons, Alternative 3' splicing, Alternative 5' splicing, Intron retention, choice of an alternative promoter, Alternative Polyadenylation. Intron retention is the major alternative splicing event in rice, whereas exon skipping is the most frequent alternative splicing in humans.

Alternative splicing has a central regulatory role in the gene expression pathway. It dictates several biological functions through the entire lifespan of an organism. It has been shown that the higher eukaryotes display the higher proportion of alternatively spliced genes indicating that alternative splicing is an indispensable feature of genomic evolution. Several splicing events are conserved among different species along with many splicing variants which are

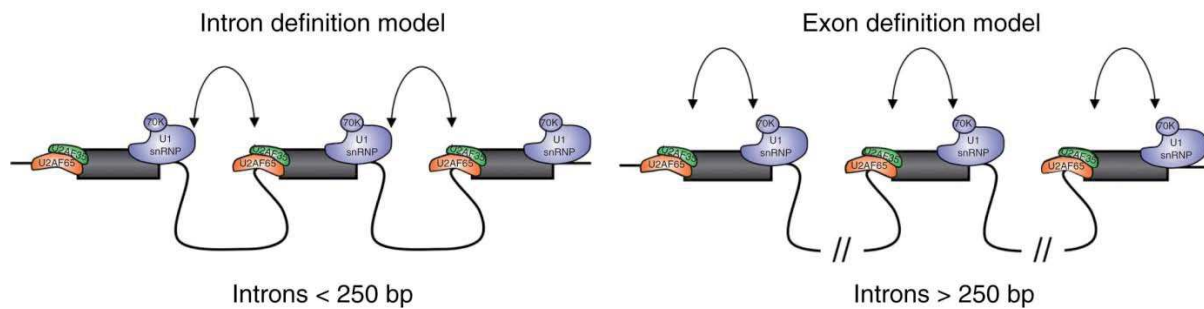
species-specific indicating that alternative splicing plays a significant role in species differentiation along with genomic evolution.

### **1.3 Regulators of Alternative Splicing:**

#### **1.3.1 Exon and Intron definition:**

The spliceosome directs both constitutive and alternating splicing with high fidelity. The average exon size is small in higher eukaryotes (approx 170nt on an average) while Introns could be up to tens of thousands of nucleotides long and harbor several “cryptic splice” sites. It is, therefore, a huge task for spliceosome to identify *bona fide* sites from the *pseudo* or cryptic ones. An understanding of how spliceosome accomplishes such task comes from the following models.

The **exon definition** model postulates that that exon, rather than introns are the basic unit of recognition (Exon definition may facilitate splice site selection in RNAs with multiple exons), and the spliceosome assembles across the exon. In contrast, in lower eukaryotes such as yeast and fly where the introns are much smaller, early spliceosome assembly is centered around the introns, which is referred to as “**intron definition**” (Talerico & Berget, 1994). The intron definition model postulates that components of the spliceosome assemble and interact across small introns, where the 5’ and 3’ splice sites are close to each other. Transfection splicing assays have shown that as intron length rises above 250 nucleotides, splicing becomes quite inefficient (Fox-Walsh et al., 2005). In intron definition, the spliceosome recognizes the splice sites across the intron, provided that this intron is not too large (e.g., <200-250 nt) and beyond this length exon definition prevails (Fox-Walsh et al., 2005) (Figure 7). Furthermore, the authors of this report found that the inclusion of an RNA *cis*-element known as an exonic splice enhancer (ESE) could dramatically increase splicing efficiency of even longer introns. While, on the other hand, Intron definition mediated splicing is an exception in mammals, it is a rule in plants, fungi, and invertebrates (Talerico & Berget, 1994) (X. Xiao, Wang, Jang, & Burge, 2007).

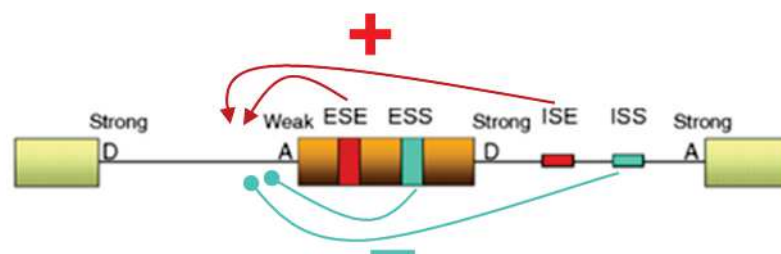


**Figure 7 Exon and Intron definition models:**

The left panel depicts the Intron definition model according to which pairing between the splice sites takes place across an intron when long exons are separated by short (<250 bp) introns. On the other hand, the right panel shows the Exon definition model where the splice site communication occurs across exons when they are separated by long (>250 bp) introns. Adapted from (De Conti, Baralle, & Buratti, 2013)

### 1.3.2 Cis-acting elements regulating Alternative splicing:

The mammalian junctions that define an exon are weakly conserved and more degenerate with respect to yeast canonical *cis*-elements. These elements are necessary but not sufficient to define exon/intron junctions. Internal exonic sequences far from the 5' and 3' splice sites were essential for exon recognition. These additional elements are called splicing regulatory elements (SREs), *cis*-acting elements which are present in the pre-mRNA and varies in terms of location and effect. They are known as ESE (exonic splicing enhancers), ESS (exonic splicing silencers), ISE (intronic splicing enhancers) and ISS (intronic splicing silencers). In general, SREs recruit trans-acting splicing factors that can act as repressor or activators of splicing. As described earlier, Introns possess several sequence elements required for pre-mRNA splicing: 5' and 3' splice sites, BP and PPT. These splice sites are often termed as “weak” or “strong” splice sites. Splice site strength depends on the complementarity between splice site sequences and U1 and U2 snRNPs binding to them. The more the degree of similarity the more the strength of a splice site (Figure 8) (Roca, Sachidanandam, & Krainer, 2005). (De Conti, Baralle, & Buratti, 2013)



**Figure 8 Splicing regulatory elements:**

SREs can enhance and repress the splice site selection by the spliceosome. Splicing is governed by *cis*-regulatory sequences in the pre-mRNA; exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs)



Generally, constitutively spliced exons possess stronger splice sites whereas alternatively spliced exons have weak splice sites; thus these splice sites are recognized less efficiently (Hertel, 2008). It has been shown that in general, splicing rate is modulated by the strength of splice sites and presence or absence of splicing enhancers (Hertel & Maniatis, 1998)(Erkelenz et al., 2013)

In addition to strength, the relative position of splice sites also affects the splicing outcome. Closer splice sites are both favored and paired quicker (Nogués, Muñoz, & Kornblihtt, 2003). However, often very close splice sites prevent the neighbor exons from being spliced together favoring mutually exclusive exon splicing (Smith & Nadal-Ginard, 1989).

The SREs also regulate alternative splicing in addition to constitutive splicing by specifying the constitutively or alternatively spliced exon.

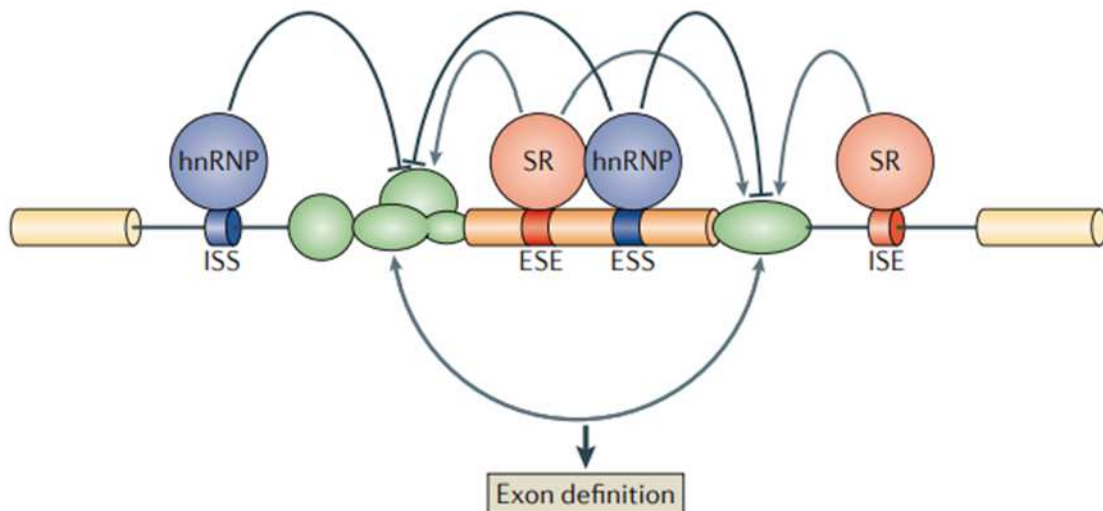
### **1.3.3 Trans-acting factors regulating Alternative splicing**

SREs recruit *trans*-acting proteins which can either activate splicing or repress it. The best-known splicing factors are the proteins belonging to the SR (serine-arginine rich) and hnRNP (heterogeneous nuclear ribonucleoproteins) protein families. Proteins belonging to SR protein family are known to enhance splicing. SR proteins have one or two RRM (RNA recognition motif) protein domains and an RS (arginine-serine rich) domain. The RRM domain mediates SR protein binding with intronic splicing enhancers (ISEs) and Exonic splicing enhancers (ESEs) on the RNA, while RS domains promote spliceosome assembly and activate the splicing. In addition to the SR protein family, there are other SR-related proteins, that also activate splicing, e.g. these include Tra2 $\alpha$  and Tra2 $\beta$  proteins (Roland Tacke, Tohyama, Ogawa, & Manley, 1998). The mechanism by which splicing activation can happen is by splicing regulator proteins that can interact with and stabilize the binding of U1 snRNP or other spliceosomal components on pre-mRNA that initiate splicing. As another possibility, splicing activators can interact with other splicing factors (like TIA proteins), and indirectly stimulate their binding to U1 snRNP and splicing. Splicing can also be activated when regulatory proteins interfere with intronic or exonic silencer elements dependent (Witten & Ule, 2011).

On the other hand, Exonic splicing silencers (ESSs) and intronic splicing silencers (ISSs) are repressors of splicing and are recognized by hnRNPs. One proposed mechanism through which hnRNPs repress splicing is to prevent the assembly of general splicing factors and SR proteins. In particular, protein multimerization along an exon is initiated by the binding of an hnRNP to a strong ESS that subsequently recruits more hnRNPs to adjacent weak ESSs



(Martinez-Contreras et al., 2007) (Zhu, Mayeda, & Krainer, 2001). Alternatively, hnRNPs can also repress splicing by looping out entire exons blocking the recruitment snRNPs (Nasim, Hutchison, Cordeau, & Chabot, 2002) (Damgaard, Tange, & Kjems, 2002). Initially identified as positive and negative regulators of splicing, respectively, it is now known that their effect on a particular splicing event is heavily context-dependent (Witten & Ule, 2011).



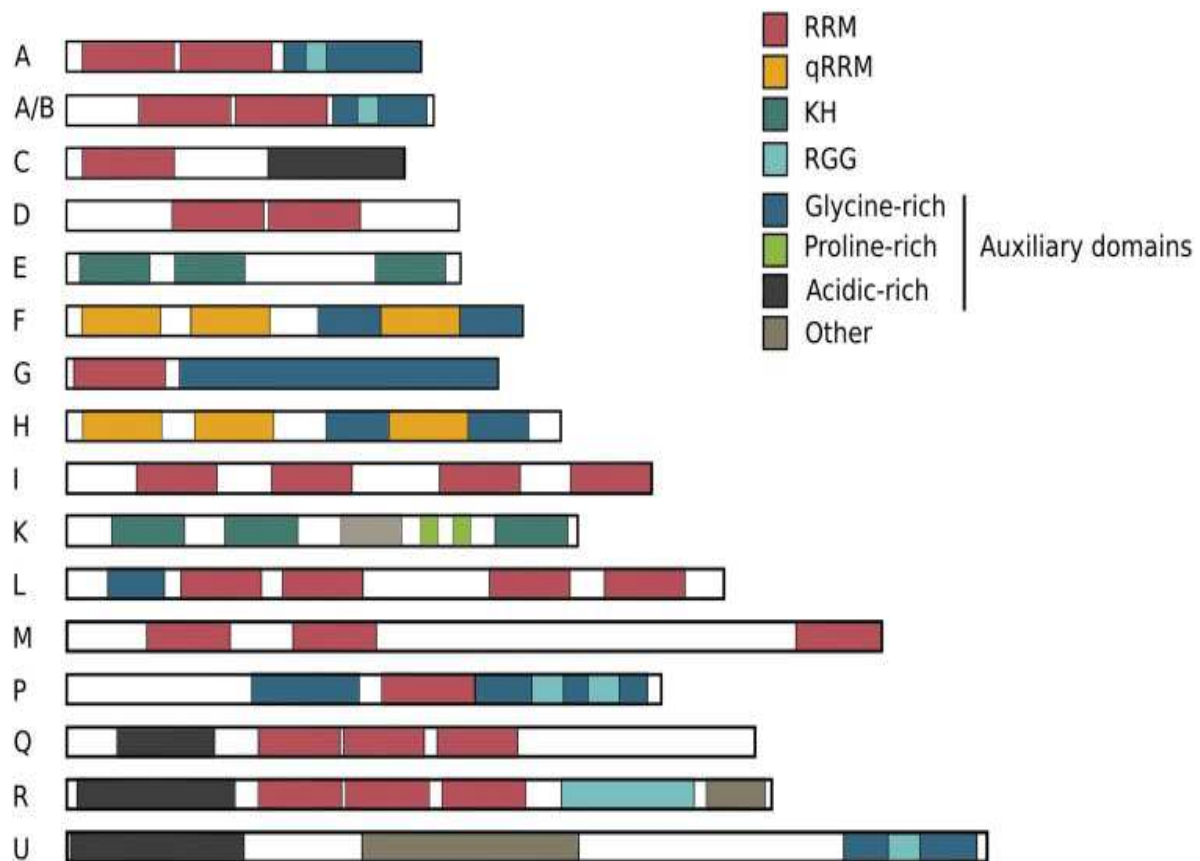
**Figure 9 SR proteins and hnRNPs:**

These are two major families of alternative splicing regulatory proteins, which are recruited by splicing enhancers and silencers. These regulatory proteins target components of the spliceosome (shown in green) that associate with both the 5' and the 3' splice sites flanking the alternative exon and can have either activating or inhibitory effects on the recognition and use of that site. In addition, interactions among components of the spliceosome that are recruited to the 3' and 5' splice sites can mediate exon definition. Adapted from (Kornblihtt et al., 2013)

### 1.3.3.1 Heterogeneous ribonuclear proteins (hnRNPs)

HnRNPs represent a large family of RBPs that contribute to multiple aspects during mRNA processing post-transcription. A typical hnRNP usually contain one or more RNA binding domains (RNA recognition motif, i.e., RRM domain), a qRRM (quasi-RNA recognition motif), a glycine-rich domain constituting an RGG box (Arg-Gly-Gly repeats) and a K-homology (KH) domain (Geuens, Bouhy, & Timmerman, 2016).

The hnRNP protein family consists of at least 20 proteins in humans that have been characterized as components of protein complexes bound to pre-mRNA (hnRNP complexes) (Dreyfuss, 1993) (Cartegni, Chew, & Krainer, 2002) (Figure 10). The molecular weight of the hnRNP family ranges from 34 to 120 kDa, and they have been named alphabetically from A to U. (Piñol-Roma & Dreyfuss, 1992).



**Figure 10 The hnRNP family:**

The hnRNPs are named alphabetically from hnRNP A1 to hnRNP U. The members of the hnRNP family are built up of four unique RNA-binding domains (RBDs): *RRM* RNA recognition motif, *qRRM* quasi-RNA recognition motif, and *KH* K-homology domain, *RGG* RNA-binding domain consisting of Arg-Gly-Gly repeats. Image adapted from : (Geuens et al., 2016)

Although hnRNPs are expressed in all tissues, but the relative amount of different hnRNPs varies among cell types and exhibit stage-specific expression patterns, for an example, some hnRNPs are extremely abundant (~100 million copies per nucleus), while others are present in a lower amount. Together, hnRNPs are similar in abundance to histones in growing cells (Dreyfuss, 1993) (Kamma, Portman, & Dreyfuss, 1995). The function of hnRNPs depends upon their localization and upon undergoing PTMs such as methylation, phosphorylation, ubiquitination and sumoylation they can change their subcellular localization and thus function (S. P. Han, Tang, & Smith, 2010) (Chaudhury, Chander, & Howe, 2010)

hnRNPs play an essential role in both constitutive and alternative splicing. hnRNPs generally repress splicing through binding to the silencer sequences (ESSs or ISSs) in the pre-mRNA and suppress splicing.

Studies have uncovered a variety of ways in which hnRNPs can modulate splicing. Mechanisms that repress exon usage involve binding that competes with the recruitment of positive splicing factors (for an example SR protein), as well as the inhibition of protein

interactions involved in exon or intron definition. This has been extensively demonstrated for hnRNPA1, A2/B1, and hnRNP. A multitude of biochemical studies too have demonstrated that hnRNP binding can promote interactions that facilitate exon inclusion, highlighting their potential to positively or negatively regulate exon usage (Martinez-Contreras et al., 2007). It appears that the ability to regulate exon usage both positively and negatively is a general feature of hnRNPs. (Huelga et al., 2012) reported that hnRNPs from diverse families can positively and negatively regulate thousands of exons by binding to proximal introns by using splicing-sensitive microarrays coupled with CLIP. They reported that hnRNPs are similar and cooperative in the roles that they play in the the regulation of AS and their RNA targets overlap. The negative of positive regulation of AS could be attributed to the binding of hnRNP in different positions relative to the exon, a phenomenon already reported for SR and others RBPs (Witten & Ule, 2011). Position-dependent AS regulation by hnRNPs could be via looping out the intervening RNA (Blanchette & Chabot, 1999). Indeed it has been shown that hnRNP A1 and hnRNP H collaborate to create a RNA loop so as to repress the internal splice sites and while activating external splice sites which are brought in a closer proximity. The authors used bioluminescence resonance energy transfer (BRET) technology to show the homotypic and heterotypic interactions between these two hnRNPs in live cells (Fisette, Toutant, Dugré-Brisson, Desgroseillers, & Chabot, 2010). The binding on opposite sides of an intron represses exon usage while looping events within an intron can bring together pairs of splice sites to promote spliceosomal interactions across an intron (Chabot, 2015).

Besides splicing family of hnRNPs have been thoroughly documented to play several roles such as HnRNP A1 and A2/B1 play a role oligodendrocytic and neuronal mRNA trafficking (Shan, Munro, Barbarese, Carson, & Smith, 2003). (Villarroya-Beltri et al., 2013) have shown that hnRNPA2B1 specifically binds miRNA-198 and miRNA-601 and enable their loading in exosome. HnRNP C too has been shown to play a role in sorting of transcripts according to their size (McCloskey, Taniguchi, Shinmyozu, & Ohno, 2012). (L. Y. Chen & Lingner, 2012) showed that hnRNP D plays a role in telomere maintenance via stimulating the transcription of telomerase reverse transcriptase (TERT) gene. Further, hnRNP I have been shown to regulate the neonatal immune response in studies conducted in intestinal epithelial cells in mouse and helps in preventing colitis and onset of colorectal cancer (Z. Jin, Liang, Yang, & Mei, 2017). Likewise, hnRNP-L has been shown to be critical for hematopoietic stem cell's (HSCs) survival and integrity via activation of caspase-dependent death receptor pathways (Gaudreau et al., 2016). hnRNP-Q is recently implicated in cell proliferation and tumor initiation in colorectal cancer via enhancing Aurora-A translation (C. H. Lai et al., 2017) By

doing individual nucleotide-resolution cross-linking and immunoprecipitation (iCLIP) studies, (Briese et al., 2018) found around 3500 RNA targets of hnRNP R including significant interactor, a noncoding RNA 7SK which is essential in regulation of axon maintenance by hnRNP R.


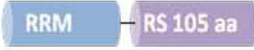
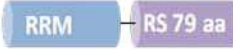









### **1.3.3.2 SR Protein Family:**

An essential class of RBP involved in splicing is the serine/arginine (SR)-rich SR proteins. The SR proteins represent a family of splicing factors that are remarkably conserved in vertebrates and invertebrates and also have been discovered in plants. Members of the SR family in mammalian cells were discovered in the early 1990s by the identification of factors associated with purified spliceosomes (X. D. Fu & Maniatis, 1990) (X. D. Fu & Maniatis, 1992). SR proteins are ubiquitous in higher organisms and present in many other diverse species, such as plants and fungi, reflecting an early emergence in evolutionary history (Twyffels, Gueydan, & Kruijs, 2011). Budding yeast is devoid of “true” SR proteins but has three SR-like proteins: Npl3, Gbp2 and Hrb1, which likely represents the ancestral basis from which SR proteins subsequently evolved (Busch & Hertel, 2012).

SR proteins are defined by the presence of one or two RNA recognition motifs and RRM homolog (RRMH) (a more degenerate pseudo RRM) at the N terminus and an RS domain (arginine/serine-rich dipeptide domain) at the C terminus. RRM domains are widespread among RNA binding proteins, and their flexibility permits diverse modes of binding between proteins (Maris, Dominguez, & Allain, 2005). In humans, Twelve canonical members of the SR protein family share this characteristic domain structure. RRMs ascertain RNA-binding specificity, whereas the RS domain functions as a protein-protein interaction module by recruiting components of the core splicing apparatus to promote splice site pairing (Jane Y. Wu & Maniatis, 1993). There are 7 homologs of these SR proteins in *Drosophila*. These essential splicing factors can bind RNA directly, and their RS and RRM domains act to promote protein-protein and protein-RNA interactions, facilitating the recruitment and assembly of the spliceosome (S. H. Xiao & Manley, 1997).

SR-like proteins also exist, such as Tra2 $\alpha/\beta$ , which contain an RS domain but lack the highly repetitive characteristic of true SR proteins or an RRM motif, although many of these are able to bind RNA via different domains and mechanisms (Long & Caceres, 2008). Genome-wide analyses revealed that RS-domain containing proteins participate in numerous aspects of gene expression regulation, including those involved in the cell cycle (Boucher, Ouzounis, Enright, & Blencowe, 2001)

The SR family nomenclature is historically diverse but in the beginning of decade an agreement was reached to have consensus terminology to identify them (Twyffels et al., 2011) (Manley & Krainer, 2010), thus they have several aliases, however it is not surprising to find researchers using old style names even now.

Aliases	New protein /gene symbol	Diagram	Homologue in <i>Drosophila</i>
ASF, SF2, SRp30a	SRSF1		dASF/SF2
SC35, PR264, SRp30b	SRSF2		SC35
SRp20	SRSF3		RBP1, RBP1-like
SRp75	SRSF4		
SRp40, HRS	SRSF5		
SRp55, B52	SRSF6		B52
9G8	SRSF7		d9G8 / dx16
SRp46 (human only)	SRSF8		
SRp30c	SRSF9		
TASR1, SRp38, SRp40	SRSF10		
p54, SRp54	SRSF11		SRp54
SRp35	SRSF12		

**Figure 11 Domain configuration of human SR proteins:**

SRSF1–12 are members of the canonical SR protein splicing family that is defined by N-terminal RRM followed by a downstream RS domain. The RRM is responsible for RNA binding, while the RS domain mediates protein/protein interactions. Left: Aliases of the SR proteins. Right: *Drosophila* homologs.

### 1.3.3.2.1 Techniques to identify bindings of SR protein:

The search for RNA target sequences of SR proteins in genes known to be regulated by SR proteins first led to the discovery of exonic regulatory sequences of the *Drosophila doublesex* gene (M. Tian & Maniatis, 1993), the mouse *IgM μ* gene (Watakabe, Tanaka, & Shimura, 1993), the bovine growth hormone gene (Q. Sun, Hampson, & Rottman, 1993) and the fibronectin gene (Lavigne, La Branche, Kornblihtt, & Chabot, 1993).

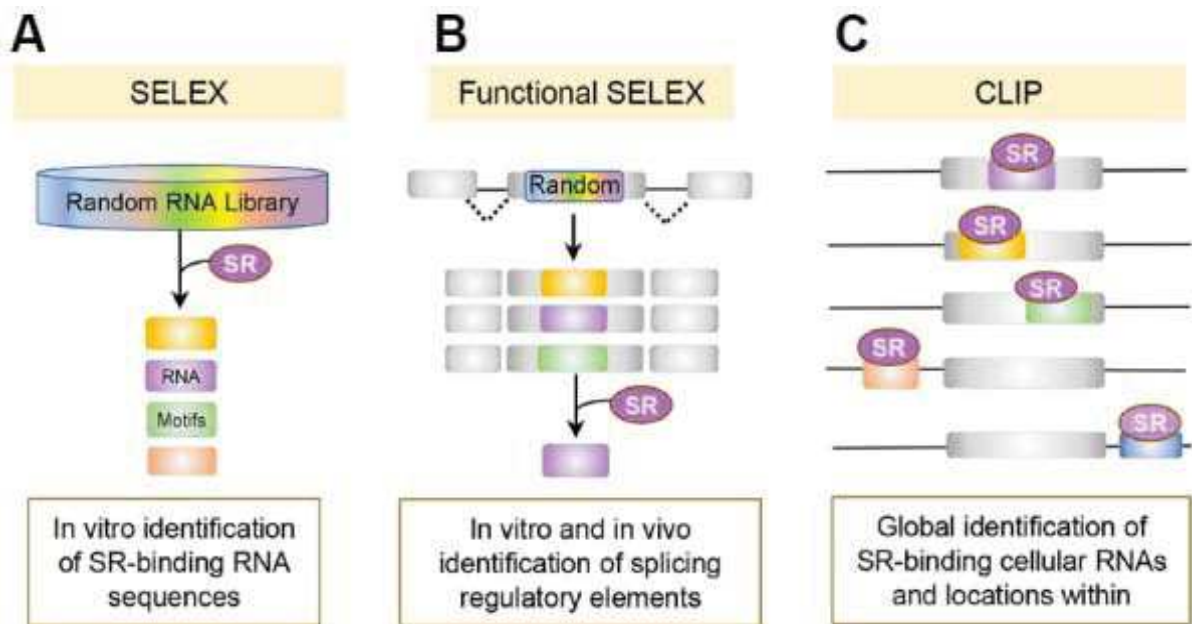
The identification of many ESE sequences of individual SR proteins was done via systematic evolution of ligands through exponential enrichment (SELEX) protocols (Tuerk & Gold, 1990) (Figure 12).

The binding SELEX consists of binding high-affinity RNA targets from a pool of RNAs with 20 random nucleotides between PCR amplifiable sequences to the immobilized SR protein. RT-PCR then amplifies the bound RNAs. This cycle is repeated at least five times before the remaining RNAs are sequenced. The idea behind the binding SELEX is that high-affinity sequences are also strong exonic splicing enhancers. The binding SELEX generally yields one or two similar high-affinity sequences (Bourgeois, Lejeune, & Stévenin, 2004).

Functional SELEX is an updated and modified version of SELEX that has been developed to identify the SREs in the cells. Random RNA sequences are added at the site of enhancer sequences. Exons included after the splicing *in vivo* or in a cell extract are RT-PCR amplified. After multiple selection cycles in the presence of one particular SR protein, the amplified ESEs are sequenced (Bourgeois et al., 2004).

However, despite the advent of SELEX it is challenging to identify the new SRE sequences and identify the RBPs and their contribution in splicing. Thus, to identify the RBP-RNA map on a global scale, CLIP (cross-linking and immunoprecipitation) analysis has been developed. In the CLIP, UV radiation is utilized to cross-link direct RBP-RNA interactions within a very short distance ( $\sim 1$  Å). Further, a CLIP-Seq also referred to as HITS-CLIP (high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation) is carried out to identify the RBP-RNA interactions precisely. In order to increase the efficiency of the cross-linking of RBP-RNA interaction PAR-CLIP (Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) was developed. Also, iCLIP (individual-nucleotide resolution CLIP) was developed that allows single-nucleotide resolution of RBP binding sites. Reviewed in (Jeong, 2017)





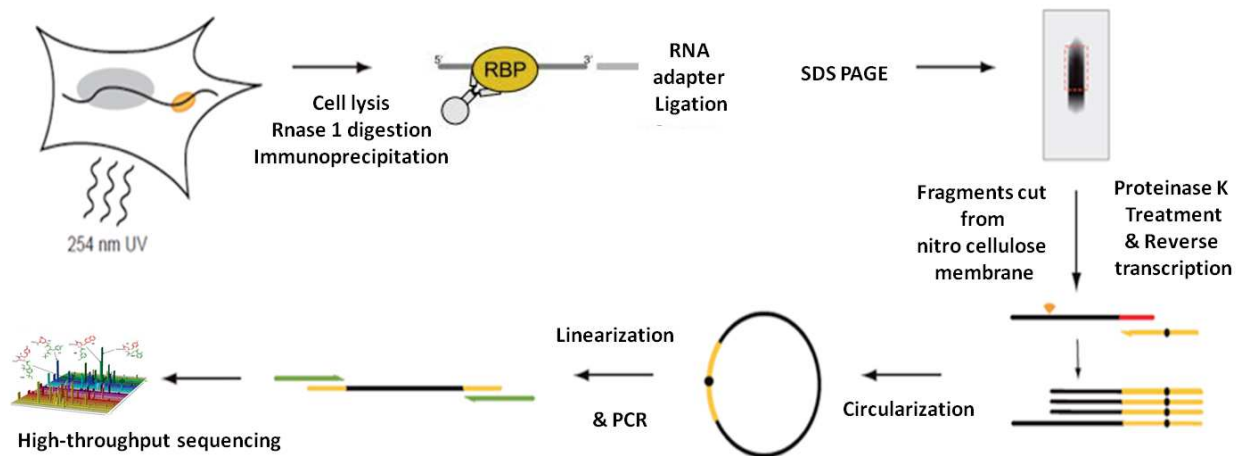
**Figure 12 Techniques to identify binding of SR proteins:**

(A) SELEX (systematic evolution of ligands by exponential enrichment) for *in vitro* identification of SR-binding RNA motifs. A random RNA library was used for the selection of binding RNA sequences. (B) Functional SELEX. Reporter-based *in vitro* and *in vivo* identification of splicing regulatory elements. ESEs (Exonic Splicing Enhancers) can be selected in the reporter as shown here. (C) CLIP-Seq (Cross-linking and immunoprecipitation-sequencing) for the global identification of SR-binding motifs in target RNAs

**iCLIP** is CLIP combined with high-throughput sequencing has been used to identify transcriptome-wide binding maps of several RNA-binding proteins such as by (Ule et al., 2003). However the method identification of binding sites with CLIP has some disadvantages; for an example, it relies on the analysis of overlapping sequence clusters and distances of less than 30 nucleotides are not resolved. Further, the requirement of reverse transcription to pass over residual amino acids that remain covalently attached to the RNA at the cross-link site is also a disadvantage. Primer extension studies have shown that the significant number of cDNAs prematurely truncate immediately before the ‘cross-link nucleotide’ (Urlaub, Hartmuth, & Lührmann, 2002). iCLIP, however, employs a different cDNA cloning protocol which allows identification of the cDNAs that truncate at the cross-link sites therefore, the position of cDNA truncation allows iCLIP to identify the cross-link sites and provide nucleotide-resolution precise protein–RNA interaction map (König et al., 2010).

In both CLIP and iCLIP, protein and RNA are crosslinked via UV, and then the protein of interest is purified, ligate the 3' adaptor, purify the protein-RNA complex, and digest the protein by proteinase K. However, In CLIP, a 5' adaptor is ligated to the RNA before reverse transcription. Therefore, CLIP can only amplify cDNAs that read through the cross-link site. However, since the cross-linked nucleotides are covalently bound to the amino acid residue, a proportion of cDNAs truncate at the cross-link site. In iCLIP, truncated cDNAs are captured

by circularization and subsequent linearization. Thus iCLIP is capable of studying a more extensive repertoire of RBPs (Figure 13).



**Figure 13 A simplified schematic of the iCLIP protocol:**

UV irradiation to covalently cross-link *in vivo* protein-RNA complexes followed by Cell lysis and partial RNA digestion (step 1). After lysis, the crosslinked RNA is fragmented by the limited concentration of RNase I and RNA fragments are then co-immunoprecipitated with the RBP (step 2), followed by ligation of a 3' adapter (step 3). After SDS PAGE purification (step 4), the crosslinked RBP is removed through proteinase K digestion and purification of RNA fragments (step 5). Reverse transcription is performed with a primer that includes a barcode (orange) containing both an experimental identifier and a unique molecular identifier (UMI) (step 6). The peptide that is on the crosslink site impairs reverse transcription and commonly leads to truncation of cDNAs at the cross-link site. Therefore, two types of cDNAs are generated: truncated cDNAs and readthrough cDNAs. In iCLIP, the cDNA library is prepared in such a way that both truncated and readthrough cDNAs are amplified (step 7). After PCR amplification and sequencing (step 8), both truncated and readthrough cDNAs are present. Figure adapted and modified from (Zünd, Gruber, Zavolan, & Mühlemann, 2013)

However (Maticzka, Ilik, Aktas, Backofen, & Akhtar, 2018) developed uvCLAP (ultraviolet cross-linking and affinity purification) method which they describe as a fast, robust technique to identify and characterize targets of RBPs *in vivo* without having to resort to labor-intensive techniques that use radioactive substances. Their technique uses a tandem affinity purification tag that consists of two  $6 \times$  His tags and an *in vivo* biotinylation signal peptide, which allowed tagged RBPs to be sequentially purified by nickel and streptavidin beads. More recently, (Ya Zhao et al., 2019) have developed a technique called spyCLIP that is a comparatively faster way to detect RBP-RNA interactions by further eliminating the need of SDS PAGE separation, membrane transfer and RNA labeling for visualization thus limiting the loss of RNA during purifications steps. They do so by using a SpyTag (a 13 aa peptide) fused to RBP and a SpyCatcher system. The covalent binding of SpyTag-Spy-catcher allows harshest washing steps which could not be done with CLIP, iCLIP or uvCLAP methods. Overall, these newly developed techniques will further allow understanding the RBP interactions and help to elucidate their role on a genome-wide scale.



#### **1.3.3.2.2 Role of SR in constitutive splicing and alternative splicing:**

SR proteins contribute to the dynamic sequential process of spliceosome assembly via recognition of ESEs. During the 1st step of splicing, i.e., the formation of commitment complex (E complex) SR proteins regulate the recruitment of U1 snRNP at 5' splice site and small subunit U2AF at 3' splice site (S. Cho et al., 2011).

Further, in A complex SR proteins facilitate the binding of U2 snRNP at branchpoint sequence by neutralizing the negative charge at phosphodiester backbone (H. Shen, Kan, & Green, 2004). At the next step, SR proteins help in the recruitment of the U4/U6.U5 tri-snRNP (Rosciigno & Garcia-Blanco, 1995). Additionally, RS domains of SR proteins are hypothesized to associate with the phosphodiester backbone near the 5' splice site to promote U6 binding (H. Shen & Green, 2004). Finally, molecular rearrangements coupled with dephosphorylation of SR proteins leads to the formation of catalytically active C complex and eventually results in splicing of introns and ligation of exons together (W. Cao, Jamison, & Garcia-Blanco, 1997). In addition, SR proteins can mediate splicing of minor U12-introns by functioning analogously to recruit the machinery of the minor spliceosome through interactions with the U5 snRNP (H. Shen & Green, 2007).

Besides playing a role in constitutive splicing, SR proteins mediate complex alternative splicing regulation. SR promotes the inclusion of the alternative exons which have characteristics short length and weak splice site to be adequately recognized by splicing machinery. The role of RBPs in AS is defined by the context and location (X. D. Fu & Ares, 2015), for an example, SR proteins associated with exonic sequences mainly act as enhancers, but SR proteins associated with intronic sequences can act as suppressors (M. Shen & Mattox, 2012). SR proteins have also shown to associate with constitutive exon adjacent to the alternative exon in order to mediate splicing and. For an example, SRSF1 has been shown to promote skipping of exon 16 by associating with constitutive exon 17 in CamKII $\delta$  highlighting the association of SR protein with constitutive exon can affect AS of alternative exon upstream or downstream (J. Han et al., 2010).

As said earlier, SR proteins can also act to inhibit splicing. SRSF9 has been shown to induce skipping of exon 7B in hnRNP A1 pre-mRNA (Simard & Chabot, 2002). Similarly, it has also been shown that SRSF11 binds to exonic splicing silencer to promote skipping of exon 10 in tau pre-mRNA (J. Y. Wu, Kar, Kuo, Yu, & Havlioglu, 2006). A few years ago (Pandit et al., 2013) showed that SR proteins not only promote but can also repress the alternative splicing of cassette exons.

### **1.3.3.2.3 Role of SR protein besides splicing:**

It is indicated that mRNA processing events are indeed coupled with transcription (Bentley, 2014). SR proteins co-localize with RNA polymerase II in nuclear speckles, an interaction mediated by the Pol II C-terminal domain (CTD) in a serine phosphorylation-dependent manner (Misteli et al., 1998) (Sapra et al., 2009). It has been shown by (De La Mata & Kornblihtt, 2006) that CTD is absolutely required to recruit SRSF3 for exon exclusion. Additionally, SRSF1 and SRSF3 have been shown to interact with H3 and associate with chromatin thus giving an insight into the regulation of SR protein's function via histone modification and nucleosome occupancy (Luco, Allo, Schor, Kornblihtt, & Misteli, 2011).

SR proteins can also directly regulate the elongation rate of RNA polymerase II. It has been shown that depletion of SRSF1 or SRSF2 can induce the accumulation of RNA polymerase II at gene loci and attenuate elongation (S. Lin, Coutinho-Mansfield, Wang, Pandit, & Fu, 2008). SRSF2 which is an essential non-shuttling SR protein has been shown to be a part of the 7SK RNP complex that facilitates the release of paused Pol II which often is paused near gene promoter (Ji et al., 2013).

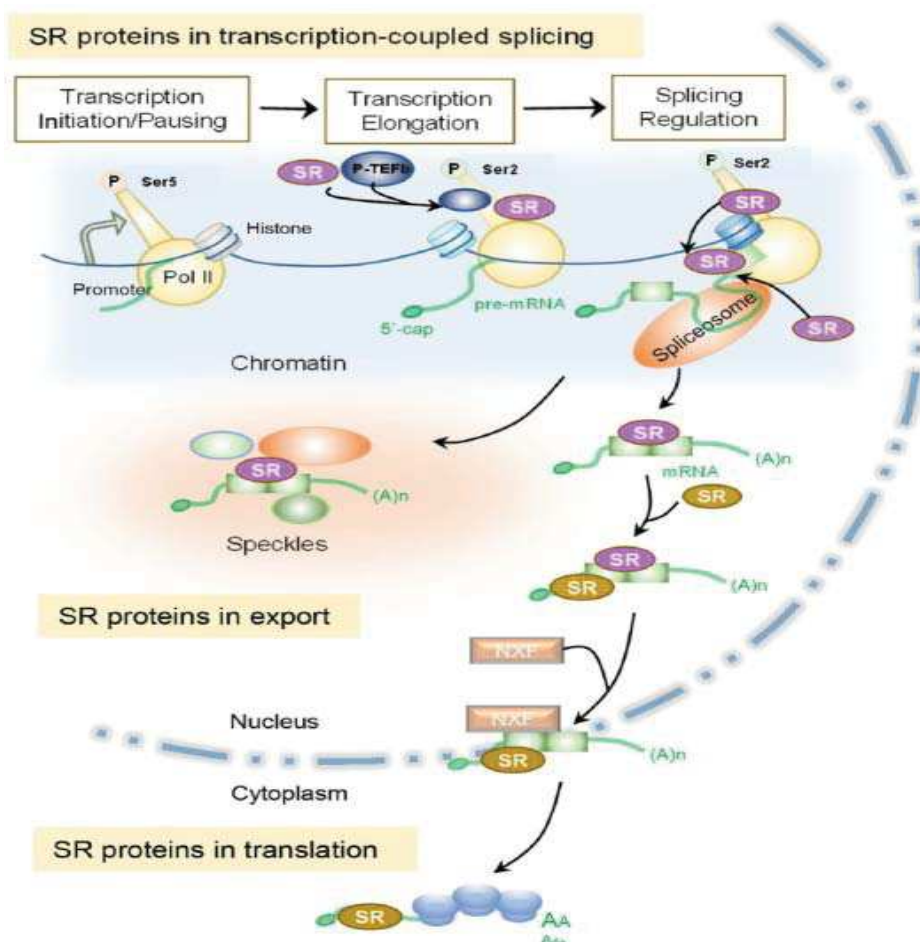
SR proteins have been shown to shuttle between nucleus and cytoplasm and they play a role even beyond splicing (Cáceres, Sreaton, & Krainer, 1998). Indeed SRSF3 and SRSF7 are shown to be involved in mRNA export and by recent CLIP analysis some SR proteins (SRSF1 to SRSF7) are shown to interact with export receptor Nuclear export factor 1 (NXF1/TAP) highlighting a role of SR proteins in export of specific mRNAs from nucleus to cytoplasm (Hargous et al., 2006) (Müller-McNicoll et al., 2016).

SR protein is also linked with mRNA translation as SRSF1 is detected in polysomal fractions of HeLa cells cytoplasm (Sanford, Gray, Beckmann, & Cáceres, 2004). Further, (Michlewski, Sanford, & Cáceres, 2008) have demonstrated that SRSF1 initiates translation via interactions with components of mTOR pathway. They suggest that SRSF1 recruits mTOR kinase to the mRNA targets to suppress the activity of 4EBP (a competitive inhibitor of cap-dependent translation) in a phosphorylation-dependent manner. This allows the release of eIF4E, a cytoplasmic cap-binding protein and thus initiating the translation. Further, (Maslon, Heras, Bellora, Eyras, & Cáceres, 2014) have identified more than 500 mRNA translational targets of SRSF1 via high-throughput deep sequencing. It has also been shown that SRSF3 regulates Internal Ribosomal Entry Site (IRES)-mediated translation initiation (Bedard, Daijogo, & Semler, 2007) and SRSF7 is involved in translation of unspliced viral RNA that contains containing Constitutive Transport Element (CTE) (Swartz, Bor, Misawa, Rekosh, & Hammarskjöld, 2007). Similarly, (Swanson, Sherer, & Malim, 2010) have shown that SRSF5

and SRSF6 promote the translation of gag protein. SR protein also plays a role in mRNA stability for an example; SRSF1 regulates the stability of PKCI- $\gamma$  mRNA (Lemaire et al., 2002). (H. Wu et al., 2010) have described an association of SR protein with microRNA and their role in microRNA biogenesis.

(Bradley, Cook, & Blanchette, 2015) have shown that SR proteins could bind to a diverse set of RNAs including long ncRNA suggesting that SR proteins can regulate gene expression via regulating ncRNA. These reports suggest that SR proteins play various functions ranging from coordination of splicing to transcription and post-transcriptional event (Figure 14).

While on the other hand MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) which is a long noncoding RNAs (lncRNAs) has been shown to associate with SRSF1, SRSF2 and SRSF3 and its depletion leads to mislocalization of splicing factors in the nuclear speckles suggesting that SR proteins are regulated by lncRNA (Tripathi et al., 2010)



**Figure 14 Multiple roles of SR proteins:**  
During gene expression from the nucleus to the cytoplasm

#### **1.3.3.2.4 Regulation of SR proteins by Post-translational modification**

Post-translation modifications play an essential role in the regulation of the activity and localization of SR proteins. SR-specific protein kinase (SRPK) family and other CMGC kinase family members, such as Clk/Sty (cdc2-like kinase/serine, threonine, and tyrosine kinase) regulate the phosphorylation of SR proteins by phosphorylating serine residues throughout the RS domain (Gui, Lane, & Fu, 1994) (Colwill et al., 1996). It is postulated that phosphorylation of RS domain increases the RNA binding capacity as well as is important for protein-protein interaction (R. Tacke, Chen, & Manley, 2002) (S. H. Xiao & Manley, 1997)

The localization of SR protein depends upon the phosphorylation as Clk/Sty mediate phosphorylation facilitates the release of SR proteins from nuclear speckles (Colwill et al., 1996). After the spliceosomal assembly, some SR proteins are re-phosphorylated so as to participate in an additional round of mRNA splicing (i.e. they are recycled), and a few dephosphorylated SR proteins associate with mRNA to signal that mRNA is ready to be exported to the nucleus. Once the export to nucleus and translation is complete, thereby SR proteins are re-phosphorylated by SRPK so as to interact with transportin-Sr and be imported back in the nucleus (Kataoka, Bachorik, & Dreyfuss, 1999) (M. C. Lai, Lin, Huang, Tsai, & Tarn, 2000)

SR proteins also undergo other post-translational modification such as methylation and acetylation. It has been shown that blocking methylation can misregulate the localization of SR proteins and thus, it can affect alternative splicing, translation, and mRNA decay (Bressan et al., 2009) (Sinha et al., 2010) (Y.-C. Chen et al., 2010) On the other hand acetylation of SRSF2 occurs in response to genotoxic stress (Edmond et al., 2011). However, the results of post-translational modification besides phosphorylation are not clearly understood, but they seem to be essential and contribute to the regulation of SR proteins.

#### **1.3.3.2.5 Targets of SR proteins:**

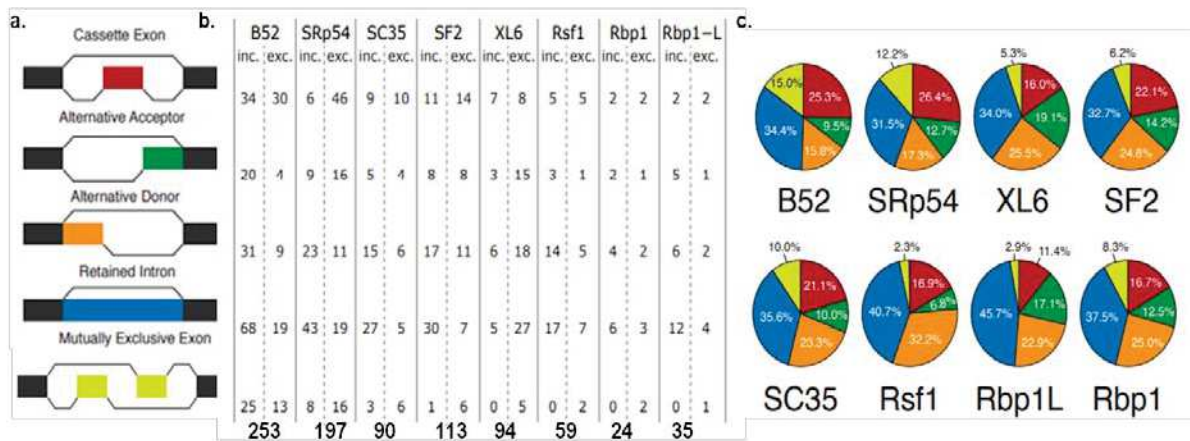
So far, we have seen the wide range of roles that SR proteins play in the process of RNA metabolism, from export to mRNA decay. In order to further elucidate the implications of SR in AS and reveal how splicing is regulated it is vital to investigate the targets of RBPs (not only SR and hnRNPs). Thanks to RNA seq strategies, now it is possible to identify genome-wide AS events and immunoprecipitation approaches such as iCLIP (Please refer to the section: 1.2.2.3.2.1 ) to identify targets of RBPs (Huelga et al., 2012) (König et al., 2010) (Brooks et al., 2011).

(Änkö et al., 2012) used iCLIP technique and reported that two SR proteins SRSF3 and SRSF4 bind to a small subset of targets but their target rarely overlap with each other, implicating that most of SR proteins targets do not overlap. While (Pandit et al., 2013) doubted the conclusions and suggested that the sensitivity of the experiments might not have been enough to capture all the SR proteins bound to the transcripts. Thus, they used CLIP-seq in combination with splicing-sensitive arrays to reveal that indeed the targets of SRSF1 and SRSF2 extensively overlap and also found that depletion of either of these SR proteins lead to both inclusion and exclusion of exon, reasserting that SR proteins play a role in both enhancing and suppression of splicing.

Further, (Bradley et al., 2015) knocked down 8 *Drosophila* SR proteins (SC35, SF2, SRp54, XL6, Rbp1, B52, Rsf1, and Rbp1-like) in S2 cells via RNAi (below 10% in each case) individually and did RNA-seq analysis in order to reveal the extent to which SR proteins targets overlap with each other and to what extent SR proteins cooperate with each other to regulate splicing. They only focused on five simplest types of AS which are cassette exons, competing donor or acceptor sites, mutually exclusive exons, and intron retention (Figure 15a).

The criteria used for assuming if an AS event is altered they used a “percentage spliced in” value (PSI or  $\Psi$ ) 10%. (PSI is generally used to determine the extent of splicing change. The percent here indicates the efficiency of splicing a specific exon into the transcript population of a gene. A PSI of 100% indicates constitutive exons that are included in all transcripts and never removed from expressed isoforms. PSI values below 100% imply reduced inclusion of alternative exons and denote the percentage of isoforms that contain the exon compared to the total transcript population) (Schafer et al., 2015).

They detected 561 events (representing 405 genes) that were altered by the depletion of 8 SR proteins, which constitute to around >10% of the simple AS events (5472) that are readily detected in *Drosophila* S2 cells. They detected that each SR protein affected a different number of AS events from B52 affecting 253 while Rbp1 altering just 24 events. Additionally, they saw that each SR protein affect all types of AS in a similar proportion (Figure 15).



**Figure 15 AS affected by individual SR proteins:**

(a.) Types of AS considered in this study. (b.) The total number of AS events regulated by individual SR proteins separated by causing more inclusion (inc.) or exclusion (exc.) of the alternative region represented individually for each AS type. (c.) Pie charts of the distribution of simple AS types differentially regulated by individual SR proteins detected by RNA-Seq. Study by (Bradley et al., 2015)

Their data reflected that SR proteins are both activators and repressors of AS events, they even found that among the 8 SR proteins 6 repressed more than half of their targets. Further, they showed that not only multiple SR proteins can affect the same AS event, but they can also have an antagonistic function in the same region. They also revealed that every AS event was affected by more than one SR protein with the only exception of B52 which seemed to regulate most of its targets (74%) individually. Moreover, on the other hand, Rsf1 regulated almost 82% of its targets in a combinatorial fashion. Finally, they show that all of the 8 SR proteins regulate the promoter selection of multiple targets and poly(A) site selection thus revealing much widespread role of SR protein in gene expression by directly affecting transcription start site and 3' end processing site choices.

Another study which got published a little later in 2015 done by (Brooks et al., 2015) complements the above-described study by (Bradley et al., 2015) in several ways. Here, the authors took a total of 56 RBPs including SR and hnRNPs and individually depleted them in *Drosophila* S2 DRSC cells by using *RNAi*. Followed to it, they did RNAseq on the polyA and RNA isolated from these 56 samples. They looked at three more types of AS events in addition to 5 that was criteria for study by (Bradley et al., 2015). To confidently assume that an AS event was altered the authors used a  $\Psi$  value of  $>10\%$ . Basing analysis on these criteria, they detected around 2876 AS events that were affected (out of 23,079 AS events) by one or more of these 56 RNA binding proteins. They found a large number of events affected by depletion of each SR protein as over 400 events alone were affected by the depletion of SR protein B52.



Similar to the works of (Huelga et al., 2012) who showed that hnRNPs too have overlaps in their targets & (Bradley et al., 2015) they reasserted the fact that SR proteins and hnRNPs promote exclusion and skipping and not work each time antagonistically according to the traditional assumption.

#### 1.3.3.2.6 SR Protein B52

In *Drosophila*, B52 is ubiquitously expressed throughout all developmental stages, and its concentration is vital to the viability and development of the organism (Kraus & Lis, 1994). This 52kDa protein was first characterized because it localized to heat shock puffs upon heat shock (puffs are the regions of visibly decondensed chromatin) on the polytene chromosome (Champlin, Frasch, Saumweber, & Lis, 1991).

Homozygous *B52* mutants ( $B52^{28}/B52^{28}$ ) is lethal at the second-instar larval stage (Ring & Lis, 1994) and overexpression of B52 leads to lethality and various defects such as reduced salivary glands and curled wings depending on the tissue where B52 is overexpressed (Kraus & Lis, 1994). In addition, they also determined that the level of B52 suddenly decreases during the first instar stage, after which it remains only 15%-20% compared to that in the embryo. From here onwards, the level of B52 remains constant for the rest of the later development highlighting its role in early development and development throughout the course of life. They also showed that B52's expression level is tissue-specific, with the highest in adult ovaries and lowest expression in the larval intestine while the imaginal disc, the brain, and ventral ganglion all have significant levels of B52.

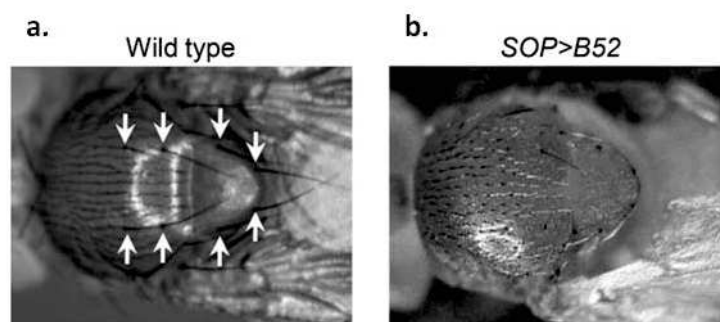
(Fic, Juge, Soret, & Tazi, 2007) showed that overexpression of B52 by using *eyeless-GAL4* (*ey-GAL4*) driver in the eye leads to severe defects of eye development while overexpression of another SR protein, dASF did not prompt any such phenotype.

Further, they also showed that B52 regulates the RNA splicing of *eyeless* (*ey*) which is a master regulator of eye morphogenesis. In another publication (Gabut, Dejardin, Tazi, & Soret, 2007) showed that expression of GFP-B52 via GMR-Gal4 results in disorganization of rod and cone cells in eye imaginal discs in the 3rd-instar larvae. These authors have also identified several B52-interacting mRNAs by performing co-immunoprecipitation of B52 proteins, using GFP overexpression as a control. Some of these targets are in agreement with the result of genomic SELEX where full-length Baculovirus-expressed B52, is used as a bait to attract potential RNA targets that have a high affinity for B52 (S. Kim, Shi, Lee, & Lis, 2003).

(Juge, Fernando, Fic, & Tazi, 2010) showed that in addition to its mRNA-splicing function, B52 is also involved in the recruitment of Topoisomerase I to the chromatin. This mechanism SR protein recruiting topoisomerase 1 is conserved in humans as recently, (Girstun, Ishikawa, & Staron, 2019) have shown that SRSF1, thanks to its distinct RS domain can recruit topoisomerase 1 to the nucleoplasm in HeLa cells. While they observed SRSF2, SRSF3, SRSF6, and partly SRSF4 exhibited an opposite effect instead. However, the mechanism of this action of SRSF1 is not yet known.

B52 has been shown, by both microarray analysis and genomic SELEX, to target a transcription factor gene BTB/POZ transcription factor longitudinal lacking, (*lola*), which controls axon extension via the transcription of Spire, an actin nucleation protein. In addition to *lola*, B52 has been shown to bind to the RNA transcripts of *Syndecan (Sdc)* and *RhoGAP16F* (Gabut et al., 2007) (S. Kim et al., 2003). These findings highlight the importance of B52 in cellular regulatory networks.

SR proteins are frequently observed to be upregulated in tumors suggesting that SR protein helps in the tumor initiation and growth and in *Drosophila* overexpression leads to severe developmental defects. (Fernando, Audibert, Simon, Tazi, & Juge, 2015) used *Drosophila* bristle cell lineage as a model to study the consequences of B52 overexpression on development and to identify factors that could rescue this phenotype as it could be of therapeutic interest. By using *SOP-GAL4* and *neuralized<sup>P72</sup>-GAL4 (neur<sup>P72</sup>-GAL4)* drivers they overexpressed B52 in the bristle lineage and by immunostaining at the pupal stage and upon observation of pharate adults, they showed that overexpression of B52 lead to partial to complete loss of microchaetes and macrochaetes (short and long bristles) (Figure 16).



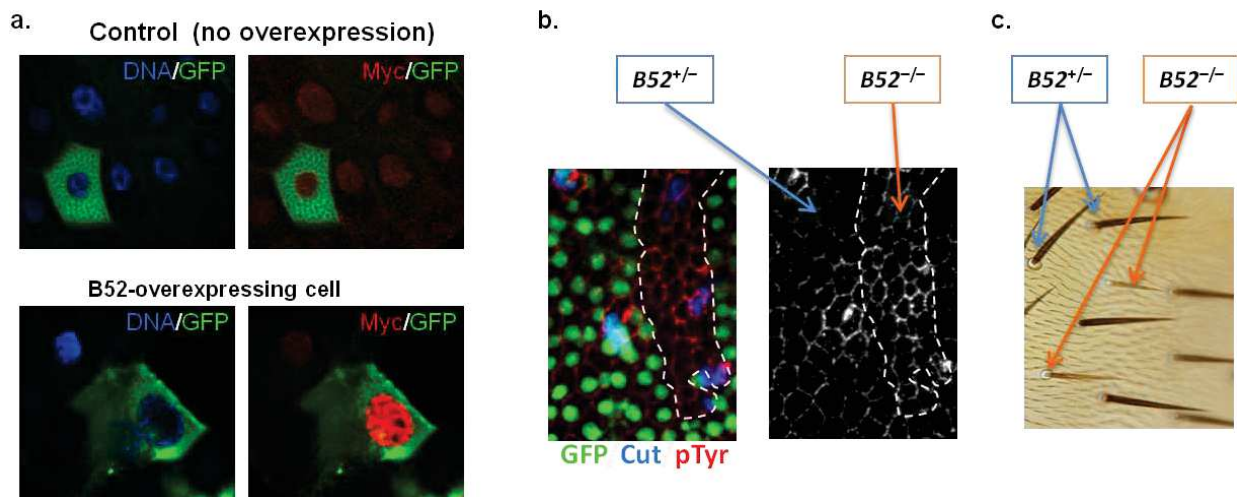
**Figure 16 Bristle phenotype:**

(a.) Wild type fly consists of eight macrochaetes on thorax which are indicated by arrows. (b.) Upon down-regulation of B52 in bristle cell lineage, there is a variable loss of macrochaetes as in this fly only one of the eight macrochaetes is remaining. Reported in (Fernando et al., 2015)

To understand the role of B52 at the cellular level, they did B52 staining at the pupal stage in the above experiment and revealed that overexpression does not affect the identity of the cells, but it increases the size of the cells which eventually induces cell death in bristle



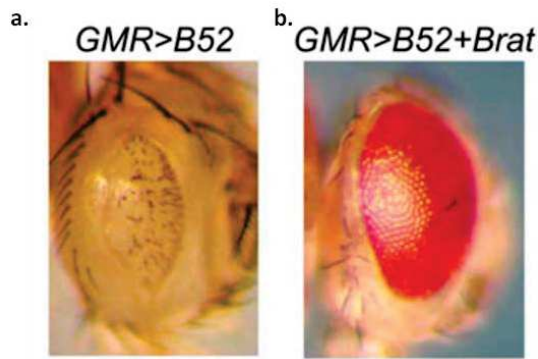
lineage. The authors further generated B52 homozygous mutant clones by somatic recombination and revealed that B52 mutant clones were smaller and eventually gave rise to smaller bristles in adults (Figure 17 b & c). They also observed that knockdown of B52 lead to delay in the development of bristle lineage and by Immunostaining, they revealed that B52 knockdown decreases Myc expression (Figure 17 a).



**Figure 17 Effect of B52 on growth:**

(a.) wildtype clone vs Increased cell size of clone overexpressing B52. (b.) Loss-of-function of B52 in the bristle lineage decreases cell growth (c.) bristle arising from wild-type clones vs from B52 homozygous mutants. Reported in (Fernando et al., 2015)

They also reported that B52 overexpression increased *dMYC* promoter activity measured by *dMYC-lacZ* reporter activity and enrichment of phosphorylated Pol II across the *dMYC* promoter. Finally, by using a genetic screen they identified several factors that could rescue the phenotype induced by B52 overexpression including *brain tumor (brat)* which is a negative regulator of growth (Frank, Edgar, & Roth, 2002) and *lilliputian (lilli)* which is a positive regulator of growth (Wittwer, van der Straten, Keleman, Dickson, & Hafen, 2001)(Figure 18). The authors further showed that mechanistically an antagonism between B52 and Brat exists as in B52 overexpression clones, overexpression of Brat reduces the transcription of *myc* to rescue the overexpression phenotype. Besides revealing the role of B52 and providing a mechanistic link between B52 and *myc* transcription this study opens a new perspective of identifying proteins that could repress the ill-effects of SR protein overexpression in mammals.



**Figure 18 Rescue of B52:**

(a.) Overexpression of B52 in the eye (b.) Co-overexpression of B52 and Brat. Reported in (Fernando et al., 2015)

Recently, (Boyin Liu & Bossing, 2016) have shown the requirement of B52 in the splicing of Choline acetyltransferase (ChAT) which encodes the enzyme required for the biosynthesis of the neurotransmitter acetylcholine. Moreover, B52 has also been shown to regulate the mid-day siesta in flies by regulating the splicing of *period* gene suggesting that this SR protein can participate/contribute in natural sleep behavior as reported in a recent study by (Zhichao Zhang, Cao, & Edery, 2018). Further studies will be needed to elucidate the complete B52 splicing program and provide an understanding of the role that B52 plays in growth and other processes.

#### 1.3.4 Additional RNA Binding Proteins that regulate splicing:

In addition to above described RBPs (SR and hnRNPs) that are ubiquitously expressed across several tissues, several reports have described some tissue-specific RBPs such as RBFOX, MBNL, PTBP2 (nPTB) and NOVA family proteins are essential regulators of tissue or cell-specific splicing (X. D. Fu & Ares, 2015). NOVA proteins are the class of RBPs that are specific to neuronal tissue, and by using CLIPseq techniques, it has been shown that NOVA regulates AS in brain (Ule et al., 2003). Further CLIP seq analysis revealed the position-dependent splicing program of NOVA, i.e., binding to downstream intron or upstream intron promotes exon inclusion or exon skipping. Mechanistically, NOVA binds to an ESS and blocks binding of U1 snRNP which inhibits the formation of E complex and promotes exon skipping. While on the other hand, when NOVA binds to ISE downstream of an intron, it promotes the formation of A complex and complexes that follow and thus it promotes exon inclusion (Ule et al., 2006). Other RBPs such as RBFOX, MBNL, ESRP1/2 and Quaking too facilitate a position-dependent splicing program. However, the underlying mechanism of the position-dependent regulation is mostly unknown.

Additional RBPs such as RBM5 regulate the splicing by inhibiting the formation of B complex in splicing assembly while RBM17 (or SPF45) inhibits splicing at the second

catalytic step during the splicing reaction (Lallena, Chalmers, Llamazares, Lamond, & Valcárcel, 2002) (Bonnal et al., 2008).

The combinatorial regulation of AS program is also reported, as is the case of splicing of exon 6 of human Fas, which encodes for a type 1 transmembrane protein. TIA-1 binding to ISE in intron 6 promotes the inclusion of exon 6 via U1 snRNP recruitment and produces an isoform that promotes apoptosis while binding of another RBP, PTBP1 to an ESS in exon 6 to inhibits the binding of U2AF65, leads to production of an isoform that lacks exon 6 and does not promote apoptosis (Izquierdo et al., 2005).

Recent studies have estimated the number of proteins encoded by humans that are capable to bind directly to RNA to be close to being in thousands (Castello et al., 2012) (Castello et al., 2016). Thus the repertoire of RBPs that regulate splicing is vastly unmapped.

### **1.3.5 Other factors affecting Alternative splicing:**

Secondary structure of RNA plays an essential role in defining interactive and functioning properties of the RNA molecule. Even though RNA is a single-stranded molecule, but it can fold back against itself to produce secondary and tertiary structures (Wan, Kertesz, Spitale, Segal, & Chang, 2011). RNA secondary structures have been reported to interfere with the interactions of splicing enhancers or silencers with proteins (Blencowe, 2000) (Smith & Valcárcel, 2000). Splicing changes occur due to RNA secondary structure formation. (Shepard & Hertel, 2008) suggested that 4 % of conserved alternative splicing are associated with secondary structure. For an example, stem-loop structures affect the 5' splice site of *SMN2*'s exon 7 and exon 10 of *Tau* (N. N. Singh, Singh, & Androphy, 2007) (Hutton et al., 1998). RNA secondary structures can even bring together distant RNA elements together. For example, secondary structure in FGFR2 pre-mRNA brings together two elements so as to activate exon IIIb (Baraniak, Lasda, Wagner, & Garcia-Blanco, 2003). In the *Drosophila Dscam* gene, that can form up to 38 000 different isoforms alternative cassette selection occurs after the formation of secondary structure between docking and selector sequences surrounding alternative cassette exons. This mechanism ensures that only one of the many alternative cassette exons is included in the mRNA (Graveley, 2005). In other instances, intronic secondary structure elements may even function as riboswitches that bind to small molecule metabolites and activate or repress splicing in response to the concentration of the metabolite. (Cheah, Wachter, Sudarsan, & Breaker, 2007) Additionally, a substantial amount of evidence has accumulated demonstrating that transcription rate and chromatin structure can affect alternative splicing.

## 1.4 Quality control of splicing:

Although “Split gene” architecture in eukaryotes is clearly advantageous such as it expands the coding capacity, but the same division of genes into exons and introns introduces a requirement of an intricate splicing regulatory network that is composed of RNA regulatory sequences, RNA protein complex, and splicing factors.

A “mistake” or “error” can be referred to the generation of any non-canonical molecular product, which either result in a nonfunctional protein product, or which induces quality control mechanisms to take action. To maintain the fidelity of gene expression cells evolved several mRNA surveillance systems (also called as quality control systems) in both the nucleus and the cytoplasm. These mRNA surveillance pathways detect not only the aberrant transcripts that may lead to toxic protein but also regulate the wild-type mRNAs (Kervestin & Jacobson, 2012). In the last decades, much advancement has been made to an understanding of these mechanisms.

Nuclear mRNA surveillance mechanisms are mainly studied in *Saccharomyces cerevisiae* (Garneau, Wilusz, & Wilusz, 2007). Before mRNAs are exported from nucleus to cytoplasm, the aberrant synthesis of mRNAs (incorrect or defective splicing, cap structure, cleavage or polyadenylation at 3' end) or the retention of mRNAs in the nucleus leads to activate different surveillance mechanisms. The nuclear retention of an mRNA that should be exported to the cytoplasm leads to decay by nuclear surveillance systems (Vasudevan & Peltz, 2003). One of the key components of nuclear surveillance is the nuclear exosome. The exosome is distributed in both nucleus and cytoplasm. To promote RNA degradation, two 3'-5' exonucleases interact with the core exosome. The nuclear exosome can lead the aberrant transcripts to accumulate at the site of transcription for degradation (Torchet et al., 2002). Cytoplasmic mRNA surveillance contains nonsense-mediated mRNA decay (NMD), no-go mRNA decay (NGD), non-stop mRNA decay (NSD) (Figure 19)

### 1.4.1 Nonsense-mediated decay (NMD)

One of the thoroughly-investigated RNA surveillance pathways is the Nonsense-mediated decay (NMD) pathway. It was identified in *S. cerevisiae* but has been shown to exist in all eukaryotes that have been investigated and is evolutionary conserved from yeast to humans (Y. H. Chen et al., 2008) (Delhi, Queiroz, Inchaustegui, Carrington, & Clayton, 2011) (Schweingruber, Rufener, Zünd, Yamashita, & Mühlemann, 2013) (He & Jacobson, 2015). The nonsense-mediated mRNA decay (NMD) pathway is a cytoplasmic surveillance mechanism that targets and degrades transcripts containing a PTC (premature termination

codon) (Leeds, Peltz, Jacobson, & Culbertson, 1991) (Peltz, Brown, & Jacobson, 1993). PTCs may arise from several sources, including DNA mutations, inaccurate transcription leading to frameshifts or single nucleotide nonsense mutations, or from alternative splicing that puts stop codons “in frame” in an inappropriate position.

A multi-subunit complex known as EJC (Exon Junction Complex) is a critical element of NMD. The EJC is essential for several cellular processes, such as nuclear export and subcellular localization (Schell, Kulozik, & Hentze, 2002) as well as translation (Nott, Le Hir, & Moore, 2004) (Wiegand, Lu, & Cullen, 2003). After splicing, the exon-exon junctions formed are bound by EJC. During the pioneer round of translation, the ribosome displaces each EJC as it moves along the mRNA. Translation terminates at the natural stop-codon, which is typically located in the terminal exon and therefore does not have any downstream bound EJC. However, if an mRNA contains a PTC within the 50 nucleotides or greater upstream of the last exon-exon junction, the ribosome stalls at the PTC, giving it the chance to interact with the downstream bound EJC. This triggers the NMD process, which leads to degradation of the PTC-containing mRNA. When the ribosome recognizes a stop-codon during translation, it recruits release factors, such as eRF1 and eRF3, which are associated with the NMD-associated protein UPF1. Recognition of PTC leads to a stable interaction between UPF1 and UPF2, bridging UPF1 with the EJC. This, in turn, leads to phosphorylation of UPF1 and recruitment of either SMG6, an endonuclease that cleaves the NMD targeted RNA, or the SMG5-SMG7 complex that is associated with uncapping and deadenylation of RNAs. Either of these pathways leads to rapid degradation of the targeted RNA.

Further, SRSF1 has been shown to stimulate NMD for mRNAs with PTC (Zuo Zhang & Krainer, 2004) Over-expression of SRSF1 greatly enhanced NMD. This activity of SRSF1 is independent of its shuttling ability but requires an intact RS domain. Recently the same laboratory pinpointed the mechanism by which SRSF1 enhances NMD. By tethering SRSF1 to transcript at the various position, they obtained evidence that SRSF1 enhances UPF1 binding to mRNA in nucleus and the increased presence of UPF1 may accelerate the NMD process (Aznarez et al., 2018).

The link between NMD and AS was first seen in a study of expressed sequence tags (ESTs) where, (B. P. Lewis, Green, & Brenner, 2003) showed that several alternatively spliced genes were found to have isoforms that would be predicted to be subject to NMD. These authors termed this phenomenon regulated unproductive splicing and translations (RUST) and this mechanism is conserved from humans to yeast (Soergel, Lareau, & Brenner, 2006). Some

splicing factors and all SR proteins autoregulate the level of their own expression through alternative splicing coupled to NMD (AS-NMD or unproductive splicing) (Lareau, Inada, Green, Wengrod, & Brenner, 2007) (Ni et al., 2007). For example, SRSF1 promotes inclusion of an intron after the canonical stop codon, which marks it as a premature termination codon (PTC) to elicit NMD of its own mRNA. These “unproductive splicing” events are highly conserved between evolutionary kingdoms. For a detailed review of NMD and mechanism it employs, one could read the following reviews by (Hug, Longman, & Cáceres, 2015), (Lykke-Andersen & Jensen, 2015) (Brognna, McLeod, & Petric, 2016) and also (Popp & Maquat, 2018).

The NMD pathway is involved in many biological processes, including cell proliferation and growth (Weischenfeldt et al., 2008) (Avery et al., 2011) (Lou et al., 2014), embryonic development and differentiation (Medghalchi, 2002) (Gong, Kim, Woeller, Tang, & Maquat, 2009) (McIlwain et al., 2010) (Bruno et al., 2011), stress response (Gardner, 2010; Sakaki et al., 2012), innate immunity (Gloggnitzer et al., 2014), and neuronal activity (Colak, Ji, Porse, & Jaffrey, 2013; Giorgi et al., 2007; He & Jacobson, 2015)

#### **1.4.2 The No-go decay (NGD)**

The No-go decay (NGD) is triggered when the elongation complex is stalled during translation. The stalling could be due to the presence of rare codons or secondary structure or poly-Lys or poly-Arg tracts, or frameshift sites in mRNA. It detects such mRNA and directs them for endonucleolytic cleavage, and subsequently, the resulting mRNA fragments are degraded by the exosome (which is a conserved multiprotein complex that degrades RNAs in the 3'-to-5' direction) and the Xrn1 5' to 3' exonuclease ( Xrn1 promotes general 5'-3' co-translational mRNA decay following the last translating ribosome) (Doma & Parker, 2006) (Passos et al., 2009)(Pelechano, Wei, & Steinmetz, 2015).

#### **1.4.3 Non-stop decay (NSD)**

Non-stop decay (NSD) is an mRNA surveillance mechanism that identifies transcripts that does not possess in-frame stop codons. These mRNAs could arise via premature polyadenylation or point mutations that disrupt stop codon and interestingly are widespread mistakes in the pathway of gene expression (Frischmeyer et al., 2002) (Vasudevan, Peltz, & Wilusz, 2002) (Cui & Denis, 2003). Because mRNAs are short of a stop codon, it leads to ribosomes to cross the poly (A) tail and remove the poly (A)-binding protein (PABP) and stall at the 3' end of the mRNA. The deed of the degradation is carried out by cytoplasmic exosome and Ski complex (Ski2p, Ski3p, and Ski8p) and Ski7p. Ski7p detects the transcript



lacking stop codon and recruits the Ski complex and exosome. Once recruited, the ubiquitin-proteasome pathway degrades the protein product generated from a non-stop transcript. (Klauer & van Hoof, 2012) (Graille & Séraphin, 2012) (Parker, 2012)

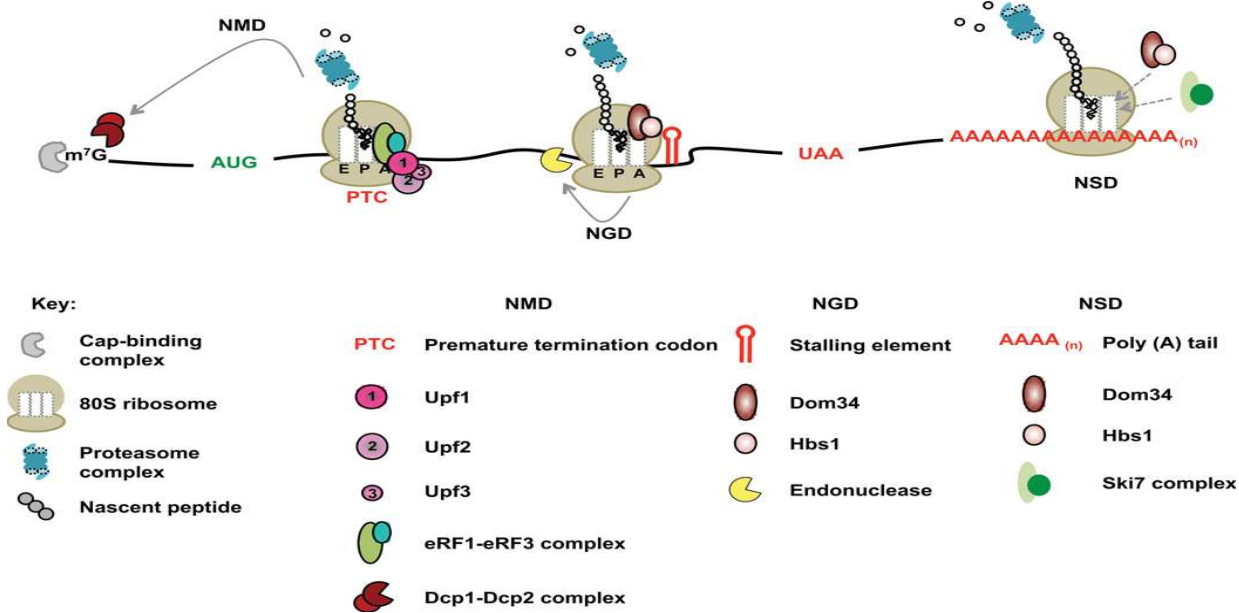


Figure 19 The three primary mRNA surveillance mechanisms: Adapted from (Roy & Jacobson, 2013)

### 1.5 Alternative splicing in diseases and available therapy

Aberrant splicing is implicated in a high number of dysfunction and human diseases (R. K. Singh & Cooper, 2012). AS has been implicated in nearly all aspects of cancer development, and therefore, is a central participant in the disease (David & Manley, 2010) (Chabot & Shkreta, 2016) (Scotti & Swanson, 2016). Alternative splicing is altered in a genome-wide manner in cancer cells and is now considered a “novel hallmark of cancer” as every common hallmark of cancer including metastasis is affected by dysregulation of splicing.

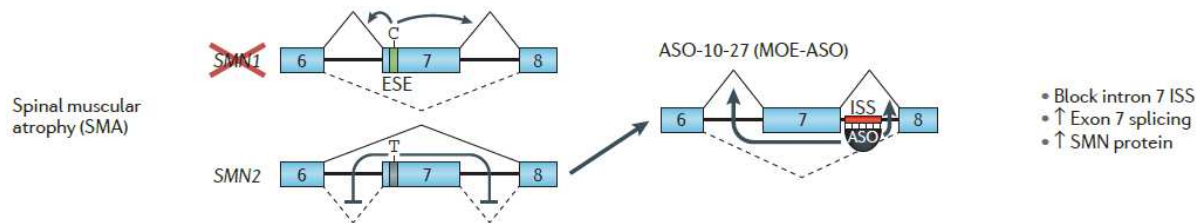
In their book, *Genetics in medicine*, (Nussbaum, Robert L; McInnes, Roderick R; Huntington, 2016) described three groups of splice defects that differ depending on their intronic or exonic location. **The first group** consists of splice junction mutations such as mutations at the 5' donor or 3' acceptor sites or in the consensus sequences surrounding these donor or acceptor sites. If the normal acceptor site is inactivated, then other intronic or exonic cryptic splice sites may elicit a splicing reaction. **The second group** of splicing defects consists of mutations in intronic cryptic sites that make it more favorable for splicing in comparison to the normal splice site. These “activated” cryptic site mutations are often referred to as “leaky” as the normal splice site is still active and produces normal gene product, thus leading to a less severe phenotype. **The third group** of mutations consists of mutations in the ORF that activate a cryptic splice site in an exon without altering the encoded amino acid (i.e., point mutations that become cryptic splice sites). It has been estimated that such *cis* splicing mutations constitute 15%–60% of human disease-causing mutations (G. S. Wang & Cooper, 2007). Additionally, mutations disrupting *trans*-acting splicing regulators cause a wide spectrum of diseases by globally compromising the splicing of many downstream target genes (Osborne & Thornton, 2006).

#### **Examples of disease caused by a point mutation in the exonic regulatory element:**

SMA (Spinal muscular atrophy) is a neurodegenerative disease that is a leading genetic cause of mortality in children (Arnold & Fischbeck, 2018). SMA is a result of deletions or mutations in the survival of motor neuron 1 (SMN1) gene, which results in SMN protein deficiency. However, humans have a centromeric copy of this gene called SMN2, which is identical to SMN1 except for a single nucleotide difference in exon 7. Thus, SMN2 cannot compensate for the loss of SMN1 as most mRNA produced from SMN2 lacks exon 7 (almost 90%). SMN2 thus encodes a truncated protein with reduced functionality. The single nucleotide mutation on exon 7, generates the binding site for hnRNP A1 which inhibits exon7 inclusion (Figure 20). Reviewed in (Sunghee Cho et al., 2014)



Antisense oligonucleotides (ASOs) are a new class of drugs that are short (15–25 nt), synthetic, single-stranded oligodeoxynucleotides that can bind to complementary target RNA and thus function to alter, reduce, restore, or modify protein expression through several distinct mechanisms. They can alter pre-mRNA splicing by sterically blocking splicing factors, or they can block mRNA translation by preventing ribosome recruitment (C. F. Bennett & Swayze, 2010). SMA is now treated by Nusinersen which is a 2'-OMe phosphorothioate ASO that can target exon 7 which in SMN2 pre-mRNA. This facilitates exon 7's inclusion and leads to the production of functional protein (Wertz & Sahin, 2016). Finally, in 2016, FDA (U.S. Food and drug administration) approved Nusinersen as an ASO to be used in SMA patients, and so far it remains as the only FDA approved the medication for SMA (Aartsma-Rus, 2017).



**Figure 20 SMA and its treatment by ASO:**

### Example of a disease caused by a mutation in the 5' splice site

**Familial dysautonomia (FD)** FD also referred to as **Riley–Day syndrome**, (hereditary sensory and autonomic neuropathy type III) is a sporadic, autosomal recessive fatal disorder caused by loss of function of the i-kappa-B kinase complex-associated protein (IKBKAP) (Slaugenhaupt et al., 2002) (F. B. Axelrod & Kaufmann, 2014). In virtually all the FD patients, 5' splice site of exon 20 carries a mutation. This mutation leads to skipping of the exon 20 thus introducing a PTC. This is detected by mRNA surveillance, and truncated protein is directed towards NMD for degradation. This further correlates with the neurological dysfunction in FD patients (Close et al., 2006). Splicing modification therapy via small molecule Kinetin (6-furfurylaminopurine) has been shown to increase wild-type *IKBKAP* mRNA and IKAP protein and thus can be used to treat FD (Felicia B. Axelrod et al., 2011).

### Example of a disease caused by an intronic mutation that activates a cryptic splice site:

It has been shown that mutations in Lamin A lead to **Hutchinson–Gilford progeria syndrome (HGPS)**, a dominant disorder characterized by the dramatic, rapid appearance of aging beginning in childhood due to translation of ageing associated protein progerin (Eriksson et al., 2003) (De Sandre-Giovannoli et al., 2003). Most of the typical Hutchinson–

Gilford progeria cases are due to a recurrent, *de novo* point mutation in LMNA exon 11. This mutation resides in a splicing enhancer sequence which as a result leads to activation of a cryptic splicing site resulting in the truncated Lamin A protein.

The use of antisense oligonucleotides to sterically block the aberrant *LMNA* splicing site in progerin production has been shown *in vitro* on human HGPS patient's cells and *in vivo* in the mouse model (Osorio et al., 2011).

#### **Example of disease caused by a mutation in core components of the spliceosome:**

**Retinitis pigmentosa (RP)** is inherited a degenerative retinal disorder that is caused by germline mutations in core spliceosomal proteins. More than 3,000 mutations in about 80 genes have been identified to be associated with RP Reviewed by (M. M. Liu & Zack, 2013). Moreover, mutations in RNU4ATAC gene which encodes U4atac, a small nuclear RNA that is a crucial component of the minor spliceosome leads to **Microcephalic osteodysplastic primordial dwarfism type 1 (MOPD I)**. Reviewed in (Nagy et al., 2012)

Although there is currently no therapy for the treatment of RP, (Nakamura et al., 2017) reported the discovery of a small drug-like molecule, called Photoregulin3, which alters the activity of a transcription factor that regulates rod genes. So far, it has been successfully tested in the mice model for RP. However, clinical trials in humans are underway.

#### **1.5.1 Conclusion:**

Breakthroughs in high-throughput sequencing technologies promise to identify and link many more human mutations to disease. Moreover, small-molecule compounds, antisense oligonucleotides, and genome-editing approaches hold promise for correcting the effects of these mutations common in human genetic variation and disease. ASO especially have a high potential in future therapy, for an example Eteplirsen, an ASO is now being used to treat DMD, ASOs to treat ALS are already being investigated and show promising therapeutic potential (C. A. Stein & Castanotto, 2017). (Le et al., 2019) have described the scope of ASOs that can target angiogenic factors and act as therapeutic molecules for the treatment of cancer. Small molecules such as a family of spliceostatins (derived from *Pseudomonas*) and herboxidienes and pladienolides (derived from *Streptomyces*) have been shown to target core spliceosome machinery and mediate anticancer properties. Reviewed in (J. C. Lin, 2018). Further, a new strategy referred to as SMaRT<sup>TM</sup> (Spliceosome-mediated RNA trans-splicing as a tool for gene therapy) is developed, it is a system used to correct a part of the mutant transcript instead of full-length cDNA. (Puttaraju, Jamison, Mansfield, Garcia-Blanco, &

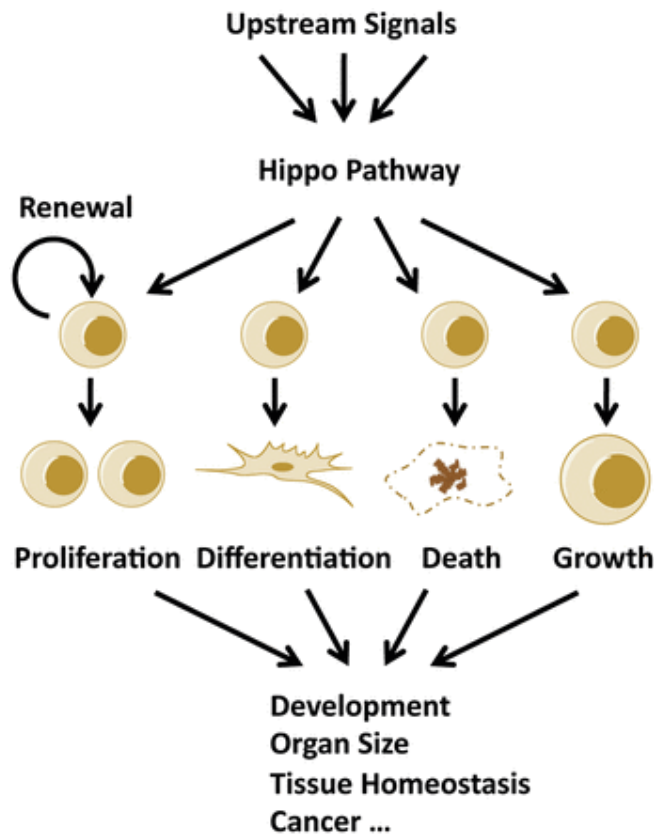
Mitchell, 1999). Recently (Dooley et al., 2018) have developed a Spliceosome-mediated RNA *trans*-splicing strategy to edit and rescue the expression of *CEP290* mRNA which is associated with Leber congenital amaurosis type 10 (LCA10)( The visual impairment) and Joubert Syndrome (JS) (a rare, autosomal recessive disorder characterized by episodes of hyperpnea, developmental delays, ataxia, and abnormal eye movements).

## 1.6 Hippo pathway and its discovery:

Researchers around the globe have a long-standing quest to underpin the exact mechanism of organ size control. Indeed it is quite intriguing to know what precise and complex set of mechanisms control the cell number during development and regeneration. Many studies have pointed towards the fact that growth to the right size and patterning to an exact shape is intrinsic information that is in possession of the organ. For example, In the *Salamander*, when eyes or limbs are exchanged between different species with different organ size, they reach a size characteristic of the donor (Twitty & Schwind, 1931). A similar experiment in *Drosophila* showed that immature imaginal discs, when transplanted in a growth-permissive environment such as the abdomen of an adult female, grow at their normal rate until they reach their final size (Bryant & Simpson, 1984).

During the course of development, an increase in cell number is required for the growth of the organ and body size. During the normal function of an organ, processes such as continuous cell turnover, apoptosis and occasional differentiation of adult stem cells to replace dysfunctional cells are required to maintain the functionality of the organ. During an injury or pathology, the process of healing accompanied by cell division and regeneration is required to compensate for the lost cells and restore the standard functionality. Although the mechanism of proliferation, apoptosis, regeneration are extensively studied, the coordinated between these events are poorly understood to this date and mechanisms underlying these molecular events are yet to be elucidated. However, the discovery and expansion of Hippo pathway have proven to be an entry point for the researcher in a quest to answering this fundamental question as Hippo signaling pathway has been shown to play a critical role in controlling organ size by regulating both cell proliferation and apoptosis. (W. Kim & Jho, 2018).

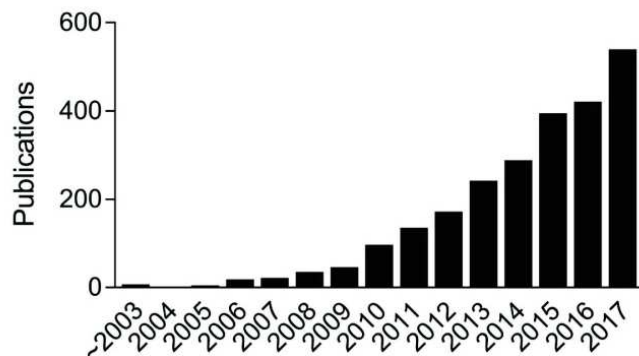
As of now, the Hippo Pathway has the status of a highly evolutionary conserved regulator of tissue growth and cell fate. Since its discovery, it has panned out as an integrator of signals from a vast network of proteins and signaling pathways and serves as an essential regulator of critical cellular processes such as organ size regulation, cell proliferation, and stem cell regulation(Harvey, Zhang, & Thomas, 2013) (Figure 21).



**Figure 21 Implications of the Hippo pathway in cell biology:**

The Hippo pathway modulates cell proliferation, differentiation, growth, and death. The coordination of different cellular processes by the Hippo pathway may contribute to various physiological and pathological conditions such as development, tissue homeostasis, and tumorigenesis.

Within the last 25 years, the field of the Hippo pathway has rapidly emerged as one of the most popular pathways due to its widespread roles in processes vital to the development. Thus it has been on the radar of researches around the globe in a race to identify its components, regulators and downstream effectors. There are a plethora of reviews with new insights and advancements in the field every year which are rapidly growing every year (Figure 22)



**Figure 22 The rise of Hippo pathway:**

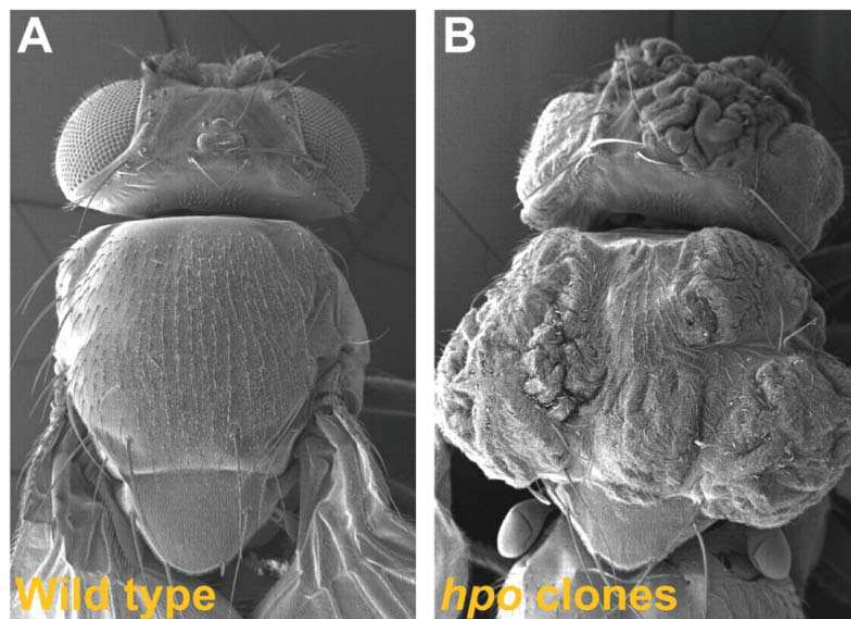
The rising number of papers being published every year in the field of the Hippo Pathway.

The Hippo pathway transduces signals from the plasma membrane to the nucleus and subsequently activates the transcription of genes involved in cell proliferation, anti-apoptosis

and cell survival (Chan, Lim, Chen, et al., 2011) (Meng, Moroishi, & Guan, 2016). The Hippo pathway is activated in response to several upstream signals such as cell-cell contact and ECM (Extracellular matrix) cytoskeleton *etc.* which are described in the forthcoming sections. Both in *Drosophila* and Mammals, The Hippo pathway functions via three interlinked parts, i.e. core kinase components, upstream regulatory elements and the downstream effectors of the pathway.

### 1.7 Hippo Pathway in *Drosophila*

Several components of the Hippo pathway were discovered in *Drosophila* during a genetic mosaic screening to look for mutants resulting in tissue overgrowth. First component of the pathway to be found was NDR (Nuclear Dbf2-related) family protein kinase Warts (Wts) in 1995, (LATS1/2) by (Justice, Zilian, Woods, Noll, & Bryant, 1995)(Xu, Wang, Zhang, Stewart, & Yu, 1995) followed by the WW domain-containing protein Salvador (Sav) in 2002 by (Tapon et al., 2002)(Kango-Singh, 2002). Later in 2003, Hippo (Hpo) (S. Wu, Huang, Dong, & Pan, 2003) (Harvey, Pflieger, & Hariharan, 2003a) and in 2005 Mob-as tumor suppressor (Mats) (Z. C. Lai et al., 2005), were identified and found to be essential tumor suppressors, loss of function of any of these genes result in tissue overgrowth (Figure 23).



**Figure 23 Hippo mutant phenotypes in flies:**

(A,B) Scanning electron micrographs of (A) a wild-type fly and (B) a fly with clones of cells homozygous mutant for *hippo* that exhibit overgrowth of the adult cuticle Adapted from (G. Halder & Johnson, 2011)

The name of *hippo* gene was established based on its mutant adult head phenotype which mirrors the creased hide of hippopotamus (Udan, Kango-Singh, Nolo, Tao, & Halder, 2003)

However, the Hippo Pathway field hit a significant milestone in 2005 when **Yorkie (Yki)**, was discovered in a yeast two-hybrid screen for Wts binding proteins (J. Huang, Wu, Barrera, Matthews, & Pan, 2005). Biochemical and genetic studies revealed that Yki is required for normal tissue growth and its activity is inhibited by Wts-mediated phosphorylation. Yki was established as a central transcriptional regulator of the Hippo pathway as downregulation of Yki lead to diminish the overgrowth phenotype caused by deleting upstream kinase Hpo or Wts. Yki acts as an vital growth promoter, with loss of Yki resulting in a restriction of tissue growth in *Drosophila* and overexpression resulted in overgrowth phenotypes that resembled mutation in *hpo*, *sav*, *mats*, and *wts* (J. Huang et al., 2005). Although, it was found that CycE and DIAP1 were transcriptional targets of Yki but the lack of DNA binding domain led to investigate the transcription factors that could mediate Yki activity. It was 2008 when at least three groups concluded that Scalloped (Sd) (TEAD family of transcription factors in Mammals) as Yki DNA binding partner via yeast 2 hybrid experiments (Q. Zhang et al., 2008)(S. Wu, Liu, Zheng, Dong, & Pan, 2008) (Goulev et al., 2008).

As shown in (Figure 24), In *Drosophila* When Hippo Pathway is in **ON state**, i.e., activated, Hpo forms a complex with Sav. This complex phosphorylates Wts which is in complex with its co-factor Mats. This phosphorylation activates Wts/Mats kinase which subsequently phosphorylates Yki as three separate phosphorylation sites, S111, S168 and S250. Phosphorylation of Yki at S168 site leads to its cytoplasmic retention as it binds to 14-3-3, therefore, inhibiting its transcriptional activation role (H. Oh & Irvine, 2009)(H. Oh & Irvine, 2008) Recently, Misshapen (Msn) and Ste-20 kinase Happyhour (Hppy) kinases were shown to act in parallel to Hpo to phosphorylate Wts (Q. Li et al., 2014)(Meng et al., 2015)(Pan et al., 2015). Two kinases Tao and Par-1 directly regulate the activity of Hpo via phosphorylation. Tao has been shown to phosphorylate the activation loop of Hpo to promote its activity (Boggiano, Vanderzalm, & Fehon, 2011) (Poon, Lin, Zhang, & Harvey, 2011). While on the other hand, polarity kinase Par-1 restricts Hpo by promoting its phosphorylation at a different site to restrict its activity. Further, Par-1 inhibits the interaction between Hpo and Sav to promote Sav dephosphorylation and destabilization, further downregulating Hippo pathway activity (H. L. Huang et al., 2013)

Recently, (X. Sun et al., 2019) showed that deubiquitinase Usp7 positively regulates Yki transcriptional program by deubiquitinating Yki. Further, they showed that Hpo or warts inhibit the Usp7-Yki interaction not only to promote Yki's nuclear accumulation but also to promote Yki nuclear degradation. This study provides new insight into how the Hippo pathway negatively regulates the activity of Yki.



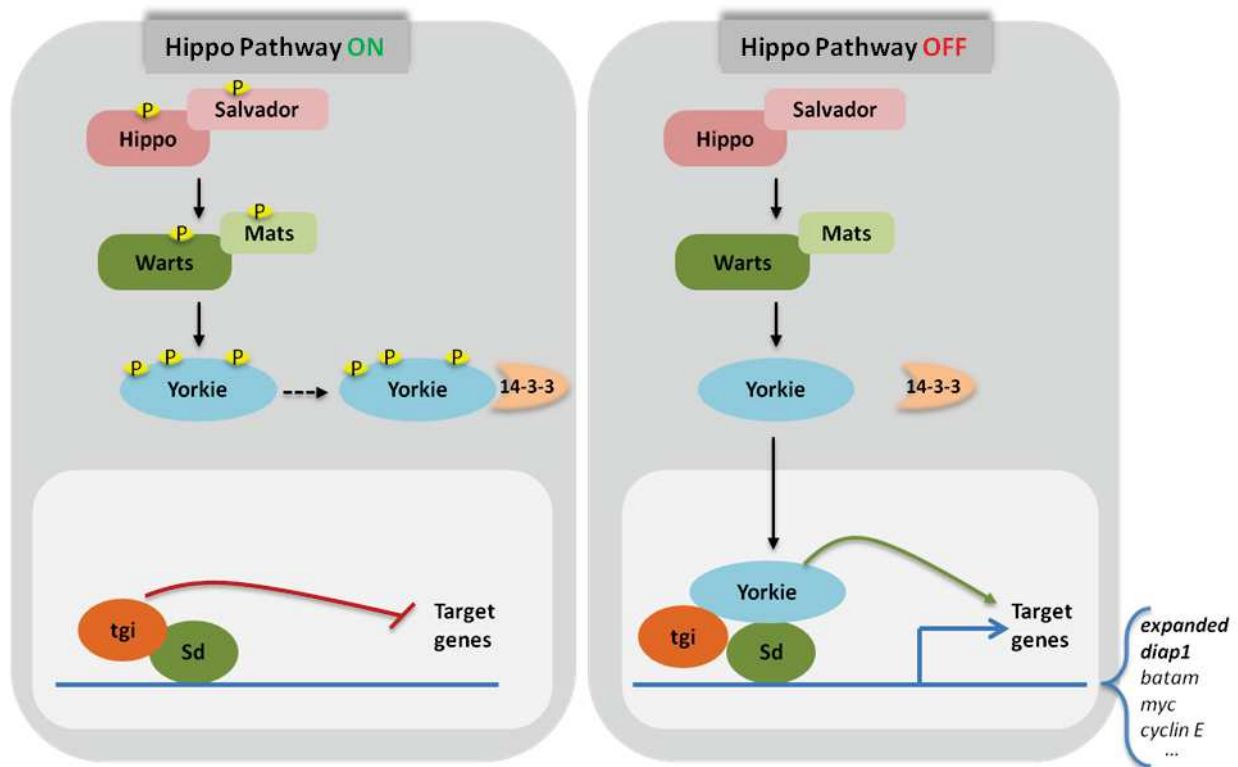


Figure 24 Schematics of the Hippo pathway in *Drosophila melanogaster*:

In contrast, when the Hippo pathway is in **OFF state**, i.e. inactivated, hypophosphorylated Yki gets translocated to the nucleus, it competes with Tondu domain-containing growth inhibitor (Tgi) to bind with transcription factor Scalloped (Sd) to regulate their transcriptional coactivator activity on several target genes (Figure 24). According to (Guo et al., 2013) Sd-Binding-Protein (SdBP) (SdBP here is Tgi) is a negative regulator of Sd-Yki complex. They show that Tgi simultaneously binds to Sd through TDU domains and Yki via PPxY motifs to disrupt the direct Sd-Yki complex and proceeds to form a transcriptionally inactive ternary complex which results in inhibition of Yki activity. They further show a competition between Tgi and Yki in binding to Sd. On the other hand, (Koontz et al., 2013) reported that Tgi binds to Sd in the absence of Yki and repress gene expression. When Yki is in the nucleus, it displaces the Tgi and converts Sd into a transcriptional activator.

Besides Sd, the transcription factor Homothorax (Hth) a TALE-homeodomain protein (the homolog of the MEIS homeodomain protein) and zinc finger transcription factor Teashirt (Tsh) has been shown to be a transcription factor of Yki in the eye disc that regulates microRNA *bantam* to promote proliferation and protect eye progenitor cells from apoptosis (Peng, Slattery, & Mann, 2009). The expression of *bantam* is also regulated by another transcriptional complex between Yki and Mad, an effector of Dpp signaling (Hyangye Oh &



Irvine, 2011). Yki interaction with different binding partners might, therefore, be tissue-dependent, and the availability of Yki partners could be a critical factor in regulating Hippo pathway activity.

The relationship between transcription and chromatin occurs at two levels. First, the nucleosome is regulated by ATP-dependent chromatin remodeling complex such as SWI/SNF family complexes mobilize nucleosomes, facilitating the access of the transcription apparatus to DNA. Secondly, the histone-modifying enzymes that add or remove chemical groups on histones leading to compaction of DNA, which prevents or enhances binding of transcriptional factors to DNA. Indeed Yki, has been shown to interact with GAGA factors (which influences the chromatin structure and) and Brahma complex (one of the *Drosophila* SWI/SNF complexes) and Nuclear receptor coactivator 6 (Ncoa6), a subunit of the Trithorax-related (Trr) histone H3 lysine 4 (H3K4) methyltransferase complex to regulate the transcription (Y. Jin et al., 2013)(Hyangyeon Oh et al., 2013) (Qing et al., 2014)

Several target genes of Yki are *cycE*, the inhibitor of apoptosis *diap1*, *myc*, miRNA *bantam* which collectively drive cell proliferation, inhibition of apoptosis and survival (J. Huang et al., 2005)(Tapon et al., 2002)(Thompson & Cohen, 2006)(Udan et al., 2003)(Q. Zhang et al., 2008)(Ziosi et al., 2010). Another interesting example for the last class of genes is the recent identification of Cactus (Cact), an effector of Toll pathway as a direct target of Yki. Cact is characterized by the presence of ankyrin repeats and functions to suppress the transcription of antimicrobial peptides introduced by gram-positive bacterial infection. This discovery further expands the regulation of which revealed an intriguing link between the Hippo pathway and innate immunity(Bo Liu et al., 2016).

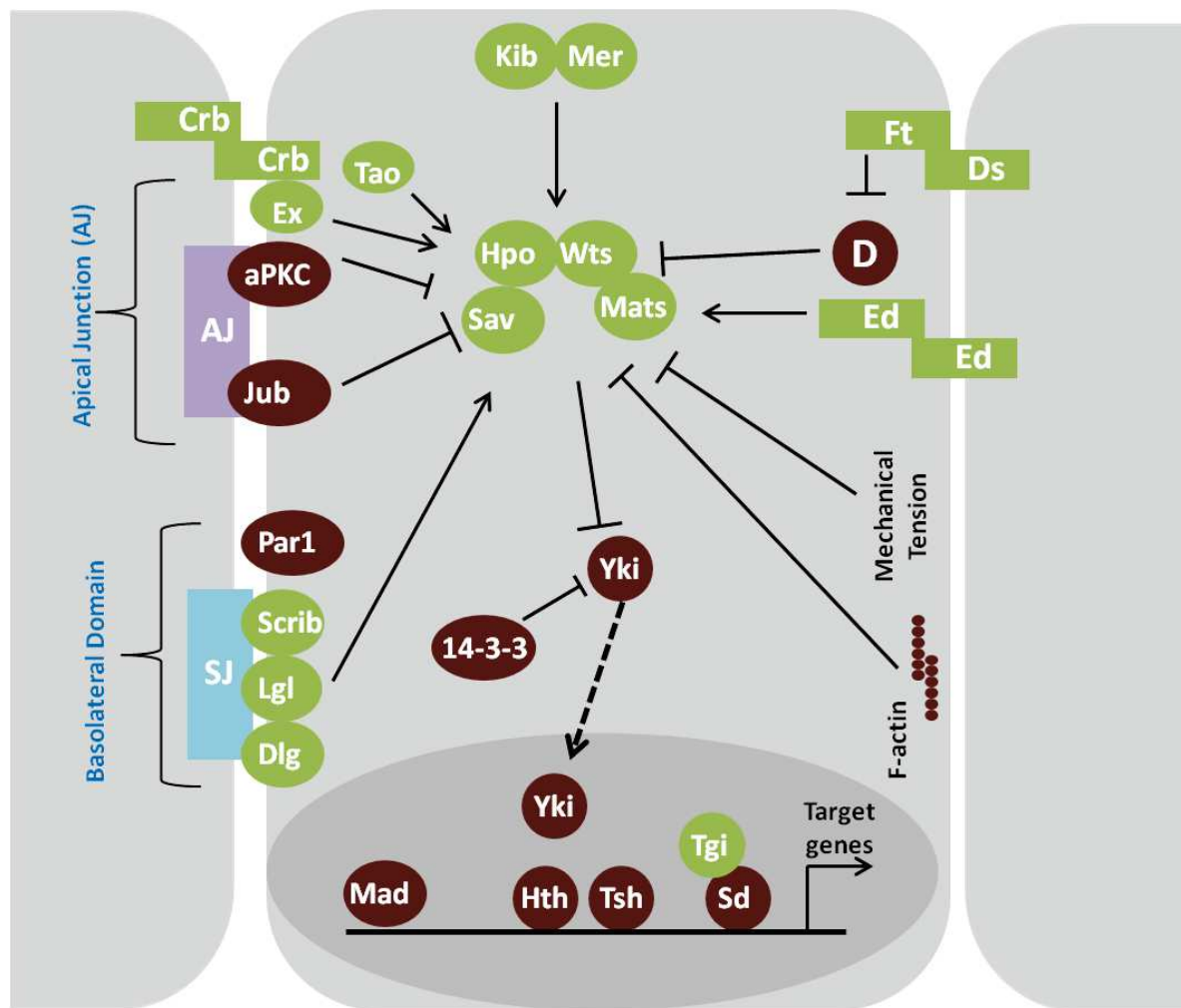
Therefore, Yki, the effector of the Hippo pathway functions to promote growth and proliferation whereas the kinase of the pathway acts as tumor suppressors as they function to suppress the activity of the Yki.

The downstream target genes of the Hippo pathway are essential in the pathway feedback. For instance, Ex, a cytoskeletal protein for cell morphology, is not only a transcriptional target of Hippo pathway but also an upstream regulator of the pathway, therefore forming a negative feedback loop in growth control regulation (Jukam et al., 2013).

### **1.7.1 Upstream regulators of *Drosophila* Hippo Pathway:**

Core components of the Hippo pathway have been studied extensively to this date. Unlike typical signaling cascades, the Hippo pathway is not regulated by a defined set of ligand-receptor interactions, but it is rather modulated by a diverse set of signals from upstream

constituents. However, what precise signals activate the kinase cascade to suppress the activity of transcriptional co-activators is not yet completely understood. Thus, several researchers were focused on to elucidate the regulators of the Hippo pathway. So far, The upstream signals that are known to regulate the Hippo pathway such as transmembrane receptors atypical cadherins Fat(Ft) and Dachous1/2 (Dchs), Kibra-Expanded/Merlin complex and apicobasal polarity complex, i.e., members of crumbs polarity complex play roles in maintaining the cell architecture such as cell polarity and cytoskeleton. The expansion of such knowledge can provide insight into what signals direct cells to adhere to a pattern of growth and when to inhibit the growth (Figure 25).



**Figure 25 Upstream regulators of Hippo pathway in *Drosophila*:**

It is regulated by various factors including the following aPKC, atypical protein kinase C; Crb, Crumbs; D, Dachs; Dlg, Discs large; Ds, Dachous; Ed, Echinoid; Ex, Expanded; Ft, Fat; Hth, Homothorax; Jub, Ajuba LIM protein; Lgl, Lethal giant larvae; Mad, Mothers against decapentaplegic (Dpp); Mer, Merlin; Scrib, Scribble; Sd, Scalloped; Tgi, Tondu domain-containing growth inhibitor; Tsh, Teashirt; Zyx, Zyxin. Growth-promoting proteins are depicted in brown and growth repressors in green. These proteins are described in text. AJ, adherens junction; SJ, septate junction. Adapted from (Irvine & Harvey, 2015)

### 1.7.1.1 Regulation by Fat: Planar cell polarity

The Fat-Dachsous signaling pathway is a cell-contact-dependent mechanism that regulates the Hippo pathway. Fat (Ft) large protocadherin (transmembrane protein) with a molecular weight of 560kDa (P. A. Mahoney et al., 1991). Ft is the receptor for Hippo Signaling pathway as it genetically interacts with the pathway (F. C. Bennett & Harvey, 2006) (E. Cho et al., 2006) (Silva, Tsatskis, Gardano, Tapon, & McNeill, 2006) (Willecke et al., 2006). Dachsous (Ds) another protocadherin acts as the ligand for Ft (Clark et al., 1995) Fat signaling pathway has a well-characterized role in regulating planar cell polarity (PCP) in epithelial tissue (Reddy & Irvine, 2008). Although, PCP itself does not regulate the Hippo pathway, but loss of Fat leads to overgrowth phenotype and up-regulation of Hippo pathway target genes such as *cycE* and *diap1*, as has been shown for multiple Hippo pathway components. Mechanistic studies have shown that Fat signaling can positively regulate the Hippo pathway via localizing Ex, an upstream regulator of the pathway to apical junction region (F. C. Bennett & Harvey, 2006) (E. Cho et al., 2006) (Silva et al., 2006) (Willecke et al., 2006). Another mechanism of positive regulation of Hippo signaling by fat signaling is via inhibition of Wts degradation thus promoting abundance of Wts mediated by the atypical myosin Dachs (E. Cho et al., 2006) (Rodrigues-Campos & Thompson, 2014) (Rauskolb, Pan, Reddy, Oh, & Irvine, 2011) (Bosch et al., 2014) In addition, (Vrabioiu & Struhl, 2015) showed that Fat signaling acts via Dachs to inhibit Wts from acquiring Mats-dependent active conformation to promote growth in *Drosophila* wing.

(Misra & Irvine, 2016) identified Vamana (Vam) to influence Dachs membrane localization and most recently same authors described Early girl as an essential component of Fat signaling that the levels of localization of Dachs and Vamana (Misra & Irvine, 2019).

### 1.7.1.2 Regulation by Ex/Mer/Kibra:

The FERM (N-Terminal Globular Domain [Band Four-point-one, Ezrin, Radixin and Moesin]) domain-containing protein Ex and Merlin (Mer) along with the C2 and WW domain-containing protein Kibra (Kib) are three important apical membrane proteins of the Hippo pathway that regulate Yki activity through the core kinase cascade (Baumgartner, Poernbacher, Buser, Hafen, & Stocker, 2010; Genevet, Wehr, Brain, Thompson, & Tapon, 2010a; Hamaratoglu et al., 2006; McCartney, Kulikauskas, LaJeunesse, & Fehon, 2000; J. Yu et al., 2010) The general role of FERM domain proteins is to link the F-actin cytoskeleton to cell membranes and membrane proteins as described by (Mangeat, Roy, & Martin, 1999).

Ex and Mer have been shown to physically interact with each other and loss of function of any of the two genes leads to overgrowth phenotype and deletion of Ex and Mer together results into even severe overgrowth phenotype due to upregulation of Hippo pathway target genes as they interact with hippo pathway components (McCartney et al., 2000) (Hamaratoglu et al., 2006). Similar to *ex* and *mer*, *kib* mutations too leads to overgrowth phenotype and up-regulation of Hippo pathway targets. Kib protein physically associates with both Mer and Ex and the three of them can co-localize at the apical junctional region, suggesting that these three proteins can function together to regulate the Hippo pathway. Interestingly Ex, Mer, and Kib can each function independently of each other in the regulation of the Hippo pathway as revealed by the genetic analysis studies (Hamaratoglu et al., 2006); (Baumgartner et al., 2010) (J. Yu et al., 2010). Studies have revealed that Ex, Mer, and Kib regulate the Hippo pathway positively by recruiting the pathway components to specific cortical domains to promote their activation. (Yin et al., 2013) demonstrated that Mer could also directly recruit Wts to the plasma membrane to activate Hippo signaling, independently of Hpo kinase activity. Generally, Ex can function as a scaffold that recruits Wts to the apical junctional region from its inhibitor Jub where it interacts Hpo and gets activated (S. Sun, Reddy, & Irvine, 2015). (Su, Ludwig, Xu, & Fehon, 2017) have demonstrated that Mer and Kib can activate Hippo signaling in parallel to Ex at a spatially distinct cellular domain, the apical cortex. Together, Ex, Mer, and Kibra may activate Hippo pathway at distinct sub-cellular locations, possibly mediating different inputs of the Hippo pathway. Alternatively, Ex can also regulate the pathway by physically binding with Yki and sequestering it outside of the nucleus acting independently of kinase cascade (Badouel et al., 2009). In addition to acting as upstream regulators of the Hippo pathway, Ex, Mer, and Kib are transcriptional targets of the Hippo pathway too (Hamaratoglu et al., 2006) (Genevet, Wehr, Brain, Thompson, & Tapon, 2010b). This suggests the existence of a negative feedback loop in which Yki activation turns on the expression of positive regulators of the Hippo pathway (Boggiano & Fehon, 2012) (Irvine & Harvey, 2015)

### **1.7.1.3 Regulation by Apical-basal polarity proteins:**

Apical polarity proteins are located in the apical cell cortex. They have been shown to regulate the Hippo pathway. Crumbs (Crb) was the first apical polarity protein found to be involved in Hippo pathway regulation. In *Drosophila* embryos, Crb is localized at the subapical plasma membrane and plays a vital role in organizing apical-basal polarity (Tepass, Theres, & Knust, 1990). Both loss of Crb and constitutive expression leads to Yki activation,

up-regulated Hippo target genes, and overgrowth. Crb has been shown to interact with Ex via its FERM domain and recruits Ex to the apical junctional (AJs) region to promote Hippo pathway activity (C.-L. Chen et al., 2010) (Ling et al., 2010) (Robinson, Huang, Hong, & Moberg, 2010) (Grzeschik, Parsons, Allott, Harvey, & Richardson, 2010). On the other hand, it has also been demonstrated that Crb promotes ubiquitin-mediated degradation of Ex via E3 ubiquitin ligase F-box protein Slimb/ $\beta$ -TrCP (Slimb) suggesting that Crb allows precise tuning of Hippo pathway activity through its dual regulation of Ex (Ribeiro, Holder, Frith, Snijders, & Tapon, 2014).

The Par complex consists of atypical protein kinase C (aPKC), its regulatory subunit Par6, the Rho-GTPase Cdc42, and the scaffolding protein Bazooka (Baz; *Drosophila* homolog of the *C. elegans* and mammalian Par3). aPKC is involved in the regulation of the Hippo pathway. Expression of a dominant-negative aPKC transgene suppresses Hippo pathway inactivation in *Drosophila lethal giant larvae (lgl)* clones (Grzeschik et al., 2010). Overexpression of aPKC can cause mislocalization of Hpo, activation of Yki, and up-regulation of Hippo pathway target genes (Grzeschik et al., 2010) (G. Sun & Irvine, 2011)

Lethal giant larvae (Lgl), Discs large (Dlg), and Scribble (Scrib) are the basolateral polarity protein genes which were first described as “neoplastic” tumor-suppressors as depletion of any of these genes in resulted in multilayered and invasive tumors in *Drosophila* imaginal discs (Bilder, 2004). As studies linked overgrowth phenotype to over-activation of Yki, these three basolateral polarity proteins are regarded as positive regulators of the Hippo pathway (Richardson & Portela, 2017)(Enomoto & Igaki, 2011).

Loss of Lgl in the eye of *Drosophila* does not disrupt cell polarity or activate Jun kinase signaling but mislocalizes Hpo, suggesting Lgl participates in localization of Hpo (Bogoyevitch, Ngoei, Zhao, Yeap, & Ng, 2010) (Grzeschik et al., 2010). While on the other hand, loss of Lgl via RNAi in wing imaginal disc, disrupts cell polarity and causes activation of JNK signaling leading to Yki activation suggesting the regulation of Yki activity via Lgl is context dependent (G. Sun & Irvine, 2011). Further, the same authors suggested that JNK activates the Wts inhibitor Jub via phosphorylation providing a link between basolateral polarity, JNK signaling and Yki activity (G. Sun & Irvine, 2013).

*scrib* mutant clones are eliminated by cell competition, a phenomenon of removal of “weak cells” by surrounding “fit cells” (Grzeschik et al., 2010) (C.-L. Chen, Schroeder, Kango-Singh, Tao, & Halder, 2012). Further,(Grzeschik et al., 2010) showed that loss of Scrib via RNAi leads to down-regulation of Hippo pathway target *diap1* suggesting that Yki is inactivated in *scrib* mutant clones. However when (C.-L. Chen et al., 2012), blocked JNK

signaling in *scrib* mutant clones they observed Yki activation and a severe overgrowth phenotype, suggesting JNK signaling is responsible for repressing Yki activity in *scrib* clones and this effect outweighs the activation of Yki by loss of Scrib. Although, the mechanism of repression of Yki by JNK signaling in this scenario is unknown, (Enomoto & Igaki, 2011) suggests that JNK can inhibit Yki activity via Wts.

#### 1.7.1.4 Cell-Cell adhesion and junctional proteins

Cell-cell adhesion and junctional proteins such as Ajuba LIM protein (Jub), Zyxin, and Echinoid regulate the Hippo pathway in *Drosophila*. Jub is localized at the apical junctions stabilizes preassembled cadherin complexes (Nola et al., 2011). Down-regulation of Jub resembles Yki down-regulation phenotypes. For example, it leads to down-regulation of Hippo pathway target genes such as *diap1* and *cycE* resulting in tissue undergrowth. Also, *jub* has been shown to genetically interact with the Hippo pathway components (Das Thakur et al., 2010). (Rauskolb, Sun, Sun, Pan, & Irvine, 2014) showed that Jub sequesters and inhibits Wts at the adherens junctions (AJs) to inhibit hippo pathway activity.

Zyxin (Zyx) which is another LIM domain protein, acts as a negative regulator of the Hippo pathway as it is required for the activity of Yki (Rauskolb et al., 2011) (Gaspar, Holder, Aerne, Janody, & Tapon, 2015). Depletion of Zyx leads to reduced Yorkie activity and undergrowth. (Rauskolb et al., 2011) also reported that at apical junctional region Zyx cooperates with Dach5 to promote Wts degradation thus inhibiting Hippo pathway activity linking Zyx to Fat signaling branch of Hippo pathway regulation. (Gaspar et al., 2015) demonstrated that Yki activity and growth promoting ability of Zyx depends upon its interaction with the actin-associated protein Enabled (Ena). More importantly, Ena promotes F-actin polymerization linking Zyx with F-actin branch of the Hippo pathway. (Hirata, Tatsumi, & Sokabe, 2008) showed that Zyx is associated with mechanical-force-dependent facilitation of actin polymerization at cell adhesive structures; thus this group was the first to hint at the possibility of mechanical cues regulating the Hippo pathway.

Echinoid (Ed) is an upstream regulator that is required for proper sub-apical localization of Sav (T. Yue, Tian, & Jiang, 2012). Ed physically interacts with Ex, Yki, Hpo, Kibra, and Mer. However, Ed only affects Sav localization without affecting Ex or Mer localization. Depletion of Ed activates Yki, and subsequently upregulating Hippo pathway target gene expression and tissue overgrowth. It is demonstrated in *Drosophila* that Ed physically interacts and stabilizes and therefore inactivates Yki activity (T. Yue et al., 2012). Echinoid (Ed) is a known receptor of the EGFR pathway (Bai et al., 2001).



The upstream regulators of the Hippo pathway include the actin cytoskeleton beneath the plasma membrane. Indeed, cytoskeleton network F-actin has been demonstrated to be a negative regulator of the Hippo pathway. Ectopic accumulation of F-actin caused by Depletion of Capping proteins Capulet (an actin-binding protein that inhibits polymerization), or constitutive expression of an activated version Diaphanous Dia; induces actin polymerization resulted in increased Yki activity and quite a substantial overgrowth (Fernandez et al., 2011) (Sansores-Garcia et al., 2011). It has also been reported that mutations in Hippo pathway genes *ex*, *mer*, *hpo*, *sav*, *wts*, and *mats* leads to an increased F-actin accumulation. This could mean that F-actin and Hippo pathway can mutually suppress each other suggesting that in normal conditions, levels of F-actin levels sustain the Hippo pathway as the activation of the Hippo pathway will suppress its negative regulator F-actin. However, regulation of the Hippo pathway by F-actin remain poorly understood to this date especially in *Drosophila*. Further, it has been observed that tension regulates the Hippo pathway in *Drosophila*.

Spectrin is a vital cytoskeleton protein that supports the integrity of cell structures. *Drosophila* encodes one  $\alpha$  subunit ( $\alpha$ -Spec) and two  $\beta$  subunits ( $\beta_{\text{Heavy}}$ -Spec or  $\beta$ -Spec).  $\alpha$ -Spec is localized along the apical-basal membrane whereas  $\beta$ - and  $\beta_{\text{H}}$ -Spec is localized at the basolateral and apical membrane, respectively. Spectrin forms hexagonal networks at the cell cortex in all animal cells and has been reported to have mechanosensory properties. Spectrin functions in parallel to the Jub-Wts pathway and loss of spectrin result in Yki activation due to increased tension mediated by activation of the actomyosin network (Deng et al., 2015) (Fletcher, Lucas, Brain, Tournier, & Thompson, 2012). This indicates that Spectrin can relay mechanical cues to the Hippo pathway.

In their review (Hariharan, 2015) further postulated on how tension can regulate the pathway. During growth *Drosophila* imaginal discs contains high levels of growth promoting morphogens in the center. One could imagine that due to this, higher growth proliferation would be focussed at the center but on the contrary uniform growth is observed in the disc, suggesting that levels of morphogen alone do not dictate the growth in the wings. As the growth takes place in the center of growing tissue, it also stretches the periphery. The resulting tension could activate Yki to promote growth. Thus, Yki activation at the periphery could compensate for non-uniform morphogen levels across tissues and promote uniform tissue growth (Hariharan, 2015) (Aegerter-Wilmsen, Aegerter, Hafen, & Basler, 2007) (Aegerter-Wilmsen et al., 2012). Another study showed that artificially increased growth in clones of wing imaginal discs reduces cytoskeletal tension, which in turn decreases the

activation of Yki. This study suggests mechanical feedback in which increased Yki activation and cell proliferation reduces tension and deactivates Yki. Such feedback could contribute to evenly distributed growth (Y. Pan, Heemskerk, Ibar, Shraiman, & Irvine, 2016)

A hippo signaling pathway is highly conserved between *Drosophila* and Humans. In order to ease further understanding, I am at this moment, giving a table to highlight the core components of the Hippo pathway in *Drosophila* and their homologs in Mammalian Pathway.

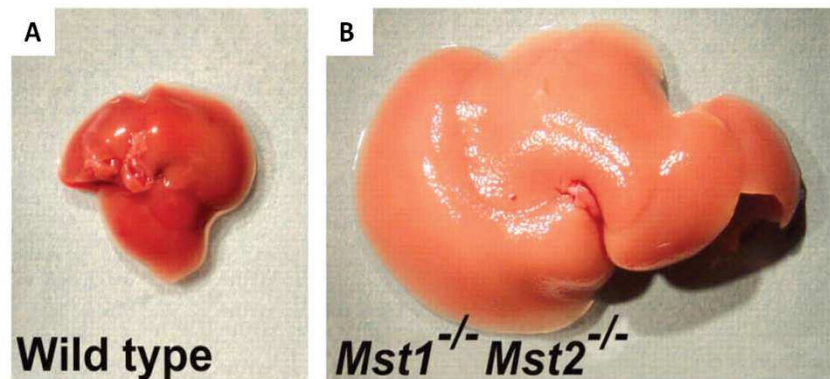
**Table 1 : Conservation of Hpo pathway core components between *Drosophila* and mammals.**

<b>Drosophila Melanogaster proteins</b>	<b>Human proteins</b>
<b>Core components</b>	
Hippo (Hpo)	Serine/threonine kinase 4/3 MST1/MST2 or STK4/STK3
Salvador (Sav)	Salvador (SAV1 or WW45)
Warts (Wts)	Large tumor suppressor Kinase 1/2 (LATS1/2)
MOB as tumor suppressors (Mats)	Mps one binder kinase activator-like 1A/1B (MOB1A/MOB1B or collectively MOB1)
Yorkie (Yki)	Yes-associated protein (YAP), Transcriptional co-activator with PDZ binding motif (TAZ) or (WWTR1)
<b>Upstream Modulators</b>	
Fat (Fat)	Angiotensin AMOT
Expanded (Ex)	Ferm domain-containing protein 6 (FRMD6)
Merlin (Mer)	Neurofibromin (NF2)
Kibra (Kib)	Kibra (KBR)
Crumbs (Crb)	CRB1–CRB3
Ras association family member (Rassf)	Ras association domain-containing protein 1–6 (RASSF1-6)
Scrib	SCRIB
Mask	MASK1, MASK2
Wbp2	WBP2
Dlg	DLG1–DLG4
Lgl	LGL1, LGL2
Jub	Ajuba, LIMD1, WTIP
<b>Downstream mediators</b>	
Scalloped (Sd)	TEA domain family member 1/2/3/4 (TEAD1, TEAD2, TEAD3, TEAD4)
Tondu-domain-Containing growth inhibitor (Tgi)	Transcription cofactor vestigial-like protein 4 (VGLL4)
Homothorax (Hth)	Meis1
Tsh	-----
Mad	Mothers against DPP (MAD).



## 1.8 Hippo Pathway in Mammals

Several components of the mammalian Hippo pathway were known prior to the formal discovery of the Hippo pathway in *Drosophila*. For example, before MOB1 was recognized as a tumor suppressor in the eukaryotes, it was known in yeast to be essential for mitosis and maintenance of the ploidy (Luca & Winey, 1998). Similarly, before the identification of *MST1* as a tumor suppressor, in yeast, it was known to be activated by caspases and mediate cell death. *Mst1* codes for the mammalian Ste20 kinase and its interaction with MAPK, p38, MKK6, MK7, and SAPK were also reported (Creasy & Chernoff, 1995). The Lats1 kinase was known as NDR kinase in yeast cells and was known to function in cell division and morphology maintenance (T. Millward, Cron, & Hemmings, 1995) (T. A. Millward, Heizmann, Schäfer, & Hammings, 1998). However, the role of these proteins in the Hippo pathway was unknown. Soon after the Hippo pathway was discovered in *Drosophila*, studies in mammals lead to the identification of orthologs of fly Hippo pathway components leading to the establishment of the mammalian Hippo pathway soon after. In 2003, MST was identified as a core kinase of the Hippo pathway in mammals (Harvey, Pflieger, & Hariharan, 2003b) (S. Wu et al., 2003). By 2008 when (Hao, Chun, Cheung, Rashidi, & Yang, 2008) (B. Zhao et al., 2007) showed that LATS1/2 Kinase negatively regulates YAP/TAZ core of Hippo pathway was considered to be complete in mammals.



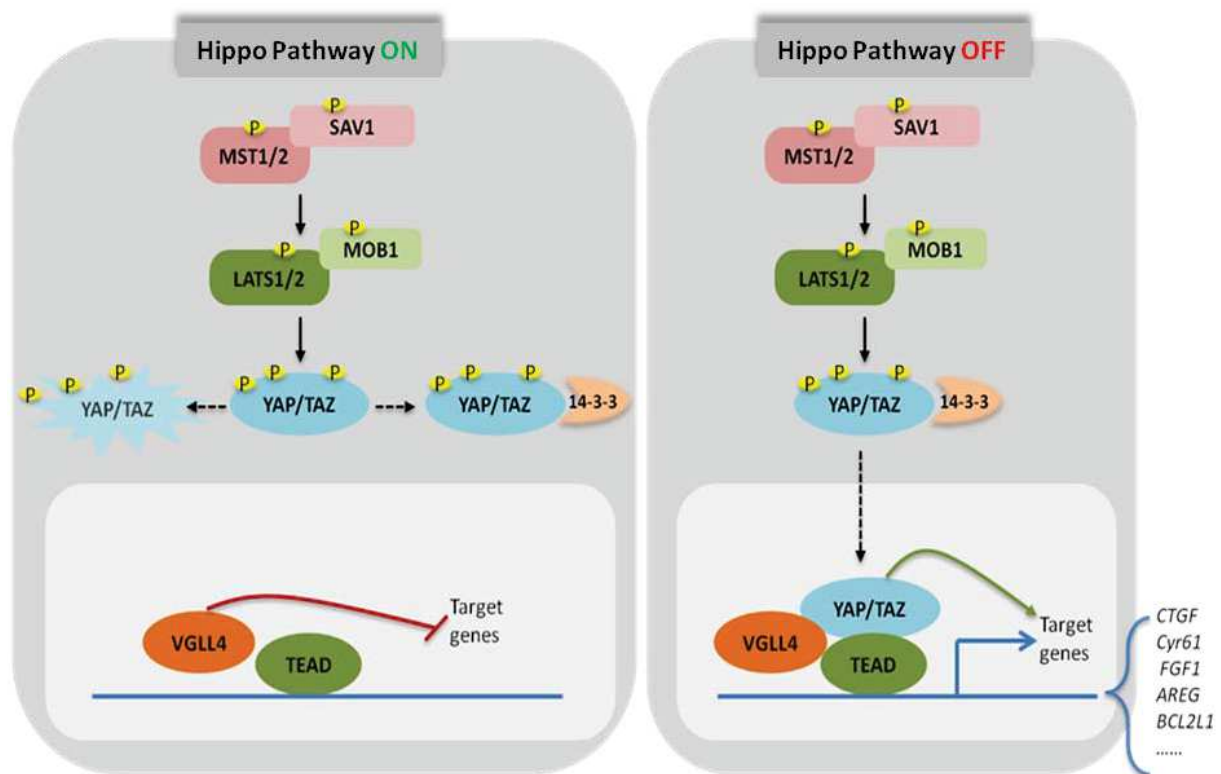
**Figure 26 (A) Effect on growth due to MST knockout :**

(A) A mouse liver at two months of age from a wild-type animal and a liver at two months of age from a mouse mutant in which both *Mst1/2* have been inactivated. The double null *Mst1/2* mutant liver is overgrown owing to an increase in cell numbers. Adapted from (G. Halder & Johnson, 2011)

**The mammalian components** of the Hippo kinase cascade consist of The MST1 and MST2 kinases, adapter protein SAV1, LATS1/2 and its protein Msp-one-binder (MOB1) and together they regulate the activity of transcriptional co-activators YAP/TAZ (Yki orthologs). YAP and TAZ are homologs of the *Drosophila* Hippo pathway effector Yki. **In ON state**, i.e.,

when the Hippo pathway is active, SAV1 interacts with MST1/2 to form a complex in order to stabilize MST1/2 which leads to autophosphorylation and thus activation of MST1/2 (Callus, Verhagen, & Vaux, 2006)(Figure 27). LATS kinase which also exists in two isoforms LATS1 and LATS2 respectively interacts with adapter protein MOB1 to form a complex. This complex is eventually phosphorylated by phosphorylation of serine 909/872 within the LATS1/2 activation loop and the hydrophobic motif (LATS1 T1079 and LATS T1041). LATS1/2-MOB1 further phosphorylates transcriptional coactivator Yes-associated protein (YAP; homologous to Yki in *Drosophila*) and TAZ (transcriptional coactivator with PDZ-binding motif, WWTR1). This phosphorylation takes place on 4 and 5 HXRXXS motifs of TAZ and YAP respectively. Once phosphorylated, YAP/TAZ interacts with 14-3-3 protein gets sequestered in the cytoplasm or degraded by ubiquitination-dependent proteasomal degradation ( $\beta$ TRCP E3) depending upon which serine residue is phosphorylated. Phosphorylation of YAP at Ser381 and TAZ at Ser311 has been linked to regulation of YAP/TAZ protein stability, as it primes subsequent phosphorylation in a phosphodegron by Casein kinase 1 (CK1 $\delta/\epsilon$ ). The phosphorylated phosphodegron then recruits the  $\beta$ -TrCP E3 ligase, leading to ubiquitination and degradation (C. Y. Liu et al., 2010) (B. Zhao, Li, Tumaneng, Wang, & Guan, 2010a) (Schäfer et al., 2005)(Dong et al., 2007)(Hirabayashi et al., 2008)(Lei et al., 2008)(Oka, Mazack, & Sudol, 2008)(Siew et al., 2008). Many components of the Hippo pathway have already been shown to be degraded by ubiquitination such as YAP, LATS1/2; Expanded(Ex) (B. Zhao, Li, Tumaneng, Wang, & Guan, 2010b) (X. Yang et al., 2011)(Salah, Melino, & Aqeilan, 2011)(B. Ma et al., 2015) (W. Li et al., 2014) (X. Ma, Richardson, Xue, Xu, & Guo, 2018).

Similar to Yki, YAP/TAZ transcriptional activators lack the DNA binding domain; thus inside nucleus they interact with transcription factors (Such as TEAD1-4) via its TEAD binding domain to be brought to the gene promoter(B. Zhao et al., 2008)(H. Zhang et al., 2009)(Chan et al., 2009). So, In the scenario when **Hippo pathway is OFF**, the un-phosphorylated YAP/TAZ gets translocated to the nucleus where it competes with VGLL4 (Vestigial Like Family Member 4; homologous to Tgi in *Drosophila*) to interact with TEAD transcription factor so as to induce the transcriptional activation of several sets of gene which play crucial role in cell growth, differentiation invasion/migration and survival. Reviewed by (Meng et al., 2016). The C-terminal domain of TEAD forms a globular structure with a  $\beta$ -sandwich fold surrounded by four  $\alpha$ -helices on one side, while the N-terminal domain of YAP wraps around TEAD, forming extensive interactions (L. Chen et al., 2010)(Z. Li et al., 2010) (W. Tian, Tomchick, Luo, Pan, & Yu, 2010)



**Figure 27 Mammalian Hippo Pathway:**

Hippo pathway is highly conserved from *Drosophila Melanogaster* to human, schematic representing ON and OFF state the pathway in mammals

YAP interacts with promoters and distal enhancers to regulate target gene expression, through mechanisms of modulating chromatin looping and Pol II pausing release(Lian et al., 2010) (Galli, Carrara, et al., 2015) (Zanconato et al., 2015). YAP/TAZ associate with a plethora of transcription factors in addition to TEAD1-4 including YAP binding partners SMAD1/2/3/7 (Ferrigno et al., 2002) (Kurisaki, Kose, Yoneda, Heldin, & Moustakas, 2001) (Alarcón et al., 2009) (Varelas et al., 2008), Runt-Related Transcription Factor (RUNX) (Zaidi et al., 2004) Erb-B2 Receptor Tyrosine Kinase 4 (ErbB4) (Komuro, Nagai, Navin, & Sudol, 2003), p73 (Strano et al., 2001) and TAZ binding partners RUNX, Peroxisome Proliferator Activated Receptor  $\gamma$  (PPAR $\gamma$ ) (J. H. Hong et al., 2005), Paired Box 3 (Pax3) (Masao Murakami et al., 2006), T-Box Transcription Factor 5 (TBX5) (M. Murakami, Nakagawa, Olson, & Nakagawa, 2005) and Thyroid Transcription Factor 1 (TTF-1) (K. S. Park et al., 2004) Further, It has been shown that the SWI/SNF (a chromatin remodeling protein complex) and (or) NCOA6 (a histone methyltransferase protein) is recruited by YAP to stimulate TEAD activity and subsequently act as co-activators of gene expression(Qing et al., 2014a) (Skibinski et al., 2014).

On the other hand, the YAP/TEAD can also act as repressors by engaging deacetylase complexes (Valencia-Sama et al., 2015) (M. Kim, Kim, Johnson, & Lim, 2015). Multiple transcriptional downstream target genes of YAP/TAZ has been identified, such as *connective tissue growth factor (CTGF)*, *cysteine-rich 61 (Cyr61)*, *fibroblast growth factor (FGF1)*, the EGF family member *amphiregulin (AREG)*, IAP family member *BIRC5*, antiapoptotic gene *BCL2L1*, *ABCB1 (ATP Binding Cassette Subfamily B Member 1)*, ANKRD (ankyrin repeat domain-containing protein), CAT (catalase), GPATCH4 (G-Patch Domain Containing 4), LMN-B2 (Lamin-B2), PTGS2 (Prostaglandin-endoperoxide synthase 2), WSB2 (WD repeat and SOCS box-containing protein 2), and TXN (Thioredoxin) (Koontz et al., 2013) (Rosenbluh et al., 2012) and described in (Venkataramani et al., 2018). Most of the identified targets of YAP/TAZ are indispensable to growth, proliferation, control cell migration, cell lineage commitment viability, tissue specification, morphogenesis and angiogenesis embryonic development, tissue turnover, and injury repair.

A variety of upstream signals that regulate the YAP/TAZ have been identified as we have seen in the case of *Drosophila* Hippo pathway. Also, signals that affect levels of TEAD also affect the functional output of the Hippo pathway (K. C. Lin et al., 2017)

### **1.8.1 Upstream regulators of Mammalian Hippo Pathway:**

#### **1.8.1.1 Regulation by Fat1-4:**

Unlike in *Drosophila*, The Ft branch of the mammalian Hippo pathway is not well very understood. Mammals have four Ft orthologs; Fat1-4 and two Dachous orthologs; Hchs1-2. Ft4 shows maximum homology to *Drosophila* Ft. Ft4, and Hchs1 mutants in mice show only planar cell polarity defects but do not show YAP or LATS related defect (Mao, 2006). Ft1 loss in Zebrafish activates YAP through Scrib (Skouloudaki et al., 2009). However, Dachs is not conserved in mammals.

#### **1.8.1.2 Cellular polarity, Cell-cell contact, and morphology:**

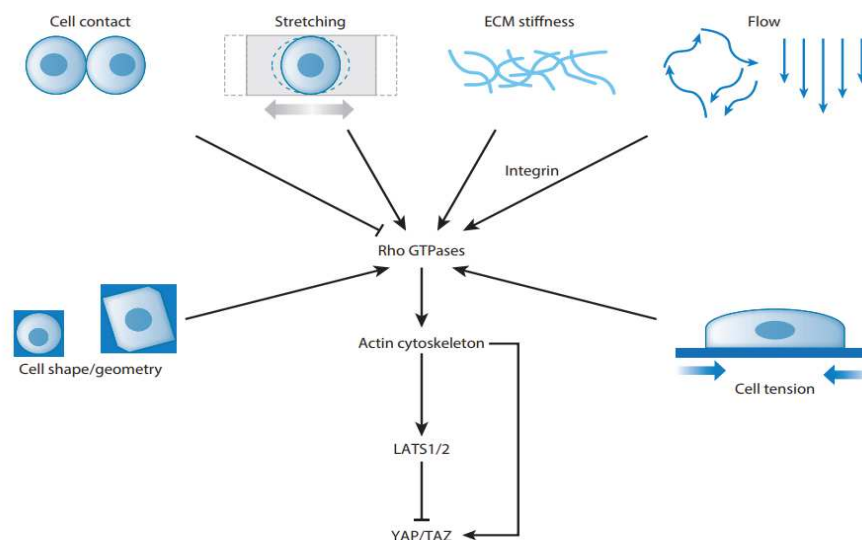
Cellular polarity is important in maintaining the structure and function of epithelial cells. ABCP (apicobasal cell polarity) proteins define the polarity of the cell. Proteins that establish cell polarity in epithelial cells at cell-cell junctions can activate the core Hippo cascade and thus play an important regulatory role (Hansen, Moroishi, & Guan, 2015) (F. X. Yu & Guan, 2013). Similarly, proper cellular adhesion is important in maintaining structure and function, and loss of proper connectivity in either type can cause cellular distress and promote tumorigenesis. Contact inhibition is also a vital factor in controlling tissue growth and

homeostasis. When cells come into contact with neighboring cells, normal proliferating cells typically undergo contact inhibition and stop proliferating in order to avoid overgrowth (McClatchey & Yap, 2012). Components of mammalian tight junction, or adherens junction (AJ) complexes promote mammalian Hippo signaling. For example, loss of Crb3, PALS1 PATJ leads to YAP/TAZ activation in mammalian cells along with polarity defects (Varelas et al., 2010)

Several cell junction proteins such as AMOT (angiomin) family proteins, LIN7C,  $\alpha$ -catenin, PATJ or MPDZ or E cadherin ZO1, ZO2, NPHP4, and LKB1 too have been identified to interact with core components of the Hippo pathway (Chan et al., 2013) (Chan, Lim, Chong, et al., 2011)(Dai et al., 2013)(Hirate & Sasaki, 2014)(Michaloglou et al., 2013)(B. Zhao et al., 2011).  $\alpha$ -Catenin, an adaptor protein between the membrane and actin cytoskeleton for adherens junction (AJ) formation, is another known ABCP regulator that influences Hippo pathway activation.  $\alpha$ -catenin links the actin cytoskeleton to other adherens (to external structures). When the Hippo pathway is ON,  $\alpha$ -catenin binds with YAP and sequesters it at the adherens junction, thereby preventing it from moving into the nucleus (F. X. Yu & Guan, 2013) (W. Wang et al., 2012). Interestingly members of the Angiomin (AMOT) family of tight junction proteins regulate YAP/TAZ activity either by binding and activating the Hippo core kinase LATS2 (Paramasivam, Sarkeshik, Yates, Fernandes, & McCollum, 2011), hence promoting YAP/TAZ phosphorylation, or by directly interacting with YAP/TAZ independently of Hippo core kinases (Chan et al., 2013) (Chan, Lim, Chong, et al., 2011) (B. Zhao et al., 2011) (W. Wang, Huang, & Chen, 2011). This mechanism is not conserved in *Drosophila*. AMOTs have also been identified as direct substrates of LATS1/2 kinases and LATS-mediated phosphorylation has been shown to promote stabilization and abundance of AMOTs, thereby restricting YAP/TAZ activity in a feed-forward mechanism (Adler et al., 2013). Mutations in E-cadherin, another protein located at the adherens junction, can lead to the loss of proper cell-cell contact, and promote Epithelial-mesenchymal Transition (EMT) which is a hallmark of cancer (Hansen et al., 2015)(Harvey et al., 2013). Other adherens junction proteins such as Protein Tyrosine Phosphatase 14 (PTPN14) are also able to bind to and sequester YAP in the cytoplasm. The ability of these proteins to sequester phosphorylated YAP in the cytoplasm prevents transcription of YAP/TAZ target genes, and thus they are essential regulators of the Hippo pathway (F. X. Yu & Guan, 2013) (W. Wang et al., 2012). Scribble (SCRIB), a basolateral protein SCRIB responds to changes in cell density by promoting the assembly of the Crb complex at the apical border of the cell at tight junctions that promotes YAP/TAZ phosphorylation by LATS1/2 and subsequent cytoplasmic retention.

### 1.8.1.3 Mechanotransduction:

The process by which cells sense external forces and spatial constraints and translate them to biochemical signals that elicit an adaptive cellular response is called mechanotransduction. The mechanical forces generated from cell-cell contacts, cell-ECM (extracellular matrix) interaction, and the microenvironment have been shown to regulate gene expression; thus they participate in growth, proliferation, and apoptosis. Evidence of YAP/TAZ as two critical mechanoregulated transcriptional effectors are mounting up. Environmental/physical cues, such as cell contact, surface texture, and mechanical forces also affect the transcriptional activities of YAP/TAZ. It has been shown that cells cultured on soft matrices or substrates with limited surface area lose their actin stress fibers which result in cytoplasmic localization of YAP/TAZ and transcriptional inactivation (Dupont, Morsut, et al., 2011) (Wada, Itoga, Okano, Yonemura, & Sasaki, 2011). The key player that relays the information about ECM stiffness, cell-cell contact, tension, cell shape is RhoGTPase. Mechanistically active RhoA promotes actin polymerization and stress fiber formation to promote nuclear localization of YAP/TAZ (Figure 28). Therefore, disruption of actin stress fiber using small molecules that inhibit the activity of RhoA, ROCK or myosin type II result in cytoplasmic translocation and inactivation of YAP/TAZ. (Dupont, Morsut, et al., 2011) (Wada et al., 2011) (B. Zhao et al., 2012).



**Figure 28 Mechanotransduction:**

Mechanical cues regulate YAP/TAZ activities through Rho GTPase and the Hippo pathway. Cell-cell contact, cell stretching, cell tension, extracellular matrix (ECM) stiffness, and changes in cell geometry modulate activities of Rho GTPases, which in turn lead to actin cytoskeleton remodeling. The actin cytoskeleton can control nuclear-cytoplasmic shuttling and transcriptional activities of YAP/TAZ through both LATS1/2-dependent and LATS1/2-independent mechanisms.

Shear stress can also activate YAP and TAZ, allowing cells to respond to physical injury directly. Wound healing experiments demonstrate that injured tissues have higher YAP and



TAZ activity that drive cell proliferation, cell differentiation, and repopulation of the injured sites to maintain tissue homeostasis (Zanconato, Cordenonsi, & Piccolo, 2016a)

At low cell densities, YAP/TAZ are localized in the nucleus and drive the transcription of target genes responsible for the proliferation, anti-apoptotic roles. While on the other hand at higher cell densities, the kinase cascade of the pathway phosphorylates the YAP/TAZ leading the cytoplasmic translocation/retention (H.-L. Zhao et al., 2008) (Figure 29).

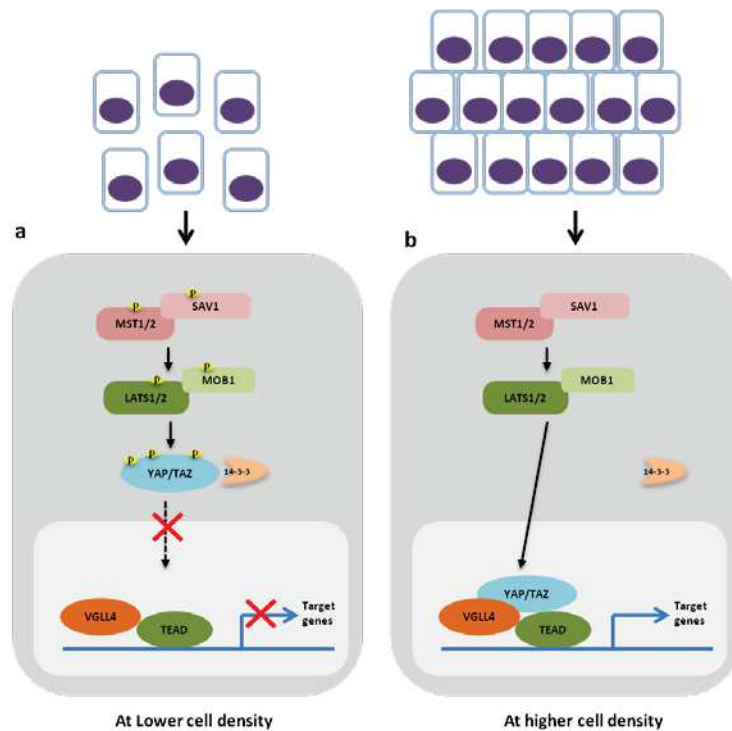


Figure 29 Localization of YAP/TAZ at lower vs higher cell density:

Moreover, the rearrangement of actin cytoskeleton and microtubules in response to mechanical cues were additionally established to influence YAP and TAZ activity. Cells at low cell density are characterized by F-actin abundance whereas cells were grown at high density present low to rare levels of this linear microfilament. A positive correlation between the presence of F-actin stress fibers and the nuclear localization of YAP/TAZ in cells was observed. Disruption of stress fibers caused cytoplasmic translocation of YAP and TAZ. Moreover, the microtubule cytoskeleton was revealed to be required for detachment-induced YAP phosphorylation (Dupont, Morsut, et al., 2011) (Wada et al., 2011) (B. Zhao et al., 2012)

Further, the spectrin network is also linked as an upstream regulator of the Hippo pathway mammalian along with *Drosophila* pathway (Deng et al., 2015)(Fletcher et al., 2015)(Wong et al., 2015).



These findings revealed an essential role for cell architecture and mechanical forces in regulation of the Hippo pathway signaling. Cell morphology mediated mechanism potentially co-acts with a cell-cell contact mediated regulation to encourage density-dependent control of cell proliferation intermediated by the Hippo pathway. This regulation may play a fundamental role in normal developmental processes as well as in the progression of diseases such as cancer.

#### **1.8.1.4 Soluble factors as upstream signals:**

YAP/TAZ have been shown to be regulated by extracellular soluble factors. This breakthrough was based on speculation that extracellular molecules such as growth factors or hormones might contribute to regulating hippo signaling in order to control tissue growth and homeostasis. Growth factors mitigate the regulation of the pathway via G-protein coupled receptors (GPCRs) (Miller et al., 2012)(F. X. Yu et al., 2012). These two independent studies have confirmed that two serum components, lysophosphatidic acid (LPA) and Serum-borne sphingosine-1-Phosphate (S1P) can inhibit LATS1/2 via activating Rho GTPases and eventually activating YAP/TAZ activity. (X. Zhou et al., 2015) showed that estrogens could hyperactivate YAP via activating specific GPCRs. Glucagon, epinephrine has been shown to inhibit YAP via PKA mediated LATS1/2 activation. The omega-3 polyunsaturated fatty acids, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) too are found to inhibit YAP/TAZ via specific GPCRs (K. Zhang et al., 2016). Wnt ligands also have been shown to regulate YAP activity via GPCRs binding to Frizzled receptors (H. W. Park et al., 2016).

Since GPCRs act through G $\alpha$  proteins to transduce signals to their primary effector proteins, different G $\alpha$  proteins have been found to either activate or repress the Hippo pathway. For instance, G $\alpha_{12/13}$ -, G $\alpha_q/11$ -, and G $\alpha_i/o$  proteins activate YAP and TAZ transcriptional activities; while G $\alpha_s$  represses YAP and TAZ both by modulating Hippo pathway. This provides an exciting gateway to manipulate the Hippo pathway signaling with small diffusible molecules since GPCRs are highly druggable. However, to this date, the mechanism by which these extracellular signals and cell surface receptors mitigate and regulate the Hippo pathway remains elusive.

#### **1.8.1.5 Components of the cell cycle as upstream regulators:**

LATS1/2 is shown to be phosphorylated in a cell cycle-dependent manner. Although the precise links are yet to be shed light upon, it has been demonstrated that CDK1 and Aurora A phosphorylate LATS1/2 during mitosis checkpoint (Toji et al., 2004) (Morisaki et al., 2002) (Yabuta et al., 2000) (L. Zhang et al., 2015). It was also shown by (Ganem et al., 2014) that

the presence of extra centrosome leads to activation of LATS2 and subsequently inhibiting YAP/TAZ transcriptional activity and stabilization of p53.

Furthermore, (S. Yang et al., 2013) used taxol or nocodazol to arrest the cells in G2/M cycle and investigated what member of the Hippo pathway are regulated during mitosis. They reported that YAP is phosphorylated at T119, S289, and S367 by cell cycle kinase CDK1 revealing an additional regulator of YAP mediated activity.

#### **1.8.1.6 Stress signals as upstream regulators:**

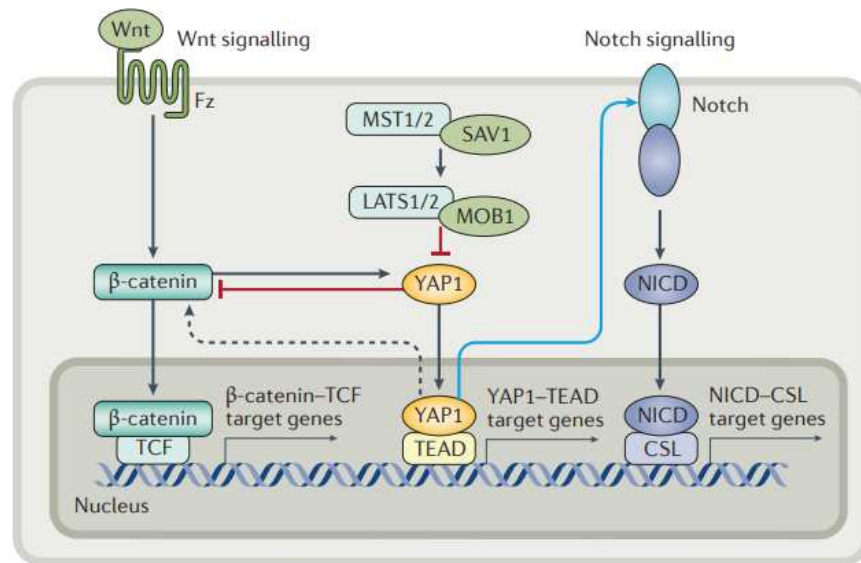
Cells adapt and overcome to constant internal and external stresses that endanger their integrity. So it is not surprising to find that several stress signals can modulate Yki/YAP/TAZ activity in vivo. In 1996 when (Taylor, Wang, & Erikson, 1996) had determined MST1/2 is activated by a high concentration of arsenite or heat shock, the components of the pathway were still not yet grouped together; thus a little attention was paid. Later in two decades when the Hippo pathway was established, it incited new interests in looking for stress signals that can modulate the kinase cascade of the Hippo Pathway. Further, MST1/2 is shown to be activated by oxidative stress caused by H<sub>2</sub>O<sub>2</sub> (Lehtinen et al., 2006)(Geng et al., 2015). YAP interacts with FoxO1 (forkhead box proteins) and activates transcription of manganese superoxide dismutase (MnSOD) and eventually help in reducing oxidative stress and ischaemia/reperfusion (I/R)-induced injury in the heart (Shao et al., 2014).

Cells depend upon carbohydrates for metabolism. Recently, it has been demonstrated that nutritional/energy stress caused by glucose deprivation leads to inhibition of YAP/TAZ activity via LATS1/2 and AMP-activated protein kinase (AMPK) (W. Wang et al., 2015) (Mo et al., 2015). (DeRan et al. 2014) showed that this inhibition is enhanced by phosphorylation of AMOTL1 by AMPK which can regulate LATS1/2 and YAP simultaneously. It was also demonstrated that this phosphorylation could further interfere with the interaction between YAP and TEAD and eventually inhibiting TEAD mediated transcription activation program. Finally, it was also described that during the state of hypoxia, E3 ubiquitin ligase SIAH2 inhibits LATS2 which in turn promotes YAP onco-activity. Loss of SIAH2 in tumor cells was shown to restore the tumor suppressor function of a Lats2 kinase (B. Ma et al., 2015). Collectively, these studies established a previously unknown role of the Hippo signaling in glucose metabolism.

#### **1.8.1.7 Crosstalk with other pathways:**

In addition, to the discussed upstream regulators, the Hippo pathway has been shown to cross talk with Wingless/Ints (Wnt), bone morphogenetic proteins (BMPs), Notch, and Hedgehog

(Hh), epidermal growth factor (EGF), as these signals have been shown to control the activity of YAP/TAZ (Figure 30).



**Figure 30 Crosstalk:**

Crosstalk between the Hippo pathway and Wnt and Notch signaling in the intestine: When the Hippo pathway is activated, cytoplasmic YAP1 inhibits Wnt signaling by sequestering β-catenin in the cytoplasm. When the Hippo pathway is inactive, nuclear YAP1 upregulates Wnt signaling via an unknown mechanism. Also, Wnt activation leads to β-catenin-induced activation and upregulation of YAP1. Activated YAP1 transcriptionally upregulates expression of Notch receptors, leading to increased activation of Notch signaling and translocation of the NICD to the nucleus. Notch is considered to be a mediator of the Hippo pathway. Adapted from (A. W. Hong, Meng, & Guan, 2016)

These findings established the role of cell architecture and mechanical forces in the regulation of the Hippo pathway. Cell morphology mediated mechanism potentially coacts with a cell-cell contact mediated regulation to encourage density-dependent control of cell proliferation via the Hippo pathway. This regulation may play a fundamental role in normal developmental processes as well as in the progression of diseases such as cancer.

#### **1.8.1.8 A subtle difference between regulation of the Hippo pathway in *Drosophila* and Mammals:**

Recently, in studies in Mammals lead to the identification of integrin signaling as an underlying mechanism for Hippo pathway regulation via mechanical cues such as cell morphology and cell-ECM contact area (N. G. Kim & Gumbiner, 2015) (Si et al., 2017) (Elbediwy, Zoé, et al., 2016).

Although Integrin and Src homologs are present in *Drosophila*, Yki protein does not respond to integrin stimulation, while in mammals integrin signaling promotes YAP/TAZ activity. One possible explanation for this different behavior may be that the N-terminus of Yki is missing a domain necessary to bind PDZ-containing proteins, which is found in its human

counterpart YAP and is necessary for the activation of the integrin-Src adhesion branch of the pathway (Elbediwy & Thompson, 2018).

However, (Elbediwy & Thompson, 2018) also proposed another exciting explanation for this difference. They draw their conclusions based on a comparative analysis of the Yki protein and the evolution of the different epithelia: In mammals, the epidermis lacks the apical domain so it must sense the basal integrin to regulate the cell shape/growth (Refer to the diagram). While in the case of *Drosophila*, the epithelial cells are columnar and they can sense the apical signals, this makes the integrins unnecessary for the activation of the Hippo pathway (Figure 31).

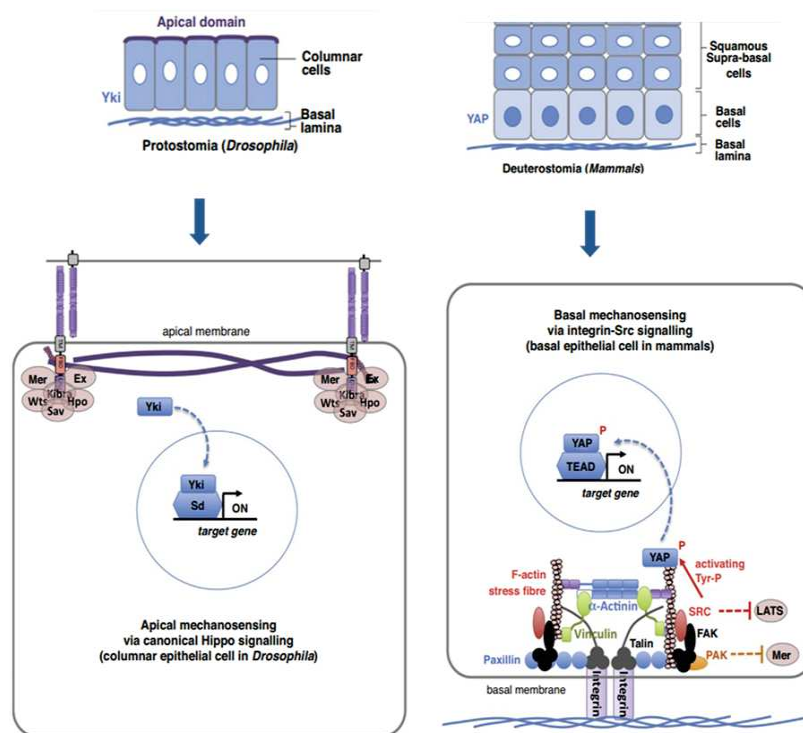


Figure 31 Apical versus basal mechanotransduction via Yki or YAP in different animals:

### 1.9 Effectors of the Hippo pathway: Yki/YAP/TAZ

There are two major classes of isoforms of YAP, namely YAP1(1 $\alpha$ /1 $\beta$ /1 $\gamma$ /1 $\delta$ ) and YAP1(2 $\alpha$ /2 $\beta$ /2 $\gamma$ /2 $\delta$ ). The isoform with a single WW domain is called often just referred to as YAP1, and the isoform with two WW domains is referred to as YAP2. These two isoforms are consequences of Alternative Splicing. In total, there are eight isoforms of YAP which are named as YAP1-1 $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and YAP1-2  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  according to the number of amino acids. Alternatively, in a letter also suggested the nomenclature based on the number of amino acids by directly mentioning the number of amino acids next to YAP 1 or YAP2 in the format YAP1-XXX (XXX = number of aa) (Figure 32)

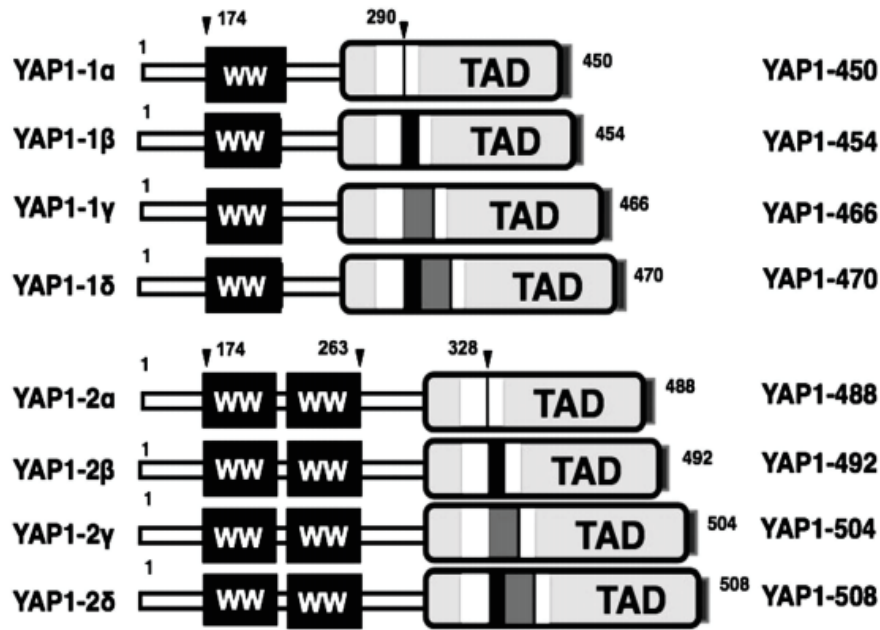


Figure 32 The eight described isoforms of YAP:

YAP was initially identified in 1994 by (Fang, Barker, Sudol, & Hanafusa, 1994) as a mammalian homolog of Yki. It was YAP's accumulation in the cytoplasm as a complex with 14-3-3 protein led to the assumption that even Yki might interact with 14-3-3. The WW domain has two highly conserved tryptophans separated by 20-23 amino acids (Salah, 2012), which mediated the protein-protein interaction at N terminus. The WW domain recognizes a PPxY motif (proline/proline/any amino acid/tyrosine) that is found in a variety of proteins, many of which control TAZ/YAP localization and activity (Figure 33).

The YAP proline-rich domain facilitates the interaction with RNA and DNA-binding protein HNRNPU in the nucleus(Howell, Borchers, & Milgram, 2004). The YAP SH3-binding motif (PKQPPPLAP) also facilitates interactions with other kinases and adaptor proteins such as SRC and NCK1/2, respectively (Sudol, 1994). Depending upon the Size of the isoforms, YAP could range from 450aa to 508aa. YAP also contains a C-terminal PDZ-binding motif, which mediates interactions with PDZ domains. PDZ domains are 80-90 amino acid protein-interaction domains that are found in several proteins, many of which are transmembrane or cytoskeleton-associated (Ye & Zhang, 2013). YAP has been shown to interact with promoters and distal enhancers in order to promote gene regulation via modulation chromatin looping and Pol II pausing release(Lian et al., 2010) (Galli, de Laat, et al., 2015)(C. Stein et al., 2015)(Zanconato, Cordenonsi, & Piccolo, 2016b).

In addition, TAZ is 395aa (50KDa) protein that is one and only paralog of YAP. It shares close to 50% sequence identity with YAP. The *WWTR1* gene encodes the TAZ protein, and it

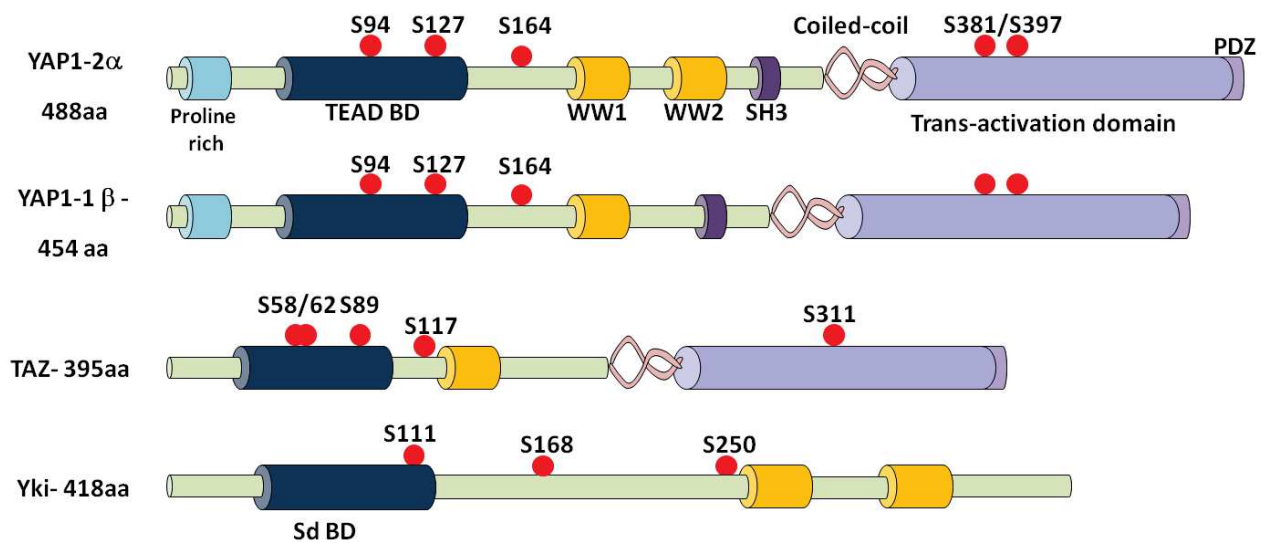
contains a single WW domain along with a transactivation domain and a PDZ binding motif in its C terminus and a coiled-coil region. TAZ actually resembles the YAP1 as it contains only one WW domain, This is the reason why YAP/TAZ often have similar function such as both of these coactivators interact with RUNT family protein RUNX2 (J. H. Hong et al., 2005) and with well studied TEAD family proteins (TEAD1-4) (Vassilev, Kaneko, Shu, Zhao, & DePamphilis, 2001)(W. M. Mahoney, Hong, Yaffe, & Farrance, 2005).

However, there are slight differences in the YAP/TAZ such as YAP can interact with the SH3 domain of YES protein and has a proline-rich region while TAZ lacks this feature(Kanai et al., 2000). TAZ binds SMAD transcriptional modulators via a C-terminal coiled-coil domain, thereby facilitating TGF $\beta$  signaling by promoting SMAD nuclear accumulation (Varelas et al., 2008) while YAP interacts with inhibitory Smad7 (Ferrigno et al., 2002) and BMP-regulated Smad1 (Alarcón et al., 2009). YAP but not TAZ was shown to interact with p73 to induce pro-apoptotic transcriptional program. Similarly, TAZ but not YAP binds to Pax3, which is involved in the embryo limb formation (Masao Murakami et al., 2006).

Structurally, Yki (*Drosophila* homolog of YAP) is closely related to mammalian YAP (Sudol, 1994) (Figure 33). The Yki peptide shares 31% homology with YAP. Similar to YAP, Yki consist of WW domain that allows it to interact with the PPxY (Where P stands for Proline, X is any amino acid and T is Tyrosine) motifs of the partners. Notably, the corresponding SH3 binding region is absent in the *Drosophila* Yki protein.

Although initially restricted to Sd the number of partners of Yki are increasingly growing in numbers, recently (Bohère et al., 2018) showed that transcription factor Shavenbaby (Svb) interacts with Yki to activate the expression of *Diap1*. Thus, the various protein binding domains present in Yki/YAP/TAZ provide a mechanism for the regulation of their localization and activity.





**Figure 33 Schematic diagram of YAP1 and 2, TAZ, and Yki:**

Red dot denotes phosphorylation sites.

They include Proline-rich region TEAD-binding domain, Sd (*Drosophila* homolog of mammalian TEADs)-binding domain, WW: WW domain, C-C: coiled-coil region, TAD: transactivation domain, PDZ BD: PDZ-binding domain. Below is the short description of these domains.

**Proline Rich domain:** The N terminus of YAP1/2 consists of a proline-rich domain which facilitates the interaction with HNRNPU which is the only known partner to utilize this domain for interaction. TAZ lacks this domain

**TEAD Binding domain:** This domain is conserved in both YAP and TAZ. YAP/TAZ bind TEAD (TEA domain) transcription factors via TEAD factor-binding domain located at N-terminal region (at amino-terminal region located between amino acid 47-154). Although TEAD4 requires similar TEAD C-terminal sites to interact but it requires different residues on YAP and TAZ.

**WW Domains:** The WW domain facilitates protein-protein interactions via recognition of proline-rich peptide motifs (PRM) and phosphorylated serine/threonine-proline sites. Aptly named, WW domain consists of ~35–40 amino acid residues, including two highly conserved tryptophan (W) residues separated by 20–23 amino acids in the polypeptide chain. WW domain allows YAP/TAZ to bind to PPXY motifs, where P is proline, x is any amino acid and Y is tyrosine.

**SH3 domain:** YAP was originally identified via a screen for proteins that bind the SH3 (Src homology domain 3) domain of the non-receptor tyrosine kinase YES1. The YAP SH3-binding motif (PKQPPPLAP) facilitates interactions with other kinases and adaptor proteins such as SRC and NCK1/2, respectively. This domain is not present in TAZ.

**Coiled-coil domain:** This domain is present in C-terminal of both YAP and TAZ but not partners of YAP binding via this domain is known yet. TAZ utilizes this domain in heterodimer formation with TEAD. TAZ binds SMAD transcriptional modulators too via this domain to facilitate TGF- $\beta$  signalling.

**14-3-3-binding domain:** TAZ was identified in a screen for 14-3-3 interactors. YAP and TAZ interact with 14-3-3 when they are phosphorylated at Ser127 and Ser 89 respectively. Interaction with 14-3-3 sequester YAP/TAZ in cytoplasm.

**Transcriptional activation domain:** The YAP/TAZ C-terminal region is rich in serine, threonine and acidic amino acids. This domain has strong intrinsic transcription stimulation activity.

**PDZ-binding domain:** Both YAP and TAZ consist of a small PDZ (Postsynaptic density 95/Disc large/Zonula occludens-1) at extreme C terminal region. PDZ binding domain is important for subcellular localization of YAP and TAZ.



### 1.10 Hippo pathway in diseases:

Hippo pathway has vital roles to play in both organism development and progression of the disease. As we have already seen, Hippo signaling determines organ size, regulates cell number via proliferation and apoptosis, plays a role in embryogenesis, cell fate specification after mitosis (Jukam et al., 2013), tissue-specific regeneration of progenitor cells (Yu Wang, Yu, & Yu, 2017) mechanotransduction (Dupont, Enzo, et al., 2011) (Georg Halder, Dupont, & Piccolo, 2012) (Morgan, Murphy, & Russell, 2013) and apical and basal signaling (Elbediwy, Vincent-Mistiaen, & Thompson, 2016). It is not surprising to that alteration or perturbation of such a complex pathway eventually leads to cell proliferation, inhibition of apoptosis, misregulation of cellular differentiation which is are critical aspects of cancer development (Figure 34).

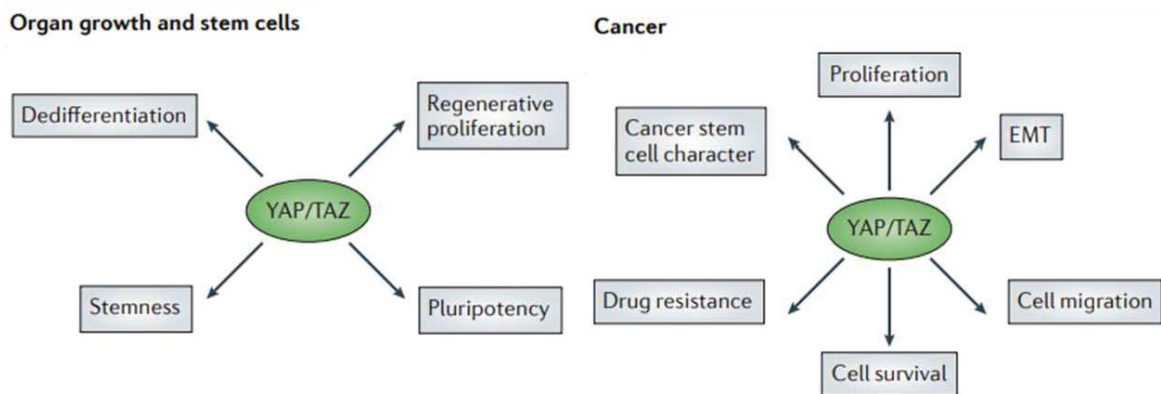


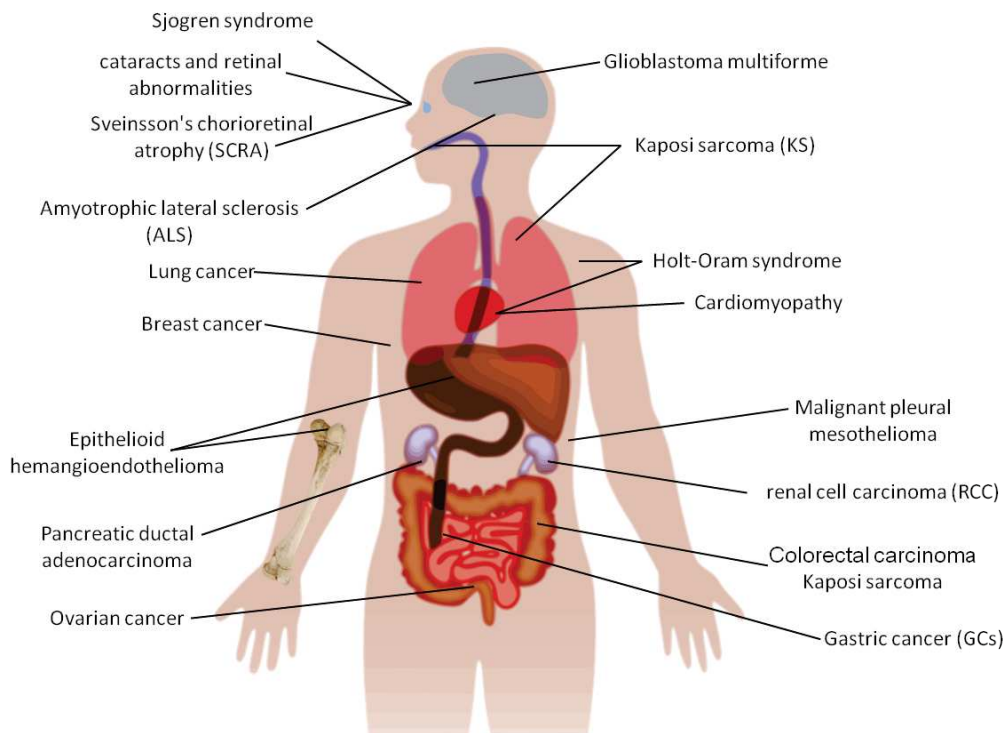
Figure 34 Cellular function of YAP/TAZ:

Along with cancer, perturbed hippo pathway is implicated in several non-cancer disease processes. I will, however, give a short introduction to this topic. For more extensive knowledge one can refer to the following reviews by (Plouffe, Hong, & Guan, 2015) (Zanconato et al., 2016b) (Xiaodong Zhang et al., 2018) (Figure 35).

#### 1.10.1 Implications of the Hippo pathway in cancer:

Due to its complexity and range of upstream signals, the Hippo pathway could be dysregulated by a range of mechanisms. The core of the pathway plays a tumor suppressor role and the effector of the pathway, i.e., transcription coactivator is responsible for the oncogenic events. Mechanisms such as deletion or mutation of an upstream signal such as GPCR's, aberrant YAP/TAZ amplification or even by a cross-talk with any of the known (such as Wnt) or unknown (yet to be linked) signaling pathways could lead to dysregulation of growth, proliferation, and inhibition of apoptosis. Normal functioning cells depend upon

aerobic oxidation of glucose as their primary source of energy, but cancerous cells switch to anaerobic glycolysis to grow and survive, even in the presence of sufficient oxygen, which is known as the “Warburg effect” and is a prominent hallmark of cancer. Reviewed in (Gatenby & Gillies, 2004). Indeed, (Enzo et al., 2015) showed that a critical enzyme of glycolysis, phosphofructokinase 1 (PFK1) interacts with transcription factor TEAD1 so as to regulate the activity of YAP/TAZ providing new insight into how YAP/TAZ pro-oncogenic activity be controlled by glycolysis. Many such mechanisms could lead to the onset of disease and cancer of which several are identified to this date. A few of these are described below:



**Figure 35 :** Implications of the Hippo pathway in various cancers and non-cancer diseases:

First described in 1982, **Epithelioid hemangioendothelioma (EHE)** is a rare malignant vascular neoplasm. EHEs usually occur in soft tissue, bone, skin and some parenchymatous organs. Recently, it has been shown that YAP/TAZ chromosome translocations arise in virtually all EHE cases. This translocation results in a fusion of TAZ to the calmodulin-binding transcription activator 1 (CAMTA1). Although this translocation occurs in almost all the EHE cases, the oncogenic mechanism of this fusion is yet to be elucidated, but it is a strong indication that dysregulated YAP/TAZ fusion proteins may act as cancer drivers (Tanas et al., 2011) (Flucke et al., 2014).

YAP/TAZ activity has been correlated with an increased danger of metastasis and reduced survival across all **human breast cancer subtypes** (Cordenonsi et al., 2011). Studies done on

breast cancer tissues have suggested that LATS1/2 mRNA is often poorly expressed and eventually leads to increased tumor size followed by lymph node metastasis (Visser & Yang, 2010) TAZ has been observed to be highly expressed in invasive breast cancer cell lines and primary breast cancers. It has been demonstrated that TAZ overexpression is sufficient to induce cell proliferation, transformation, and epithelial-mesenchymal transition in breast cancer cell lines (Lei et al., 2008) (Siew et al., 2008). YAP, however, is shown to impart metastatic abilities to benign tumors in mammary tumor cell lines in the mouse as shown by (H. Liu et al., 2012). On the other hand, (Q. Chen et al., 2014) demonstrated that hyperactivating YAP alone leads to differentiation of secretory cells during lactation, but it cannot induce mammary tumorigenesis *in vivo*. They speculated that additional mutations would be required for ultimate tumor-inducing capabilities of YAP. So more investigative studies are required to decide whether these clashing observations are due to cell type-specific differences.

**Lung cancer**, accounting for 12.3% of all tumor prevailing in humans is by far the most prevalent form of cancers (as of 2018), and it also ranks up in cancer with meager survival rate. WHO has categorized lung cancer in two categories: non-small cell lung cancer (NSCLC) (accounting for 85%) and **Small-cell lung cancer (NSCLC)** (accounting for 15%) (Oser, Niederst, Sequist, & Engelman, 2015). Lung adenocarcinoma (LAC), Lung squamous cell carcinoma (LSCC), and Large-cell carcinoma, all belong to the NSCLCs (Yokota & Kohno, 2004). At the start of this decade, (Yang Wang et al., 2010) and (Z. Zhou et al., 2011) showed that YAP/TAZ are highly expressed in non-small cell lung cancer (NSCLC), and either of their knockdown in NSCLC cells is adequate to suppress proliferation, tumor growth and metastasis in the mouse model. It has also been demonstrated that hyperactivating YAP is sufficient to drive lung cancer progression *in vivo* (Lau et al., 2014). Similar to studies in breast cancer, (Wenjing Zhang et al., 2015) described that YAP/TAZ activation alone might not be sufficient to drive NSCLC genesis, but they showed that ectopic expression of YAP/TAZ could transform small adenomas to high-grade LAC in the mouse model.

**Malignant pleural mesothelioma (MPM)** cancer of the mesothelium is one of the rarest cancers, but it is an aggressive one which does not respond to current therapy thus survival rate is lower in patients detected with this form of cancer. It is cancer associated with the pleura, the lining which covers many of the body's internal organs. This cancer is associated with exposure to asbestos, and recently identified germline mutation is also said to predispose patients to MPM (Takahashi & Landrigan, 2016) (Cheung et al., 2013). Homozygous deletion or inactivating mutations in NF2, SAV1, or LATS2 are often observed in human malignant

mesothelioma tissues and cell lines (H. Murakami et al., 2011). (Sekido et al., 2011) have shown that nearly 50% of all mesothelioma tumors have inactivation mutation in *NF2* gene. Further, Ajuba LIM protein (AJUBA) can inactivate YAP through signaling via LATS kinase, and down-regulation of AJUBA led to induction of malignant mesothelioma in MM line (Tanaka et al., 2015). The downregulation of AJUBA subsequently activates YAP which regulates the transcription of cell cycle promoting gene *CCND1*(G1/S-Specific Cyclin-D1) and *FOXM1* (Forkhead Box M1; a transcription factor targeting both G1/S and G2/M progression regulators) to induce malignant mesothelioma in MM cell lines (Mizuno et al., 2012). Thus, the Hippo pathway has been strongly implicated in cancers of mesothelioma, and further research could provide a way to be able to develop a therapy to which this type of cancer can respond to.

**Pancreatic ductal adenocarcinoma (PDAC)** is classified as a very aggressive form of cancer that is often detected only in a very advanced stage as the patient does not experience any noticeable symptoms until it is too late. PDAC arises from the progression of pancreatic intraepithelial neoplasias (PanINs) which are precursor lesions in fully grown metastatic cancer (Hruban, Goggins, Parsons, & Kern, 2000). Mutations in *KRAS* genes are known to be one of the initiating factors for PDACs as studies have shown that *KRAS* mutation is present in 95% of PDACs (Biankin et al., 2012). Biopsies from patients with PDAC revealed an increased YAP level and higher nuclear localization. Moreover, YAP knockdown in PDAC cells resulted in reduced proliferation and reduced anchorage-independent growth, suggesting YAP's role in PDAC (Weiyang Zhang et al., 2014).

**Kaposi sarcoma (KS)** is cancer that appears on the outer skin, lymph node or some other organs as cell masses. It is caused by the infection of Kaposi sarcoma-associated herpesvirus (KSHV) also called as HHV-8 in immunodeficient patients, such as patients with AIDS. (G. Liu et al., 2015) observed elevated levels of YAP/TAZ as KSHV encodes a vGPCR to inhibit LATS1/2 and subsequently suppress the Hippo pathway. Although the implementation of antiretroviral therapy has led to increased patient survival but still this form of cancer remains to be a killer, and YAP/TAZ can be a future target for therapies (Ledergerber et al., 1999).

**Colorectal carcinoma (CRC)** is a cancer of the digestive system. Although CRC responds to the therapy, this cancer is still a morbid one due to the frequency of reoccurrence; thus long term therapies need to improve and develop. YAP is often found overexpressed and YAP/TAZ activity is associated with poor patient outcome (F.-X. Yu, Meng, Plouffe, & Guan, 2014). Hypermethylation of *LATS1* has been shown as a cause of CRC which can explain the elevated YAP activity (Wierzbicki et al., 2013).

YAP has also been implicated in **renal cell carcinoma (RCC)** which is one of the most common cancers of the urinary tract. This cancer is distinct from other cancers as it is highly vascularized and resistant to conventional chemo or radiotherapy. Elevated YAP levels are associated with RCC too. Recently it was shown that LATS1 promoter is hypermethylated in RCC and therefore reducing the LATS and activating YAP (K. H. Chen et al., 2014) (Rybarczyk et al., 2017). (J. J. Cao et al., 2014) supported the role of YAP in RCC by showing that knockdown of YAP in ccRCC (clear cell Renal Cell Carcinoma) cell lines inhibits proliferation and initiate apoptosis.

Most of **Gastric cancer (GCs)** are gastric adenocarcinomas (GAC) that derive from the glandular epithelium of the stomach. It is the fifth most common cancer and the third leading cause of deaths worldwide due to cancer (Garattini et al., 2017). The dysregulation of Hippo signaling is associated with GAC from its initiation to metastasis (Ramos & Camargo, 2012). Elevated YAP activity and nuclear localization of observed in all kinds of gastric cancers (X. Hu, Xin, Xiao, & Zhao, 2014). TAZ too is highly expressed and nuclear-localized in human GAC lines further supporting the YAP/TAZ association in GACs (G. Yue et al., 2014)

Apart from the above-described cancers overexpression of TAZ is observed in high-grade brain tumors, i.e., **Glioblastoma multiforme** (T. Tian et al., 2015). Immuno histo-chemistry studies on patients samples revealed high levels of YAP in both low and high grades of gliomas. This associate with shorter survival of patients with gliomas (Orr et al., 2011). Several studies have already described how mutations in RTK-PI3K-PTEN and ARF-MDM2-p53 INK4a-RB pathways contribute to the strengthening of the oncogenic capacity of advanced gliomas (Zheng et al., 2008). Recently (Rivas, Antón, & Wandosell, 2018) have shown an actin cytoskeleton-associated protein WIP (WASP-interacting protein) as a driver of cancer progression via stabilizing YAP/TAZ.

**Ovarian cancer** ranks third in the list of most common malignancies in the female reproductive system (Tworoger, Shafirir, & Hankinson, 2017). 5-8% of all ovarian tumors are a result of Granulosa cell tumors (GST). In a study published in 2014, (D. Fu et al., 2014) reported that GST's show significantly higher levels of YAP when compared to healthy ovarian tissue. Although at this point the mechanism by which YAP regulates the proliferation of granulosa cell tumor is unknown.

So far we have seen that YAP/TAZ acts as oncogenes in the majority of cancers. It is worth mentioning that the Hippo Pathway plays a crucial role in lymphocyte apoptosis and YAP plays the role of tumor suppressor in hematological cancer such as multiple myeloma, lymphoma, and leukemia. It has emerged that YAP/TAZ plays a context-dependent role in

hematopoietic cancers. It has been observed that YAP is often deleted or down-regulated in patients' samples suffering from early to advanced stages of MM (Cottini et al., 2014). On the one hand, YAP acts as a tumor suppressor by regulating DNA damage response via interacting with ABL1 to induce p53-independent apoptosis while on the other hand, (T. S. Kim et al., 2012) and (Hartmann et al., 2010) have reported the loss of upstream components of Hippo in leukemia. Recently, TEADs too have been shown to activate the transcription of the oncogenic program by reprogramming enhancers to drive B cell transformation in leukemia (Y. Hu et al., 2016). These reports have now cautioned scientists to look at YAP/TAZ in a context-dependent manner and consider a possibility of a tumor suppressor role as the current paradigm is that YAP/TAZ are pro-oncogenes in all cancer-related malignancies.

### 1.10.2 Hippo pathway in non-cancer diseases:

Given the broad range implications of the Hippo pathway, it is not surprising to find its role in diseases beyond cancers too. Thus it is essential for current researchers to study the various mechanistic involvements in such diseases in order to open the possibility of treating such diseases via therapy based on the input signals or the components of this pathway.

Patients suffering from a rare genetic disorder Neurofibromatosis 2 (NF2) are predisposed to an additional risk of developing **cataracts, and retinal abnormalities** and about half of them proceed to develop them (Parry et al., 1996)(Parry et al., 1994) (Parry et al., 1996). YAP is required for normal ocular development (J. Y. Kim et al., 2016). Association of Hippo pathway has been made in ocular abnormalities such as ocular colobomas optic fissure closure defect (Oatts et al., 2017) (Williamson et al., 2014) , Sveinsson's chorioretinal atrophy (Kitagawa, 2007) (Fossdal et al., 2004) and retinal degeneration (Hamon et al., 2017).

**Sveinsson's chorioretinal atrophy (SCRA)** is an autosomal dominant disease linked to the eye that is described to be caused by the mutation in TEAD1. It is a rare condition characterized by choroid and retinal degeneration in humans. The study done (Fossdal et al., 2004) by using genome-wide linkage analysis describes a missense mutation in the TEAD-1 gene that results in the substitution of tyrosine with a histidine (Tyr421) in the C-terminal of TEAD1 protein in Icelandic patients. This mutation limits the TEAD-YAP complex formation which results in SCRA.

In a study from 2013, (Enger et al., 2013) have shown evidence that Hippo pathway is implicated in **Sjogren syndrome (SS)**, a chronic autoimmune disease which causes



hyposalivation and ocular dryness in salivary and lacrimal glands respectively. In a study using non-obese diabetic (NOD) mice (a mouse model for SS), they showed that salivary glands of NOD mice phenocopies that salivary glands with LATS 2 inhibition. The IHC in salivary glands of human Sjogren patients exhibits nuclear staining of TAZ along with upregulation of transcriptional target of TAZ.

Hippo pathway is also associated with cardiac disease. Studies on the sample from patients with hypertrophic **cardiomyopathy** and transverse aortic constriction (TAC) in mice, have shown elevated levels of YAP and decreased phosphorylation of Ser127 suggesting that nuclear YAP is implicated in the hypertrophic heart (P. Wang et al., 2014). In the same year (Z. Lin et al., 2014) showed elevated YAP levels and phosphorylated LATS in the heart samples from patients with ischaemic or nonischemic heart failure suggesting increased hippo activity.

According to (Basson et al., 1997) a mutation in TBX5 of T-Box family of transcription factors is implicated in **Holt-Oram syndrome**. It is an autosomal dominant disorder that causes the malformation of upper limbs and cardiac abnormalities. It has been demonstrated by (M. Murakami et al., 2005) that mutation in TBX5 prevents it from binding to TAZ which subsequently leads to Holt-Oram syndrome.

Apart from the above described mammalian pathologies, components of the Hippo pathway are also involved in hepatomegaly, neurogenic muscle atrophy, **Amyotrophic lateral sclerosis (ALS)**, Immunodeficiency, *etc.* however, a lot of these studies are only done on mice model, and thus it is always to kept in mind that animal model such as mice tends to develop different range of disease subtypes. Thus in the coming years, it is necessary to study the association of these pathway components in a wide range of pathologies in humans.

### 1.11 Hippo pathway as a therapeutic target

What we have seen so far is that in several of pathologies, deregulation of the Hippo signaling pathway occurs and eventually elevated YAP/TAZ activity induces expansion of tissue-specific stem/progenitor cells. This often leads to the development of tumors, and thus for future cancer therapy, it is essential to shed light on the physiological role of the Hippo signaling pathway and more specifically YAP/TAZ in tissue homeostasis and cancer stem cells.

We know that the association between cancer and Hippo pathway is very tissue or cell type-specific thanks to studies which demonstrated that skin-specific deletion of MST1/2 or LATS1/2 leads to no abnormalities in the epidermis or MST1/2 or SAV1 deletion does not



lead to elevated YAP phosphorylation of Kidney abnormalities (Schlegelmilch et al., 2011) (Reginensi et al., 2013). On the other hand, we have seen so far how dysregulation of YAP is observed in numerous forms of tumors and diseases. However, if we look the data presented in (Harvey et al., 2013) from the COSMIC (Catalogue of somatic mutations in cancer) database, it shows that so far only NF2 and TAZ are classified at cancer genes out of all the genes in the pathway. Indeed, they postulate that this information might change in the future as the volume of the genomic data increases due to the global cancer-sequencing efforts. According to most current studies, YAP, LAST1/2, GPCRs, NF2, and TAZ are related to the development of cancer. One possible explanation to the rarity of mutations in genes of Hippo pathway is so far partially due to the fact that YAP/TAZ is often affected by other pathways as we have already seen such as Wnt signaling, TGF- $\beta$ -BMP, NOTCH, EFR, and GPCR, *etc.* Thus it is critical to investigate and classify all the underlying causes of YAP/TAZ up-regulation in tumors and paint a much larger picture with the mutations in Hippo genes and crosstalk with aberrant signaling pathways that can lead to tumor formation. Hereby, I will discuss the status of targeting hippo pathway components against cancer and the strategies of the therapy.

### **1.11.1 YAP/TAZ activators in Regenerative medicine:**

Elevated YAP/TAZ incites cell proliferation in tumors, and it is obvious to target inhibition of YAP/TAZ to control the tumor. However, the cell division and proliferation also corresponds to the growth of animal tissues, and it is required for tissues to be maintained and repaired. Thus, it is not surprising to see why scientists emphasize on developing YAP/TAZ activators in the field of regenerative medicine. Now there are ample amount on studies which shows that YAP1 activators could be potentially used to promote tissue repairs after injuries. For example: (Q. Zhou, Li, Zhao, & Guan, 2015) demonstrate that inhibition of the Hippo pathway improves cardiac function after infarction in adult mice. There are also reports of YAP1 activation after hepatectomy, and its deletion leads to necrosis. Deletion of YAP1 also leads to impairment of intestinal regeneration and wound healing. TAZ is also shown to be activated right after muscle injuries (Hwang et al., 2010). Using the strategy of TAZ activation, (Hata et al., 2014) put forward a patented compound IBS008738 that activates TAZ and promotes myogenesis in mouse myoblast cells (C2C12 cells). This compound facilitates muscle repair after cardiotoxin-induced muscle injury and prevents steroid induced muscle atrophy in mice. They argue that this compound can be used realistically for old patients who suffer from sarcopenia and confined to bed. Due to such confinement, they lose

skeletal muscle in lower limbs and often become disabled. The TAZ activator, IBS008738 can be administered to such elderly patients to maintaining muscle volume until the patient can resume exercise. The short term application can further downside the worries of TAZ activation leading to oncogenesis or inducing indolent tumors.

### **1.11.2 Inhibitors of Hippo signaling as anti-cancer drug candidate:**

As seen in the plethora of studies, YAP/TAZ activity is high in several cancers. This is a reason why many studies are aiming to target the kinases of the Hippo signaling as it is often seen that MST1/2 and LATS1/2 activities are lowered in many cancers. Therefore enhancing kinase activity is an attractive therapeutic target for many involved in this research.

It has been shown that YAP /TAZ confer some degree of chemotherapy resistance to cells. We know that resistance to RAF and MEK inhibitor therapy is a major challenge in patients undergoing chemotherapy. Recently, (L. Lin et al., 2015) identified YAP as a culprit that promotes the resistance to RAF and MEK inhibitors. Thus they have opened a possibility of combined suppression of YAP and RAF or MEK as an improved therapy for enhanced treatment response and survival of patients.

(Liu-Chittenden et al., 2012) first promoted the idea of targeting YAP-TEAD interactions using verteporfin (Visudyne) after screening approximately 3000 FDA approved drugs in a screen to look for small molecules that can inhibit YAP/TEAD complex. Verteporfin (VP) is a heme analog approved for clinical use as a photosensitizer in patients with neurovascular macular degeneration. In murine models of hepatocellular carcinoma with YAP-overexpression and in Yki-overexpressing *Drosophila* S2 cells, VP was shown to inhibit YAP-Tead2/Yorkie-Sd complex formation through selective binding to YAP/Yorkie. Furthermore, with VP concentrations of 2.5 $\mu$ M-10 $\mu$ M, liver growth was substantially decreased, and mRNA expression of *diap1* was significantly reduced in S2 cells with 200nM VP treatment. Work by (Jiao et al., 2014) and (Wenjing Zhang et al., 2014) has also suggested that VGLL4, a transcription co-factor protein, can act as an inhibitor of YAP activity by competing with YAP for TEAD binding. These studies show VGLL4 expression in-vitro and in-vivo decreased tumor formation, growth, and cell proliferation in both gastric and lung cancers. Additionally, (Wei et al., 2017) have shown that verteporfin can suppress YAP activity in PDAC cells via inhibiting YAP and TEAD interaction and opened a possibility of using it as an anti-tumor drug in the treatment of pancreatic cancer (Figure36).

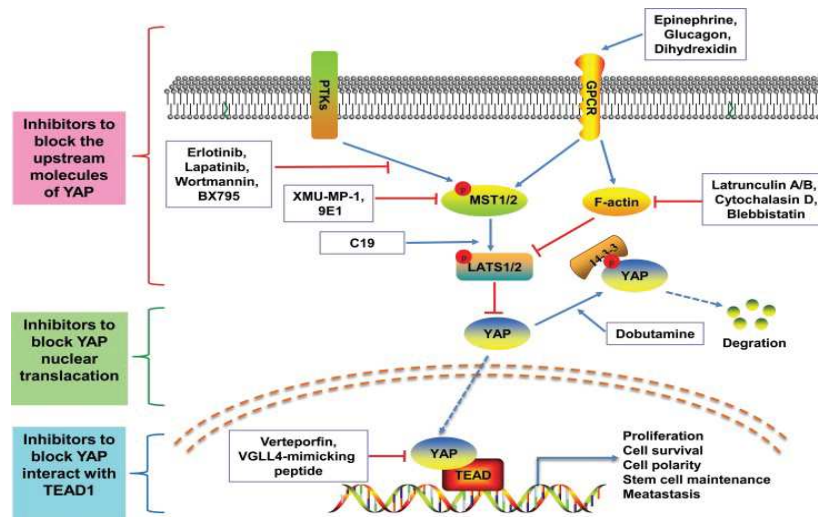
Also , Several studies have indicated that studies have shown that knockdown of YAP or TAZ genetically by RNA interference (RNAi) in various cancer cells could improve immune

response to a wide variety of chemotherapeutic drugs such as, cisplatin, anti-tubulin drugs, EGFR inhibitors, c-Abl inhibitor, RAF and MEK inhibitors (Lee et al., 2016) (L. Lin et al., 2015) (D. Lai, Ho, Hao, & Yang, 2011) (Yulei Zhao & Yang, 2015)

(Nishio et al., 2015) found that **ivermectin, an antiparasitic drug**, and its derivative, milbemycin D, inhibits YAP/TAZ activity and suppress tumor growth in MOB1- deficient mice. Further, researchers wish to find compounds that can allow them to play with the subcellular localization of YAP/TAZ since the phosphorylated YAP/TAZ and translocated to the nucleus, and unphosphorylated YAP/TAZ are degraded in cytoplasm. In 2011, (Bao et al., 2011) described that dobutamine, a class of drug used in patients who recently suffered from a cardiogenic shock can reabsorb YAP from the nucleus to cytoplasm. This opens a perspective of investigating and identifying the mechanism of inhibition of YAP-dependent gene transcription by dobutamine.

Similarly, in 2014 (Sorrentino et al., 2014) and (Z. Wang et al., 2014) described how statin (a class of drug to treat hypercholesterolemia) indirectly inhibit YAP and TAZ by reducing the downstream synthesis of geranylgeranyl pyrophosphate, which is required for Rho GTPases to inhibit LATS kinase activity. They have also shown that statin treatment of breast cancer cells resulted in lower CSC properties such as self-renewal and inhibited in vitro and in vivo growth.

Studies performed by (Moroishi et al., 2016) have shown that deletion of LATS1/2 enhances the antitumor immune response. Via High throughput screen (Fan et al., 2016) have identified a compound named XMU-MP-1 that can inhibit kinase MST1/2. They have demonstrated that this molecule can ameliorate chronic liver injury by enhancing tissue repair and regeneration. However very recently, (Helena et al., 2018) have published results in contrast with (Moroishi et al., 2016) and affirmed that TAZ promotes human cancer evasion via inducing aberrant expression of PD-L1 on cancer cells which subsequently evade the antitumor immune response. The authors speculated that this conflict might arise because Hippo pathway gene regulation may differ from species to species.



**Figure 36 Schematic demonstration of the small-molecule modulators of YAP:** These small-molecule modulators of YAP can be classified into three categories: (1) the pink area represents those regulating the upstream molecules of YAP. (2) the green area represents those modulating the phosphorylation of YAP and blocking YAP nuclear translocation, such as Dobutamine. (3) The blue area represents those inhibiting YAP interaction with TEAD1 by directly targeting YAP, such as Verteporfin, VGLL4-mimicking peptide.

### 1.12 Conclusion:

Hippo pathway integrates the signals from a verity of growth pathways, dysregulation of Hippo pathway components could lead to cancer and other diseases via vast range of alterations that eventually yield in same molecular result, expression of nuclear localized YAP/TAZ thus targeting the constitutive YAP/TAZ activation could be of therapeutic benefit as already shown in cancer model studies. On the other hand, pharmacological inhibition of Hippo kinases MST1/2 and/or LATS1/2 seems to offer potential therapeutic benefit in an organ-cell specific setting, especially in patients recovering from myocardial injury as transient YAP activation in cardiomyocytes could expand the cardiomyocyte cell pool during therapeutic heart regeneration. However, prolonged inhibition of kinase could also prove to be harmful to the immune system as increased YAP/TAZ activity result in abnormalities in various human organs. Thus, it is a must to be extremely cautious while manipulating Hippo pathway for the therapeutic benefit so that the drug does not significantly alter vital organs/tissues or pools of progenitors and any undesirable side effects are avoided.

However, right now the future for the therapies does not look so bleak as this is the exciting time when the Hippo field is expanding tremendously with every passing year. Further knowledge on the signals that regulate the pathway will prove to be beneficial for the development of therapies based on modulation of YAP/TAZ and prevention of cancer and other non-cancer diseases.

# RESULTS

## **2.0 RESULTS**

**Article in preparation**

**Yorkie alternative splicing is required  
for developmental robustness**

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The mechanisms that contribute to developmental stability are barely known<sup>1</sup>. Alternative splicing (AS) of mRNA precursors expands transcriptomic and proteomic diversity and its extent correlates with organism complexity (REFs). Nevertheless its contribution to developmental robustness has not been documented. Here we show that AS of *yorkie* (*yki*) is required for developmental stability in *Drosophila*. Yki encodes the effector of the Hippo pathway that has a central role in controlling organ growth and regeneration. We identify the splicing factor B52 as necessary for inclusion of *yki* alternative exon 3 that encodes one of the two WW domains of Yki protein. Compared to the canonical Yki2 isoform containing two WW domains, Yki1 isoform with a single WW domain, has reduced transcriptional and growth-promoting activities, decreased binding to specific partners, and lacks the ability to bridge two proteins containing PPxY motifs. Yet, Yki1 and Yki2 interact similarly with transcription factors and can thus compete *in vivo*. Flies in which *yki* AS been abrogated to express only Yki2 isoform show increased fluctuating asymmetry in the wings giving evidence of increased developmental noise. Our results provide the first experimental support that AS participates in developmental robustness and identify *yki* AS as a new level of control of Hippo pathway. Remarkably, alternative inclusion of the second WW domain is a conserved feature between Yki and its human homolog YAP, indicating that modulation of YAP AS could be a novel strategy to lower YAP activity that is frequently upregulated in cancer cells.

RNA binding proteins of the SR family are conserved proteins that play a major role in AS regulation. *Drosophila* B52 is an essential protein that belongs to this family of splicing factors. Our previous work identified a positive link between B52 and cell growth<sup>2</sup> but the underlying molecular mechanisms were not characterized. We reasoned that B52's effect on growth might be due to AS modulation of one or several genes controlling growth. To identify AS events that are robustly affected by B52 depletion, we crossed two previously published RNAseq datasets corresponding to RNAi-mediated depletion of B52 in S2 cells compared to control cells<sup>3,4</sup>. We used MAJIQ (Modeling Alternative Junction Inclusion Quantification)<sup>5</sup> to identify Local Splicing Variations (LSV) that vary more than 20% between control and B52 RNAi conditions, in both datasets (see Methods). This identified 119 AS events in 119 genes (Suppl. Fig. 1a and Suppl. Table 1). Strikingly, GO term enrichment analysis of these genes places regulation of growth at the top of the list (Suppl. Fig. 1b). Interestingly, three genes linked to Hippo pathway were identified: *yorkie* (*yki*), *WW domain*



*binding protein 2 (Wbp2)* and *Zyxin (Zyx)*. RNAseq data indicate that B52 depletion promotes skipping of exon 3 in *yki* mRNA, the inclusion of exons 6 and 7 in *wbp2* and the retention of last intron in *Zyx* (Suppl. Fig. 1c). The functional consequences of these AS events are unknown.

Hippo signaling is an evolutionary conserved pathway that regulates cell fate and cell proliferation to control organ growth and regeneration<sup>6</sup>. The core of the Hippo pathway in *Drosophila* consists of two kinases, Hippo (Hpo) and Warts (Wts), and their adaptor proteins, which phosphorylate the transcriptional coactivator Yorkie (Yki) to sequester it in the cytoplasm through binding to 14.3.3 proteins. Upon inactivation of the pathway, unphosphorylated Yki translocates in the nucleus where it binds to transcription factors such as Scalloped (Sd) and recruits partners such as NcoA6 or Wbp2 to activate transcription of its target genes.

*yki* gene contains an alternative exon (exon 3) that encodes one of the two WW domains of the protein, thus producing two isoforms that we named Yki2 and Yki1 according to their number of WW domains (Fig. 1a). We confirmed by RT-PCR that RNAi-induced depletion of B52 in S2R+ cells or in wing discs induces skipping of *yki* exon 3 and increases expression of Yki1 at the expense of Yki2 isoform (Fig. 1b,c). To test whether B52 depletion affects Yki activity, we monitored expression of two reporter genes, *ex-lacZ* and *diap-lacZ*, which are direct targets of Yki. Expression of B52 RNAi in the in the posterior domain of the wing disc decreases expression of the two reporter genes in this domain reflecting a reduction of Yki activity (Fig. 1d). This goes along with a reduction of posterior domain size. These flies are poorly viable at 25° but are viable at 18° and show a net reduction of wing posterior domain size (Fig. 1e). Using this phenotype, we tested whether *B52* interacts genetically with the Hippo pathway. Overexpression of Yki (UAS-Yki-V5, corresponding to long isoform<sup>7</sup>), as well as depletion of the kinases Hpo or Wts by RNAi, partially rescue the growth defect induced by B52 depletion (Fig 1f). Therefore increasing Yki activity antagonizes the effect of B52 depletion. Nevertheless, posterior domain size in these contexts remain smaller than the corresponding controls (overexpression of Yki or depletion of Wts or Hpo in the absence of B52 depletion, Fig. 1e and Suppl. Fig. 2). This suggests that B52 depletion affects other genes or pathways necessary for efficient growth. Moreover, the strong overgrowth induced by Yki overexpression, or by Hpo or Wts depletion, may compensate a growth defect in B52 depleted cells, unrelated to Hippo pathway. This is unlikely because depletion of Tgi, a transcriptional repressor interacting with Sd, which induces a very mild increase of posterior domain size on its own, also rescues the growth defect due to B52 depletion (Fig. 1e).

Altogether these results indicate that B52 depletion reduces growth at least in part through the Hippo pathway and suggest that it lowers Yki activity by modifying alternative splicing of *yki* mRNAs. We, therefore, explored the functional differences between the two Yki isoforms.

To compare the activity of Yki isoforms, we created *UAS-Yki1* and *UAS-Yki2* transgenic flies for GAL4-mediated overexpression, by site-specific integration. Compared to Yki2, overexpression of Yki1 isoform induces weaker overgrowth phenotypes in the posterior domain of wings (Fig. 2a) or in the eyes (Suppl. Fig. 3). We analyzed expression of Yki reporter genes *ex-lacZ* and *diap-lacZ* following Yki isoforms overexpression in the posterior domain of wing discs. Expression of both transgenes is moderately increased by Yki1 overexpression as compared to Yki2 overexpression (Fig. 2b,c). We also analyzed expression of a *bantam* miRNA sensor, which expression is inversely correlated to the level of *ban* miRNA, a direct target of Yki. Yki1 induces a weaker decrease of bam-sensor expression than Yki2 isoform (Fig. 2c). Together these results show that Yki1 has a reduced activity compared to Yki2 *in vivo*.

Yki1 and Yki2 isoforms differ respectively by the absence or presence of alternative exon 3, which includes the second WW domain (WW2). Numerous studies have demonstrated that, in the context of Yki2 isoform, WW domains are required for interaction with several partners containing PPxY motifs such as Ex, Hpo, Wts, NcoA6, Wbp2 and Tgi<sup>8-15</sup>. The absence of one WW domain in Yki1 is supposed to reduce interaction with such partners, but this assumption has not been experimentally demonstrated. Moreover binding of proteins that interact with the N-terminal part of Yki, such as Sd or 14.3.3, may also be affected if the structure of the protein is modified by the absence of the domains encoded by exon 3. It is therefore important to address these interactions in the context of full-length proteins and with a quantitative assay. To this end we developed a dual-luciferase co-Immunoprecipitation (co-IP) method in S2R+ cells inspired by the DULIP method<sup>16</sup> described in mammalian cells. Our assay monitors co-IP between a bait protein fused to Flag-tagged-Firefly luciferase and a prey protein fused to HA-tagged-Renilla luciferase (Fig. 3a). Quantification of luciferases activities ratio after IP with anti-Flag antibody gives a rapid and sensitive readout of the interaction between the bait and the prey. Flag-Firefly-Yki1 and Flag-Firefly-Yki2 were used as baits for interaction with thirteen known Yki partners fused to Renilla luciferase as preys (Fig. 3b). We used Flag-Firefly as a control for non-specific interactions. By this approach, we detected significant interactions between Yki isoforms and all proteins tested, from modest co-IP with GAF, MAD and Cbt, to high and very high interaction for the other proteins. We found that both isoforms Yki1 and Yki2 interact

similarly with the transcription factors Sd, MAD, Cbt and Hth, the chromatin-associated factors GAF and Mor, and with 14.3.3 protein (Fig. 3c). These results show that interactions with these partners are not influenced by the domains encoded by *yki* exon 3. On the other hand, we saw that interaction with Wts, Ex, NcoA6, Wbp2 and Tgi is reduced two to four times for Yki1 compared to Yki2 (Fig. 3c). All these proteins contain multiple PPxY motifs. Interestingly, we did not detect significant difference between Yki isoforms for the binding to MAD and Hpo which both contain a single PPxY motif. This is in agreement with in vitro studies reporting cooperative binding between multiple PPxY motifs and tandem WW domains of YAP and Yki<sup>17,18</sup>. Altogether, these results suggest that Yki isoforms interact similarly with TF but that Yki1 cannot efficiently recruit co-activators like NcoA6 or Wbp2 to stimulate transcription.

In addition to increasing the binding of proteins with multiple PPxY motifs, the presence of two WW domains may also allow Yki2 to interact simultaneously with two PPxY-containing proteins. To test this hypothesis we compared the ability of Yki isoforms to bridge different proteins, by monitoring their co-IP using the DULIP assay in the presence or absence of Yki. Previous reports showed that Yki2 binds to Sd through its N-terminal part and to Tgi *via* the WW domains<sup>14,15,19,20</sup>. Moreover Sd and Tgi were shown to interact directly<sup>14,15</sup>. Yki was shown to compete with Tgi for Sd binding<sup>14,15</sup>, but could also be found in a trimeric complex with Sd and Tgi<sup>14</sup>. We, therefore, monitored Sd/Tgi interaction in absence or presence of Yki isoforms in transfected cells (Fig. 3d). Using Firefly-Sd as bait, we detected co-IP between Firefly-Sd and Renilla-Tgi in absence of Yki as previously reported. Both Yki1 and Yki2 isoforms enhanced the co-IP between Sd and Tgi, with Yki2 having a stronger effect. Point mutations in the two WW domains of Yki2 abolish this interaction. These results are in agreement with the existence of a trimeric complex between Sd-Tgi and one isoform of Yki.

To further test Yki bridging activity, we analyzed the co-IP between Firefly-Sd and Renilla-Wbp2 by the same approach. Co-transfection of Yki1 or Yki2 increases the pull-down between Sd and Wbp2, with Yki2 having a stronger effect. Mutation of both WW domains in Yki2 abrogates this effect (Fig. 3e). These results indicate that, in interaction with Sd, both Yki1 and Yki2 isoforms participate in the recruitment of Tgi or Wbp2, with Yki2 being more efficient owing to its two WW domains. Finally, we tested if the two WW domains of Yki2 can interact with two different proteins carrying PPxY motifs. To this end we analyzed the co-IP between Tgi and Wbp2 using Firefly-Tgi as bait and Renilla-Wbp2 as prey. Remarkably,

Yki2 isoform allowed to pull-down Wbp2 with Tgi, whereas Yki1 isoform did not. As expected, mutation of the two WW domains in Yki2 abrogates the co-IP (Fig.3f).

Taken together our results show that Yki isoforms interact similarly with TF but differ by their capacity to bind and bridge PPxY-containing proteins. We propose the following model (Fig. 4a). Upon repression of Hippo pathway, unphosphorylated Yki isoforms enter the nucleus. Yki2 being more abundant, joins the transcriptionally repressed Tgi-Sd complex. Within this trimeric complex, Yki2 may engage only one WW domain thus being able to recruit through the second WW domain another partner such as Wbp2 or NcoA6, which finally may or not displace Tgi. This could reconcile the observation that Tgi enhances Sd-Yki interaction but decreases distance between Sd and Yki as measured by FRET<sup>14</sup> suggesting remodeling of the complex. In the B52 depleted situation, Yki1 isoform level is increased and this isoform substitute to Yki2. In a complex with Sd and Tgi, Yki1 would be unable to recruit additional partners and activate transcription.

One prediction of this model is that Yki1 should be able to compete with Yki2 for binding to transcription factors *in vivo*. To test this hypothesis we used activated forms of Yki1 and Yki2 carrying the mutation S168A that kills a Wts phosphorylation site and lowers Yki cytoplasmic retention by 14.3.3 proteins<sup>21</sup>. We verified that this mutation favors nuclear accumulation of both Yki1<sup>S168A</sup> and Yki2<sup>S168A</sup> isoforms (Suppl. Fig. 4) and increases the overgrowth phenotypes induced by overexpression of these isoforms in the eye (Suppl. Fig. 3). Of note activated Yki1<sup>S168A</sup> induces dramatically weaker overgrowth compared to Yki2<sup>S168A</sup> confirming its reduced transcriptional activity (Suppl. Fig. 3). Overexpression of Yki2<sup>S168A</sup> in the eye induces strong over-growth and remarkably, co-overexpression of activated Yki1<sup>S168A</sup>, but not non-activated form Yki1, reduces this phenotype, suggesting that Yki1 can compete with Yki2 in the nucleus (Fig. 4b). These results are in agreement with a competition model between a less-active isoform Yki1 and a fully active form Yki2. We propose that modification of the balance between the two isoforms, such as created by B52 depletion, participate in the regulation of Yki activity.

To evaluate if this balance has a developmental role, we abrogated AS of *yki* exon3 by creating flies producing exclusively Yki2 isoform. We edited endogenous *yki* locus to replace the central part of *yki* locus by a portion of *yki2* cDNA, therefore eliminating the introns surrounding exon 3 (See strategy in Suppl. Fig. 5). This allele, denoted *yki*<sup>2only</sup>, carries exon2 fused to 4 and do not contain exogenous sequence (Fig. 4c). We confirmed by western blotting that flies no more produce Yki1 isoform (Fig. 4d). These flies are viable and fertile. Interestingly we noticed that several flies in the population display asymmetric wings (Fig.

4e). The intra-individual variation between the size of right and left wings, called Fluctuating Asymmetry (FA), reflects a developmental instability. We measured wild type and *yki*<sup>2only</sup> mutant wing areas and quantified the fluctuating asymmetry index FA10 (ref) which takes into account measurement errors (Suppl. Fig. 6). *yki*<sup>2only</sup> flies display higher FA in both males and females (Fig. 4f), reflecting increased developmental variability upon abrogation of Yki AS.

We propose that *yki* AS represents an additional layer of modulation of Yki activity that participates in buffering developmental noise. Recently it has been shown that, in addition to its intrinsic effect on tissue growth, Yki is involved in systemic growth by interacting with ecdysone signaling. Yki is involved in basal expression of ecdysone<sup>22</sup>, interacts with the ecdysone receptor coactivator Taiman<sup>23</sup> and controls the expression of *dilp8* which is involved in inter-organ coordination of growth<sup>24</sup>. Significantly, genomic deletion of *dilp8* Hippo Responsive Element is sufficient to increase FA in flies<sup>24</sup> indicating that Yki's control on *dilp8* is required to minimize developmental variability. It will be important to determine to which extent AS of *yki* is dynamically regulated during normal growth and regeneration, and which signals are involved.

Our identification of B52 as a modulator of *yki* AS is a first step in the characterization of this regulation. Finally it is worth noting that alternative inclusion of the second WW domain is a conserved feature between Yki and its human homolog YAP. Moreover, YAP1 isoform containing a single WW domain is a weaker transcriptional activator than YAP2<sup>25</sup>. Therefore modulation of YAP AS could be a new strategy to decrease YAP activity in cancer cells.

## Methods

### RNAseq analysis

We used MAJIQ (Modeling Alternative Junction Inclusion Quantification) to identify Local Splicing Variations (LSV) in two previously published datasets from Bradley et al. (#GSM1552264, # GSM1552267) and Brooks et al. (#GSM627333, #GSM627334 and #GSM627343). Each dataset contains two replicates of control cells and B52-depleted RNAseq. The reads were subjected to standard quality control (QC) and filters criteria according to the following parameters: (1) trimming and cleaning reads that aligned to primers and/or adaptors, (2) reads with over 50% of low-quality bases (quality value $\leq$ 15) in one read, and (3) reads with over 10% unknown bases (N bases). We have used a software called Trimmomatic (v0.36) to remove primers and also to remove bad quality reads. After filtering, we removed short reads (<36bp), the remaining reads are called "clean reads" and stored as FASTQ format. Reads were aligned to *Drosophila melanogaster* reference genome release 6.19 using STAR (Spliced Transcripts Alignment to a Reference). STAR output were stored in BAM files. BAM files were then submitted to MAJIQ analysis pipeline. LSV definitions were generated and quantified by MAJIQ. MAJIQ applies several normalization factors to the raw values before to compute normalized PSI (Percent Selected Index) and compare them between replicates. To make a selection of best candidate genes we used a  $\Delta$ PSI threshold of 0.2. All others specified arguments were used with default values. Gene lists for each dataset were compared to select common events between them. This identified 119 AS events in 119 genes that show reproducible change of AS upon B52 depletion. GO term analysis was performed with PANTHER.

### Fly strains and Genetics

*Drosophila* were maintained on standard cornmeal-yeast medium. Experiments were performed at 25°C, except for the analysis of wing phenotype of flies expressing B52 RNAi under the control of HH-Gal4 that were performed at 18°C, as mentioned in figure legends. Inducible RNAi lines used were UAS-IR-B52 (GD8690), UAS-IR-Wts (GD1563 and TRiP.HMS00026), UAS-IR Hpo (TRiP.HMS00006), UAS-IR-Tgi (TRiP.HMS00981) and were previously validated in the literature.

UAS-Yki1 and UAS-Yki1<sup>S168A</sup> transgenes were constructed from pUAS-Yki-V5-His and pUAS-Yki<sup>S168A</sup>-V5-His clones in pUAS-attB vector, kindly provided by K. Irvine (these clones contain Yki2 isoform cDNA). A XhoI site located between UAS sequences and start

codon, flanked by two EcoRI sites, was deleted by EcoRI digestion and re-ligation of these vectors. This generated UAS-Yki2-V5-His and UAS-Yki2<sup>S168A</sup>-V5-His. The SfiI-XhoI fragment containing the C-terminal part of Yki2 was replaced by the corresponding SfiI-XhoI amplified from a *yki1* cDNA obtained by RT-PCR (third instar larvae). This generated UAS-Yki1-V5-His and UAS-Yki1<sup>S168A</sup>-V5-His transgenes. The four constructs UAS-Yki2-V5-His, UAS-Yki2<sup>S168A</sup>-V5-His, UAS-Yki1-V5-His and UAS-Yki1<sup>S168A</sup>-V5-His were inserted in attP2 site. Injections were performed by Bestgene Inc.

### ***yki* gene editing**

To edit endogenous *yki* locus by CRISPR/Cas9-mediated Homologous Recombination, a repair construct corresponding to *yki* gene without introns 2 and 3, and containing a piggyback insertion in intron 1, was created by cloning of multiple PCR fragments amplified with Q5-Taq polymerase (Biolabs). *yki* gene fragments were amplified from *yw* flies. PiggyBac transposon containing the reporter gene 3xP3-DsRed (expresses DsRed in the eye), was amplified from pHD-3xFLAG-ScarlessDsRed (DGRC #1367) and cloned into a DraI site present in *yki* intron 1, thus creating TTAA sequences at both sides of the transposon that are necessary for its excision. We used two guide RNAs targeting exon 2 and exon 4 of *yki*, cloned into pCFD4 (addgene #49411). The plasmid containing the repair construct was co-injected with the plasmid encoding the two guides into *nos-Cas9* embryos. Injection and screening for positive DsRED flies in the progeny, were performed by Bestgene Inc. Two DsRED-positive lines, validated by PCR, were selected for excision of PiggyBac transposon using a source of transposase (Bloomington #8285). For each line a single excision event was selected to establish a stock. Two independent *yki*<sup>2only</sup> alleles were obtained, called *ED1* and *ID3*. The entire *yki* locus was sequenced in these lines. We detected polymorphism in introns and silent polymorphism in exons. These variations are present between the *yw* line used to create the *yki*<sup>2only</sup> construct and the *nos-Cas9* line in which the injections were made. Both *yki*<sup>2only</sup> lines display enhanced FA.

### **Molecular biology**

cDNAs corresponding to MAD, GAF, Sd, Hth, 14.3.3, Hpo and Wbp2 were amplified from third instar larvae RNA by RT-PCR, using a forward primer stating at ATG and reverse primer located just upstream (or sometimes including) the stop codon. Forward primer contains CACC sequence upstream of ATG to orient cloning in pENTR/D-Topo (Invitrogen). All cDNAs were entirely sequenced. Other cDNAs were Cabut



(DmCD00765693, DNASU), Tgi (DmCD00765105, DNASU), Mor (Addgene #71048), Ex (kindly provided by N. Tapon). NcoA6 was amplified from a plasmid kindly provided by K. Irvine.

For DUAL-luciferase co-IP, we developed three destination vectors for the Gateway system: pAct-Flag-Firefly-RfA, pAct-HA-Renilla-RfA, pAct-RfB-Renilla-HA. The luciferase-gateway cassettes from mammalian vectors pcDNA-Flag-Firefly-RfA, pcDNA5-HA-Renilla-RfA and pcDNA5-RfB-Renilla-HA (kindly provided by E. Bertrand) were cloned into pAFW backbone. cDNAs cloned into pENTRD/Topo were transferred to the appropriate destination vector by LR recombination (Invitrogen).

### **Cell culture and co-immunoprecipitations**

*Drosophila* S2R<sup>+</sup> cells were maintained in Schneider's medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen) at 27°C. For B52 depletion, S2R<sup>+</sup> cells were treated with a mix of two dsRNA (produced by *in vitro* transcription) targeting exon 2 and exon 9 of *B52*, for 72h. RNAs were extracted with trizol (Sigma). Proteins were prepared in urea buffer. Antibodies used for western were: rabbit anti-Yki<sup>26</sup>, Rabbit anti-B52<sup>27</sup>, Mouse anti-actin (DSHB).

For dual-luciferase co-IP, S2R<sup>+</sup> cells were transfected with two plasmids (0.15µg each) in quadruplicate in 24-wells plates (400000 cells/well) using effectene (Qiagen). Typically, 4 plates were handle at the same time to perform 24 co-IPs in quadruplicate. After 48h of transfection, cells were washed with PBS and lysed with 250µl HNTG buffer (20mM Hepes pH7.9, 150 mM NaCl, 1mM MgCl<sub>2</sub>, 1 mM EDTA, 1% triton, 10% glycerol) supplemented with proteases inhibitors (Halt inhibitor cocktail, Thermo Scientific). Immunoprecipitation were performed on each lysate in 96-wells Neutravidin plates (Termo Scientific) previously coated for 2h with biotinylated anti-Flag antibody (Bio-M2, Sigma) in HNTG (2 µg antibody/well). IP were performed with 100µl of lysate/well and incubated overnight at 4°. IP were washed 5 times with 200 µl HNTG/well for 5 min at 20° on a thermomixer (Eppendorf) with intermittent shacking. Firefly and Renilla activities were then quantified with DUAL luciferase reporter assay (Promega) using 50µl of reagents/well and an InfiniteF200 reader (TECAN). To quantify input, 10µl of each lysate were transferred in a white 96 plate and quantified as the same time as IP plate with the same procedure. The level of co-IP is quantified by calculating the level of co-IP normalized to the efficiency of IP:  $(Renilla^{IP}/Renilla^{Input})/(Firefly^{IP}/Firefly^{Input})$ .

For bridging experiments between a Firefly-tagged bait and a Renilla tagged prey in absence or presence of Yki isoforms, the same procedure was used with a co-transfection of 0.15µg of Firefly plasmid, 0.15µg Renilla plasmid and 0.3µg pAct-Myc-Yki plasmid (or without Yki for control). Transfection and IP were performed in quadruplicate as described above.

### **Wing measurements**

Young flies (1-3 days) of the appropriate genotypes were stored in isopropanol. Wings were mounted in Euparal (Roth, Germany) on glass slide with coverslip and baked overnight at 65°. For measurements of posterior vs total area (experiments with HH-Gal4 driver), pictures were acquired on a Leica M80 stereomicroscope equipped with a Leica IC80 HD camera using LAS software. Quantification was performed with Omero ([www.openmicroscopy.org](http://www.openmicroscopy.org)).

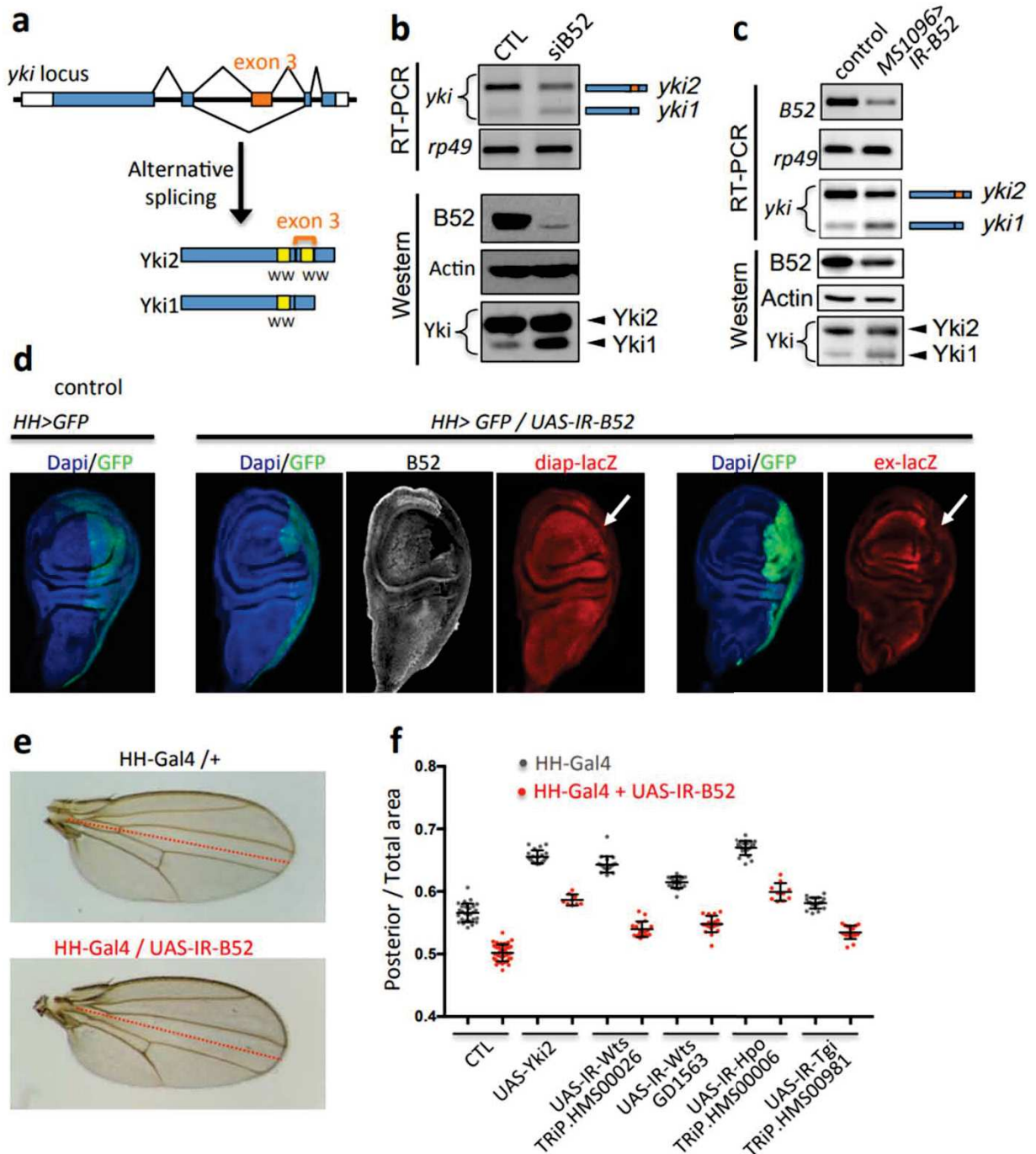
For quantification of Fluctuating Asymmetry (FA), left and right wings were mounted as pairs. Slides were digitalized using Nanozoomer (Hamamatsu). Quantification of wing size was performed with ImageJ. Each wing was measured twice in two independent sessions, by one or two persons. In rare case where the variation between replicate measurements was superior to 0.5% of total wing size, the wing was quantified again to minimize measurement error. The FA10 index<sup>28</sup> was used to estimate FA, i.e. FA corrected for measurement error, directional asymmetry and inter-individual variation. For all genotypes, the interaction individual/side was significant, indicating that FA was larger than measurement error. Conventional two-way mixed model ANOVAs were applied to area data using Prism Software. These values were used to calculate FA10 index. To compare FA10 values between genotypes, we used F-test to compare variance of the samples.

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# FIGURES FOR THE ARTICLE



**Figure 1. B52 depletion induces skipping of *yki* alternative exon 3 and lowers Yki activity.**

a. Drawing of *yki* locus and its two isoforms Yki2 (includes exon 3 and contains 2 WW domains) and Yki1 (skips exon 3 and contains 1 WW domain)

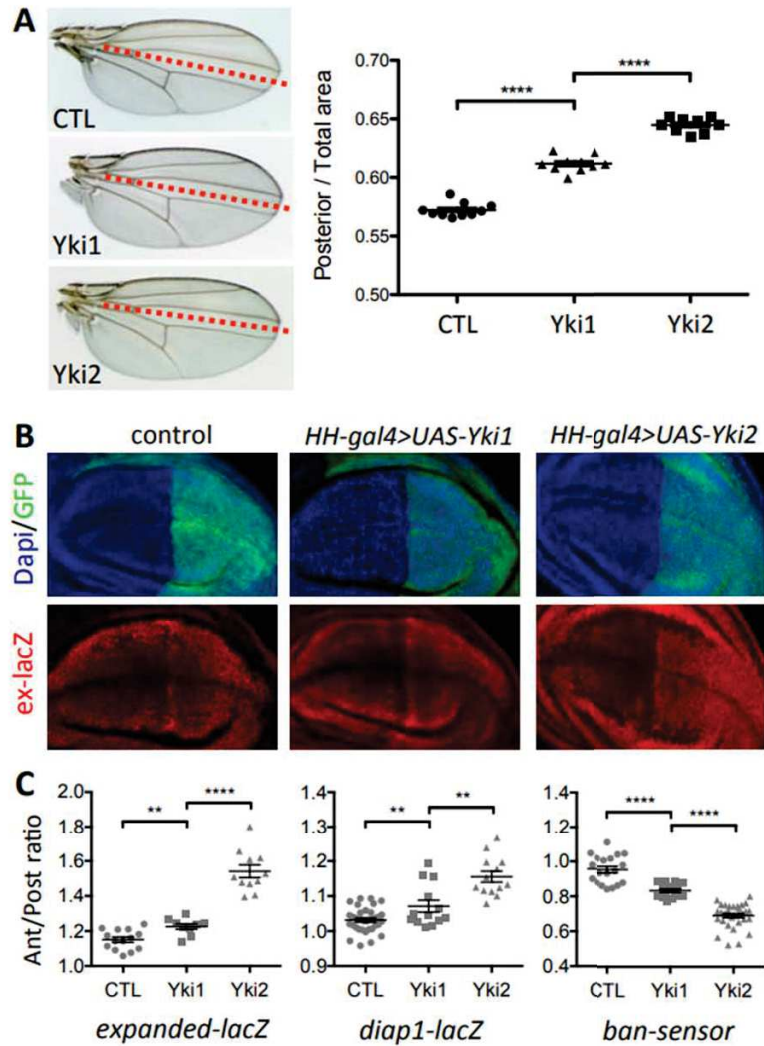
b,c. RT-PCR and western blot showing effect of RNAi-mediated B52 depletion in S2R+ cells (left) and in larval wing discs (right). Note that for the wing discs, the Gal4 driver MS1096 is expressed only in the wing pouch of the disc, thus depletion is partial. PCR primers in exons 2 and 4 are indicated in a.

d. Immunostaining of wing discs

e. Wing phenotype induced by depletion of B52 in the posterior domain (flies grown at 18°)

f. Quantification of Posterior/Total wing area in male flies. Bars represent mean with standard deviation. (flies grown at 18°)





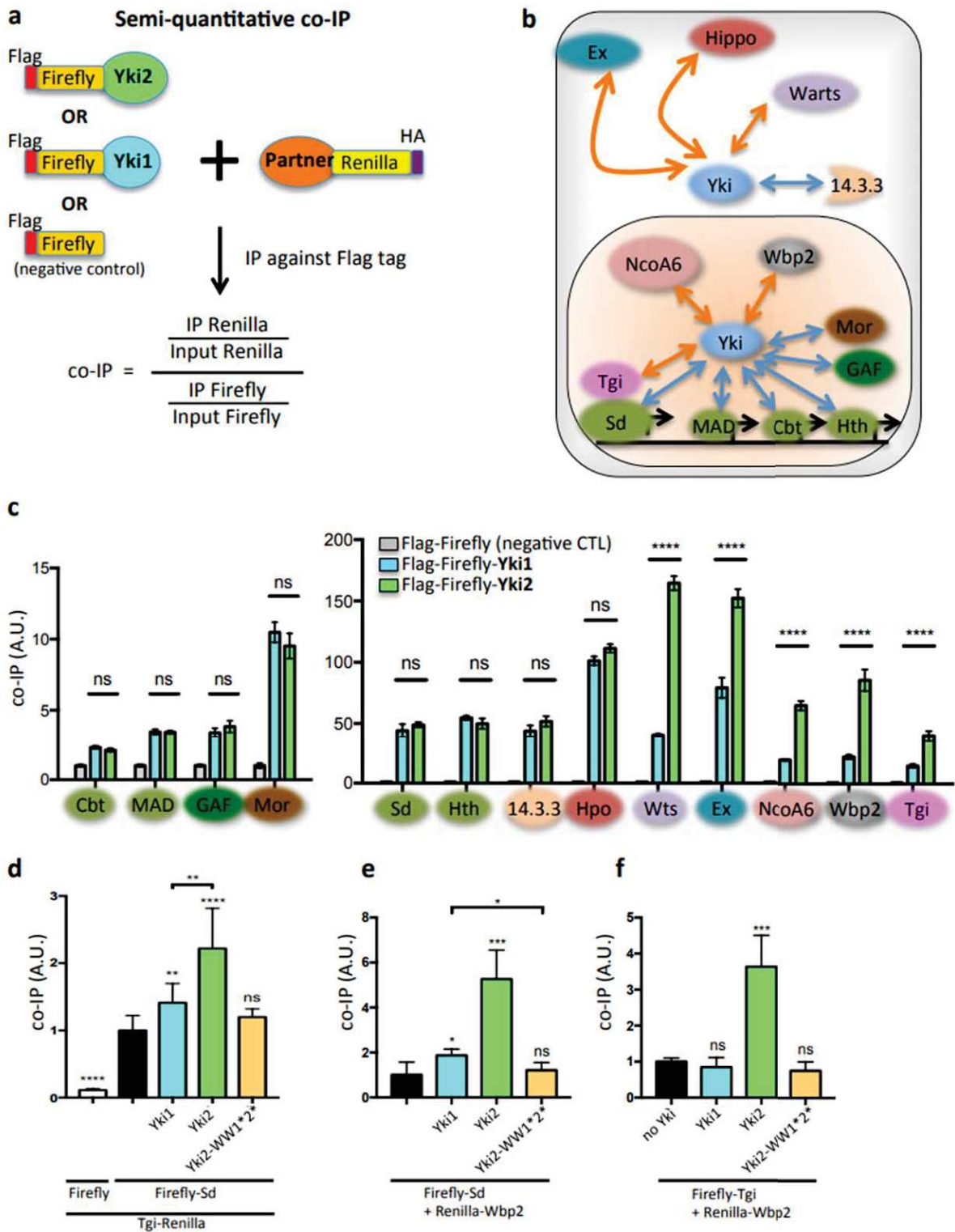
**Figure 2. Yki1 isoform is a weaker transcriptional activator than Yki2 isoform.**

a. phenotype of wings overexpressing Yki1 or Yki2 in the posterior compartment with HH-gal4 driver  
Quantification of the ratio between posterior domain size and total wing size.

b. Example of immunostaining used to quantify expression of ex-lacZ in wild type or upon Yki1 or Yki2 overexpression in wing disc posterior domain. GFP labels the posterior domain.

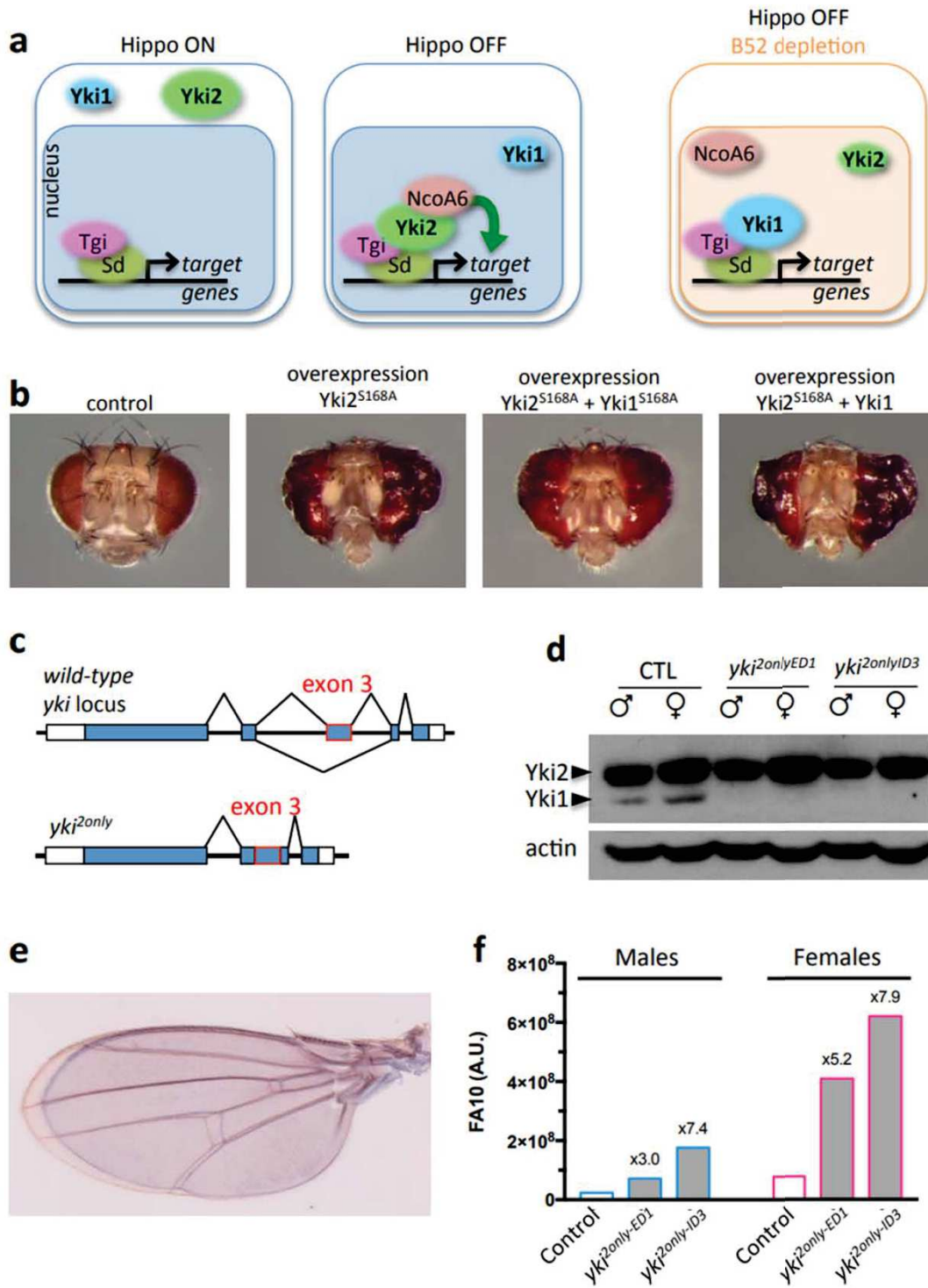
c. Quantification of yki reporter genes expression upon overexpression of Yki isoforms in the wing posterior domain. For each target gene, relative expression between posterior and anterior domain in the wing discs was quantified by immunostaining using anti-bgal (for ex-lacZ and diap-lacZ) or direct visualisation of GFP (ban-sensor, in this case posterior domain was visualized by V5-tag present in UAS-Yki transgenes).





**Figure 3.** Comparison of Yki isoforms binding capacities.

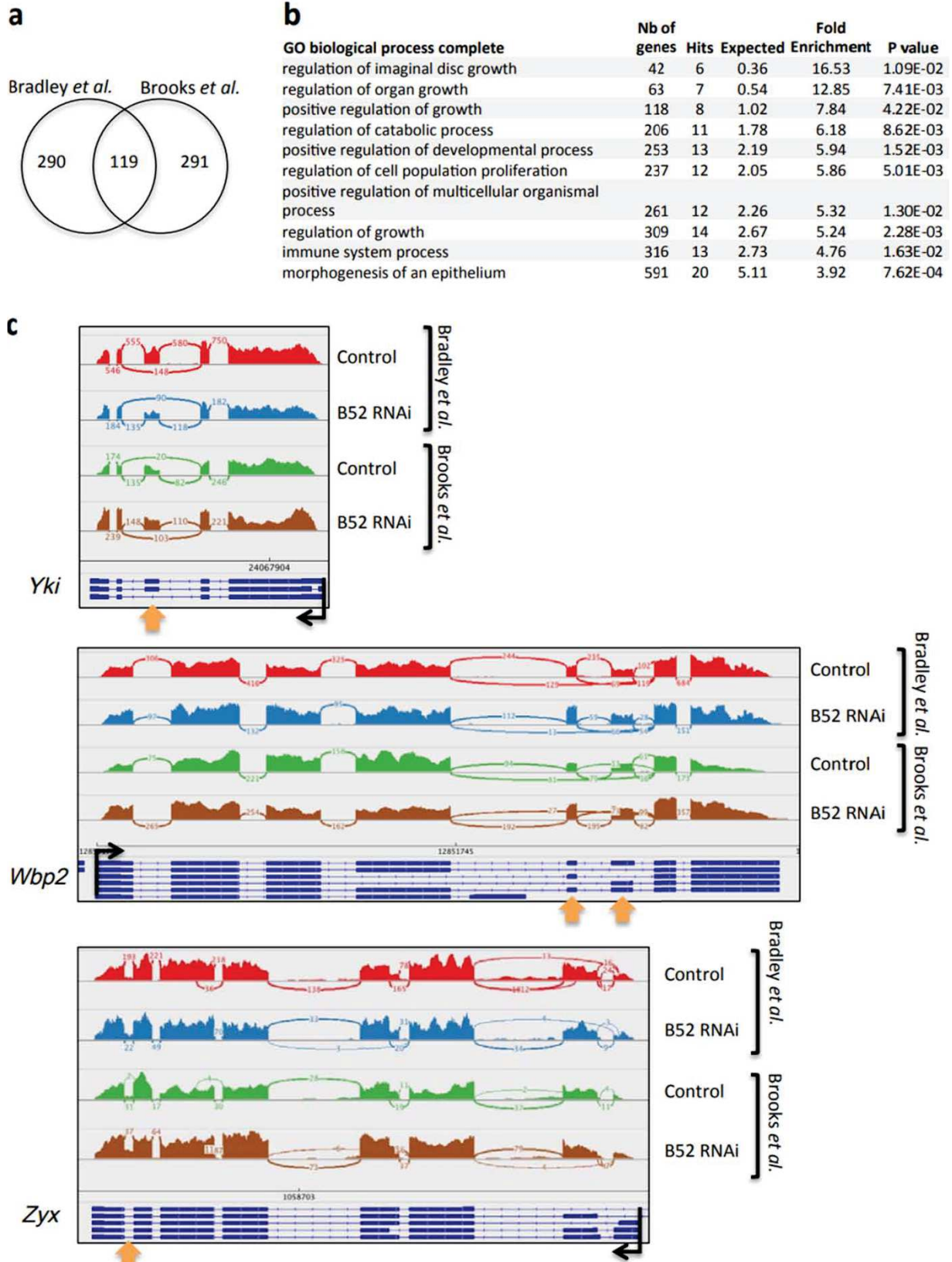
- Principle of dual-luciferase co-IP. In all experiments immunoprecipitation is performed with the flag-tag fused to the Firefly.
- Drawing of the monitored protein-protein interactions. Interactions previously shown to rely on Yki WW domains are represented by orange arrows, whereas other interactions are schematized by blue arrows.
- Results of co-IP
- Analyze of interaction between Sd and Tgi in absence of presence of Yki isoforms.
- Analyze of interaction between Sd and Wbp2 in absence of presence of Yki isoforms.
- Analyze of interaction between Tgi and Wbp2 in absence of presence of Yki isoforms.



**Figure 4.**

- a. Model of modulation of Yki activity by alternative splicing. See text.
- b. Competition between Yki isoforms in the eye. Overexpression of Yki isoform with GMR-Gal4 driver.
- c. Structure of the *yki<sup>2only</sup>* allele compared to wild type *yki* locus. Introns surrounding exon3 are removed. This allele do not contain any exogenous sequence.
- d. Western blot of adult males and females showing disappearance of Yki1 isoform in the two *yki<sup>2only</sup>* lines *yki<sup>2onlyED1</sup>* and *yki<sup>2onlyID3</sup>*.
- e. Overlay of right and left wings of *yki<sup>2onlyED1</sup>* flies.
- f. Quantification of fluctuating asymmetry index FA10 in control (*yw*) and two *yki<sup>2only</sup>* alleles corresponding to two independent homologous recombination events (see Suppl. Fig. 5)

# SUPPLEMENTARY FIGURES



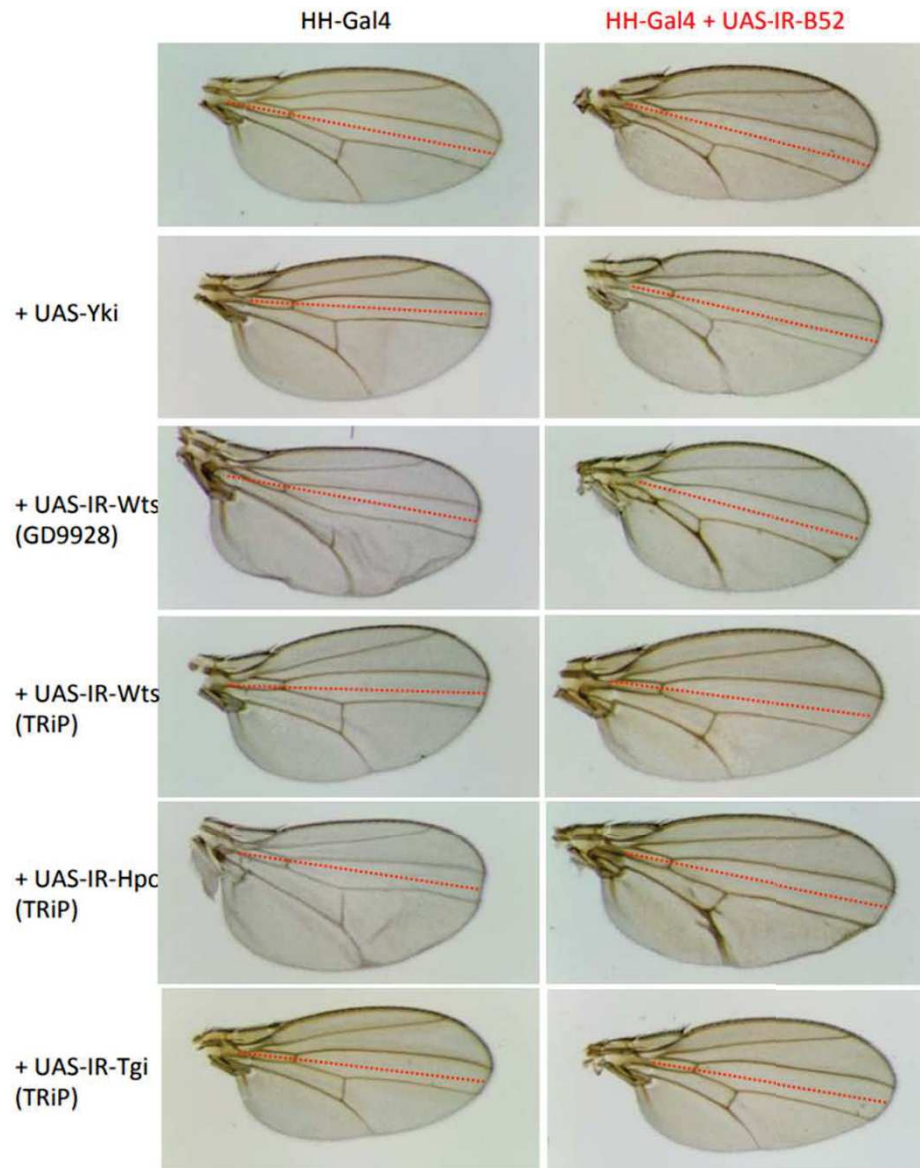
**Suppl. Figure 1.**

- Number of genes identified by MAJIQ that display 20% or more variation in splicing between wild-type and B52 depleted cells, in each dataset.
- GO term enrichment analysis of the 119 genes identified in (a), performed by PANTHER with Bonferroni correction for multiple testing.
- Shashimi plots corresponding to the three genes linked to Hippo pathway. The alternative exons or intron retention are indicated by the orange arrows.



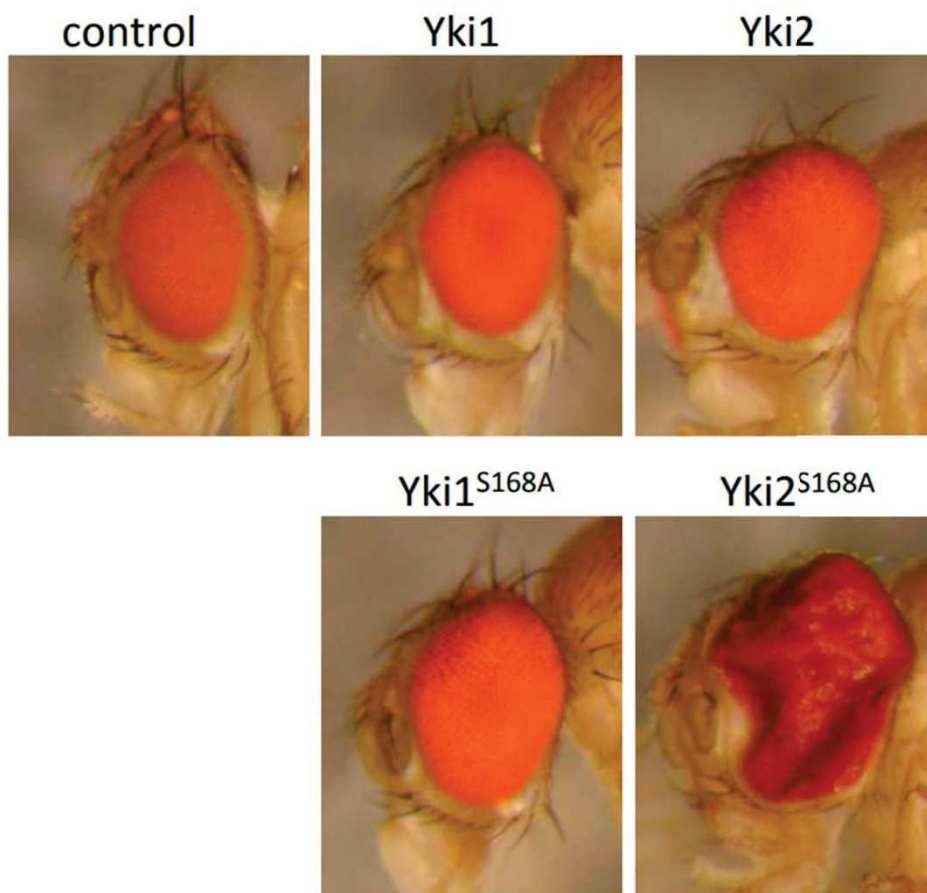
SYMBOL	NAME	FBID_KEY			
Abi	Abelson interacting protein	FBgn0020510	kuz	kuzbanian	FBgn0259984
Acn	Acinus	FBgn0263198	l(2)k09913	lethal (2) k09913	FBgn0021979
Akap200	A kinase anchor protein 200	FBgn0027932	Mef2	Myocyte enhancer factor 2	FBgn0011656
Aldh-III	Aldehyde dehydrogenase type III	FBgn0010548	mib2	mind bomb 2	FBgn0086442
Ate1	Arginyltransferase 1	FBgn0025720	mip120	Myb-interacting protein 120	FBgn0033846
Atg16	Autophagy-related 16	FBgn0039705	Mkrn1	Makorin 1	FBgn0029152
Atg18a	Autophagy-related 18a	FBgn0035850	Mlf	Myelodysplasia/myeloid leukemia factor	FBgn0034051
bip2	bip2	FBgn0026262	MP1	Melanization Protease 1	FBgn0027930
Caper	Caper	FBgn0031883	mri	mrityu	FBgn0035107
cert	ceramide transfer protein	FBgn0027569	Msp300	Muscle-specific protein 300 kDa	FBgn0261836
CG11753	-	FBgn0037603	mtd	mustard	FBgn0013576
CG13124	-	FBgn0032156	mud	mushroom body defect	FBgn0002873
CG13366	-	FBgn0025633	Nc73EF	Neural conserved at 73EF	FBgn0010352
CG1578	-	FBgn0030336	ND-15	NADH dehydrogenase (ubiquinone) 15 kDa subunit	FBgn0031228
CG1620	-	FBgn0033183	ND-MWFE	NADH dehydrogenase (ubiquinone) MWFE subunit	FBgn0085468
CG17691	-	FBgn0039993	Nipped-B	Nipped-B	FBgn0026401
CG2519	-	FBgn0037336	nolo	no long nerve cord	FBgn0051619
CG3065	-	FBgn0034946	Nrx-IV	Neurexin IV	FBgn0013997
CG31191	-	FBgn0051191	p24-2	p24-related-2	FBgn0053105
CG31855	-	FBgn0051855	Pdhb	Pyruvate dehydrogenase E1 beta subunit	FBgn0039635
CG32344	-	FBgn0052344	Pect	Phosphoethanolamine cytidyltransferase	FBgn0032482
CG3760	-	FBgn0022343	Pep	Protein on ecdysone puffs	FBgn0004401
CG41378	-	FBgn0085638	Pgam5	Phosphoglycerate mutase 5	FBgn0023517
CG4281	-	FBgn0025626	PGAP5	Post-GPI attachment to proteins 5	FBgn0031997
CG5746	-	FBgn0039186	Phb2	Prohibitin 2	FBgn0010551
CG6084	-	FBgn0086254	PMCA	plasma membrane calcium ATPase	FBgn0259214
CG6171	-	FBgn0026737	px	plexus	FBgn0003175
CG7028	-	FBgn0027587	Rad23	Rad23	FBgn0026777
CG7879	-	FBgn0035235	Rala	Ras-like protein A	FBgn0015286
CG7974	-	FBgn0035254	rdx	roadkill	FBgn0264493
CG8765	-	FBgn0036900	rin	rasputin	FBgn0015778
CG9684	-	FBgn0037583	ri	rolled	FBgn0003256
CG9705	-	FBgn0036661	RpS21	Ribosomal protein S21	FBgn0015521
CG9776	-	FBgn0027866	rush	rush hour	FBgn0025381
ctrip	circadian trip	FBgn0260794	sbb	scribbler	FBgn0285917
Cul2	Cullin 2	FBgn0032956	SelR	Selenoprotein R	FBgn0267376
CycG	Cyclin G	FBgn0039858	seq	sequoia	FBgn0028991
Dap160	Dynamin associated protein 160	FBgn0023388	shi	shibire	FBgn0003392
Dg	Dystroglycan	FBgn0034072	Sodh-2	Sorbitol dehydrogenase-2	FBgn0022359
dlg1	discs large 1	FBgn0001624	sol	small optic lobes	FBgn0003464
E(z)	Enhancer of zeste	FBgn0000629	spi	spitz	FBgn0005672
E2f1	E2F transcription factor 1	FBgn0011766	spz	spatzle	FBgn0003495
east	enhanced adult sensory threshold	FBgn0261954	stas	Stasimon	FBgn0030850
eIF4B	eukaryotic translation initiation factor 4B	FBgn0020660	Sting	Sting	FBgn0033453
eIF4E1	eukaryotic translation initiation factor 4E1	FBgn0015218	Su(z)12	Su(z)12	FBgn0020887
fdl	fumble	FBgn0011205	tacc	transforming acidic coiled-coil protein	FBgn0026620
Fer1HCH	Ferritin 1 heavy chain homologue	FBgn0015222	Taf12	TBP-associated factor 12	FBgn0011290
Flo2	Flotillin 2	FBgn0264078	teq	Tequila	FBgn0023479
Gdh	Glutamate dehydrogenase	FBgn0001098	Tif-IA	Tif-IA	FBgn0032988
hadly	hadley	FBgn0038842	Tm1	Tropomyosin 1	FBgn0003721
Hfp	half pint	FBgn0028577	tou	toutatis	FBgn0033636
HipHop	HP1-HOAP-interacting protein	FBgn0036815	TRAM	TRAM	FBgn0040340
HIPP1	HP1 and insulator partner protein 1	FBgn0037027	Trs23	TRAPP subunit 23	FBgn0260861
Hnf4	Hepatocyte nuclear factor 4	FBgn0004914	Unc-115b	Uncoordinated 115b	FBgn0260463
Indy	I'm not dead yet	FBgn0036816	unc-13	unc-13	FBgn0025726
Jupiter	Jupiter	FBgn0051363	Usp2	Ubiquitin specific protease 2	FBgn0031187
Kdm4B	Lysine (K)-specific demethylase 4B	FBgn0053182	Vha100-1	Vacuolar H[+]ATPase 100kD subunit 1	FBgn0028671
kis	kismet	FBgn0266557	vtd	verthandi	FBgn0260987
			Wbp2	WW domain binding protein 2	FBgn0036318
			yki	yorkie	FBgn0034970
			Zyx	Zyxin	FBgn0011642

Suppl. Table 1. List of genes showing reproducible AS variations upon depletion of B52 in S2 cells.



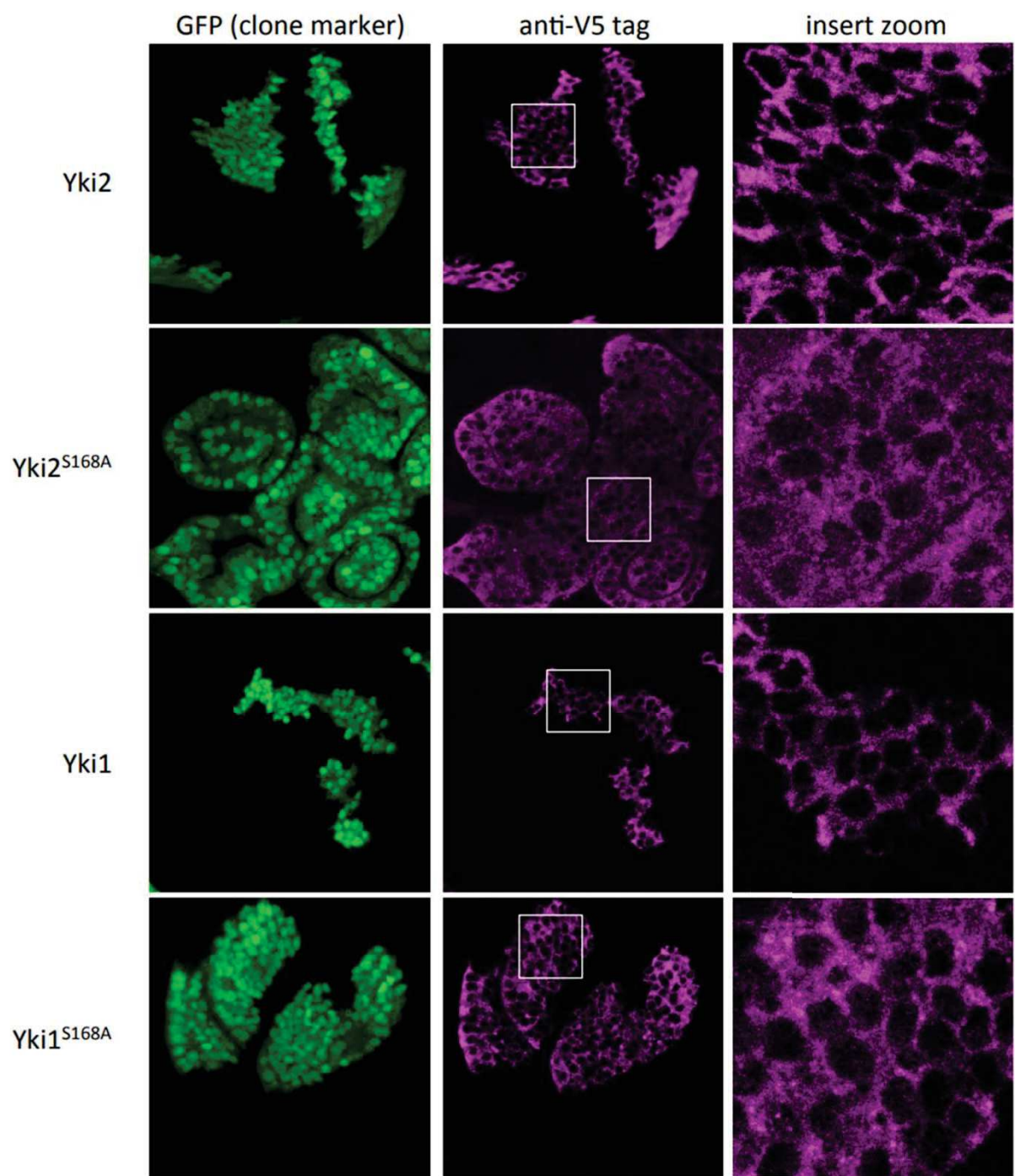
**Suppl. Figure 2. Genetic interaction between *B52* and Hippo pathway core components.**

RNAi were driven by *HH-Gal4* in the posterior domain of the wing. Each transgene is present in one copy. Flies were reared at 18°.



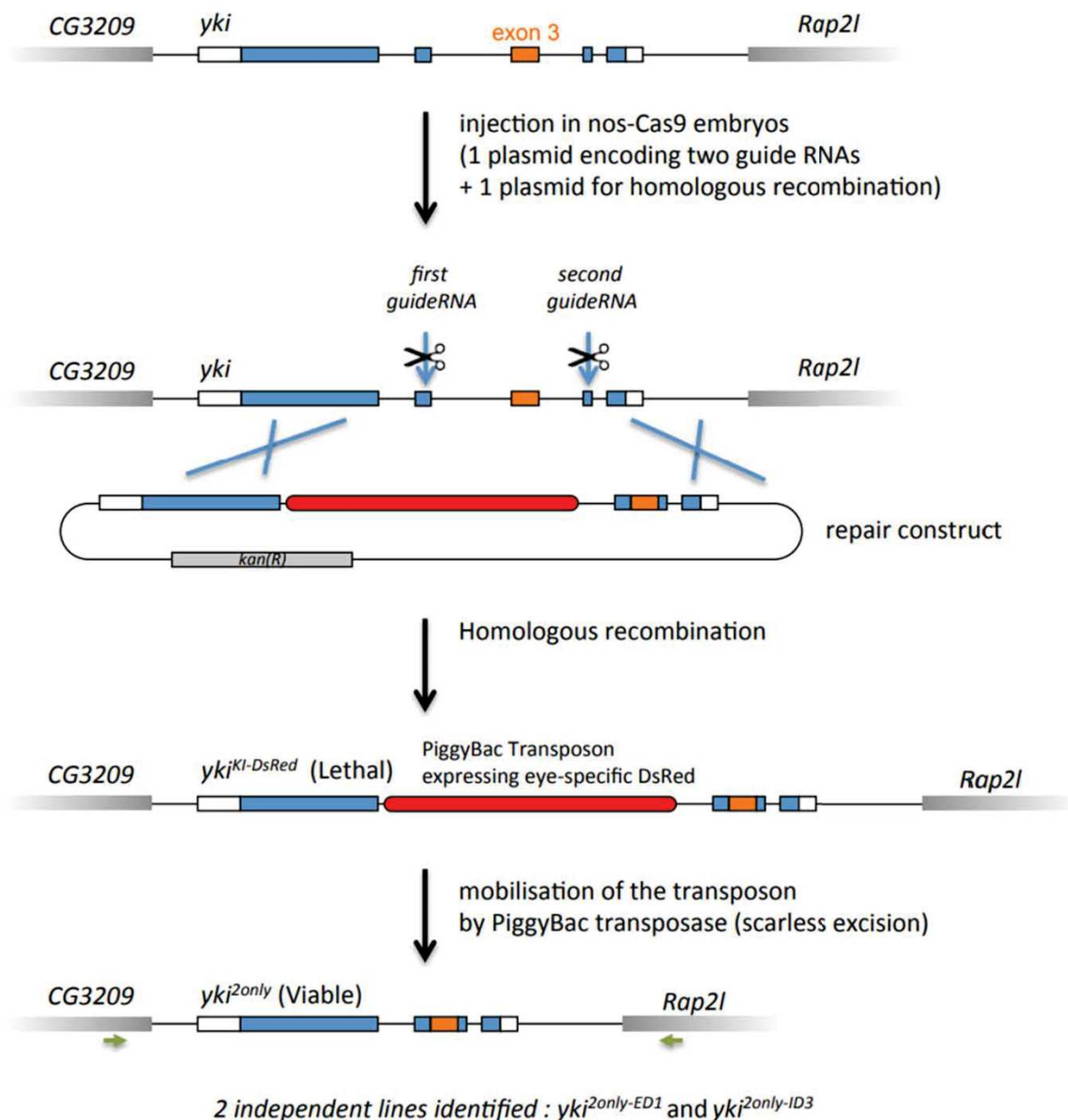
Suppl. Figure 3. Phenotypes induced by overexpression of Yki isoforms in the eye (GMR-Gal4 driver).





**Suppl. Figure 4. Subcellular localization of Yki isoforms in overexpression clones in wing discs (flip-out clones).**

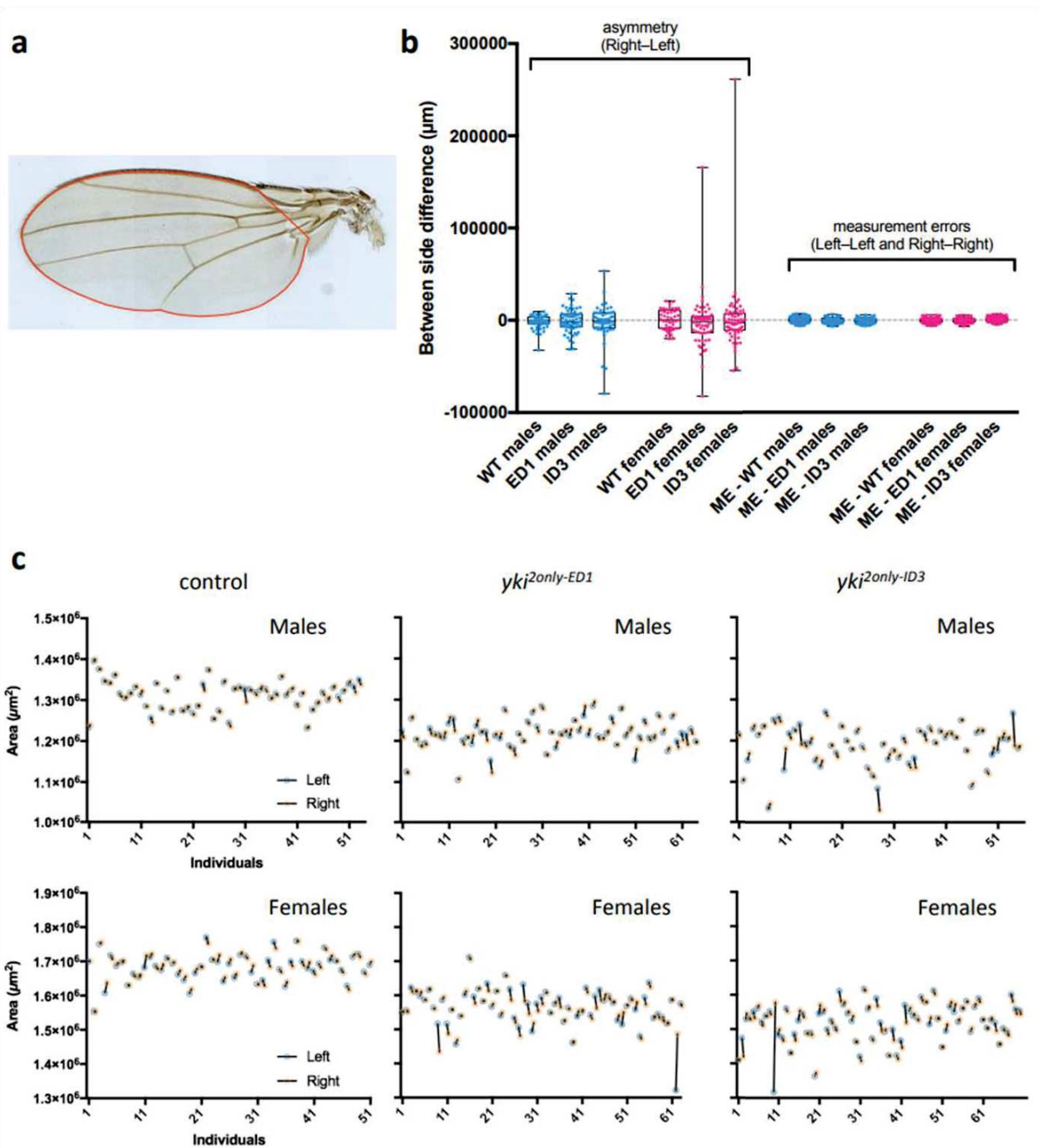
Clones are labelled with GFP. Yki proteins are visualized with V5-tag fused to each isoform.  
 Genotype: HS-FLP / Act[CD2]Gal4 ; UAS-GFP<sup>NLS</sup> / UAS-Yki (1, 2 or activated forms).



#### Suppl. Figure 5. Strategy used to create *yki<sup>2only</sup>* allele

The *yki<sup>2only</sup>* locus was first assembled and cloned in bacteria. It contains the entire *yki* locus deleted for introns 2 and 3 and a PiggyBac transposon, carrying a eye-specific DsRED maker, inserted in the first intron. This construct was used as template for gene conversion after induction of double strand breaks in *yki* locus at the level on exons 2 and 4. Following injection in Cas9 embryos, DsRED positive F1 flies were recovered and analyzed molecularly. Flies carrying the PiggyBac insertion in *yki* locus are not viable. Upon excision of the PiggyBac by a transposase provided in trans, non-DsRED flies were recovered. These flies are viable and correspond to the *yki<sup>2only</sup>* allele. Two independent lines, corresponding to two independent recombination events were analyzed).

The entire locus was sequenced. We noticed polymorphism in introns and silent polymorphism in exons that were present in the original flies (*yw*, used to create the repair construct) and nos-Cas9 in which injection was made. The positions of most distal primers used to sequence the locus are shown (green arrows).



**Suppl. Figure 6.** Quantification of fluctuating asymmetry.

a. Illustration of the domain used to quantify wing area (red line). Each wing was measured twice, to determine measurement error.

b. plot of Right–Left difference compared to Measurement Errors (corresponding to the difference in replicate measurements).

c. Distribution of wing asymmetry among the analyzed flies. Each circle represents the area of a single wing, with its replicate. The difference between Right (orange) and Left (blue) sides is schematized by a black line.

## Discussion and Perspectives



### 3.0 DISCUSSION AND PERSPECTIVES

Alternative splicing (AS) of mRNA precursors is a crucial step in the regulation of the gene expression pathway. The SR proteins constitute a family of multifunctional RNA binding proteins that play a significant role in alternative splicing regulation. Several genetic studies have reported that SR proteins are essential for animal development (Ding et al., 2004) (Feng et al., 2009) (Jumaa, Wei, & Nielsen, 1999) (H. Y. Wang, Xu, Ding, Bermingham, & Fu, 2001).

In addition to their role in splicing, several SRSF proteins have been shown to regulate transcription elongation, RNA export, decay, translation (Zhong, Wang, Han, Rosenfeld, & Fu, 2009) and for *B52*, transcriptional regulation (Juge et al., 2010).

Our group has been studying the functions and regulations of SR proteins by using mammalian cells and *Drosophila* as models, and previously showed that modulation of protein levels of B52 (which is the closest *Drosophila* orthologue to human SRSF6) can affect cell growth in *Drosophila* (Fernando et al., 2015). The study did not characterize the underline mechanism by which B52 effects growth. However, they speculated that B52 could alter the AS of genes involved in the control of growth. Indeed (Fic et al., 2007) and (Gabut et al., 2007) previously showed that B52 overexpression could alter the AS of genes involved in development.

Capitalizing on this, in my Ph.D., I aimed to explore the mechanistic link between B52 and how it can regulate growth.

#### AS program of B52:

Thanks to the advances in the ability that allows rapid characterization of genome-wide AS events by RNA sequencing and immunoprecipitation techniques, several groups attempted to reveal the precise map of several RBPs binding on the transcriptome. Two such studies were highly interesting for us. In studies by (Brooks et al., 2015) and (Bradley et al., 2015) where, among other SR proteins, B52 was depleted in *Drosophila* S2 cells and RNA seq was performed to check the affected AS events.

We decided to merge the two available datasets order to identify AS events reproducibly affected by B52 depletion. With our strategy, we identified several AS events modulated by B52 level in genes involved in cell growth, including three genes linked to the Hippo (Hpo) pathway. Among these three genes, we also found *yorkie* (*yki*), which encodes the central effector of the Hippo pathway. The second candidate gene was *wbp2* that encodes for Wbp2

protein which has been shown to bind Yki and enhance its intrinsic transcriptional co-activator activity (X. Zhang, Milton, Poon, Hong, & Harvey, 2011). The other candidate gene was *zyxin* (*zyx*) that encodes for Zyxin (Zyx) which has been shown to act as a negative regulator of the Hippo pathway as it is required for the activity of Yki (Rauskolb et al., 2011) (Gaspar et al., 2015). RNAseq data indicate that B52 depletion promotes skipping of exon 3 in *yki* mRNA, the inclusion of exons 6 and 7 in *wbp2* and the retention of the last intron in *zyx*. The functional consequences of all the identified AS are uncharacterized including that of *yki*.

### **B52 favors Yki1 isoform at the expense of Yki2:**

Over the years the Hippo pathway has emerged as a significant player in tissue growth control, stem cell function, regeneration and tumor suppression. During organogenesis, optimum levels of Hippo signaling are crucial for maintaining tissue homeostasis. Over-expression of Hippo signalling results in the formation of smaller organs due to induction of apoptosis (Harvey et al., 2003b) (Pantalacci, Tapon, & Léopold, 2003) (Udan et al., 2003) (S. Wu et al., 2003).

With Yki being the central effector of the Hippo pathway in *Drosophila* where all upstream signals and kinase cascade converge on Yki to regulate the activity of pathway, finding that *yki* AS is altered by depletion of splicing factor B52 was a major reveal for us. Several studies have shown that the regulation of the transcriptional co-activator Yki is central to the regulation of the Hippo pathway.

Indeed we confirmed this AS alteration by western, and *RT-PCR* B52 depleted S2 cells and *Drosophila* Wing disc samples. We observed that depletion of B52 induces skipping of *yki* alternative exon 3 and increases expression of Yki1 at the expense of Yki2 isoform. Thus, Yki protein exists as two isoforms containing one or two WW domains thereafter called Yki1 and Yki2 respectively.

Although the canonical isoform of Yki (Yki2) has been characterized and has been described in plethora of research papers, the presence of short isoform (Yki1) has been largely neglected since the very first paper described Yki (J. Huang et al., 2005). Thus in this field of Hippo pathway, it is not known yet if the short isoform of Yki is functional or redundant.

We believed that elucidation of the role of this isoform could better allow us to understand better how B52 controls growth via the Hippo pathway. To further test the effect of B52 on the growth we depleted it in the posterior part of *Drosophila* wing disc and observed that it

leads to a reduction of the posterior domain and in discs and in adult wings. In addition, we saw the depletion of B52 leads to down-regulation of two well-established reporter of Yki activity, *diap-lacZ* and *Ex-lacZ*.

Taking hints from this result, we hypothesized that B52 reduces growth by reducing Yki activity and if this is true then these phenotypes should be rescued by overexpression of Yki and by inactivation of the kinases Warts (Wts) or Hippo (Hpo) which phosphorylates to regulate Yki negatively. Indeed we observed the rescue of the phenotype by overexpressing Yki and depleting Wts and Hpo, but we did not attain overgrowth as observed by overexpression of Yki or RNAi Wts/Hpo in the presence of wild type level of B52, suggesting that B52 is involved in other modulation of other genes and (or) pathways controlling growth. Given the results that we obtained one could also argue that partial rescue that we see could correspond to the overcompensatory effect of Yki overexpression of Wts/Hpo depletion of defect induced by B52 depletion acting independently of Hippo pathway. To this end, we also depleted transcription factor Tgi that binds to Sd to repress the pathway in normal state and saw that its depletion alone induces a mild overgrowth, but it too rescues the growth defect inflicted by depletion of B52. However, we did not attain overgrowth as observed in wild type level of B52. Altogether our result allows postulating that although B52 is involved with other genes and pathway in the regulation of growth, it also regulates growth upto significant extent via regulating Yki activity by modulating *yki* AS.

### **Yki 1 is a weaker isoform compared to Yki2:**

At this juncture, we observed that B52 depletion favors the expression of Yki1, decreases Yki target genes and decreases growth partially through decreased Yki activity. This suggests that Yki 1 isoform could be less efficient when compared to the canonical Yki2 isoform. Indeed by overexpression assays, we observed that Yki 1 induces a weaker phenotype when compared to Yki2 in the eyes of adult *Drosophila*.

We observed that expression of the Yki reporter transgenes *ex-lacZ*, *diap1-lacZ* and *bantam* microRNA are weakly activated by Yki1 compared to Yki2. Further, we could extend these experiments by checking the upregulation of another well-established target of Yki, cell-cycle regulator CycE (Tapon et al., 2002).

Furthermore we directly compared the ability of Yki1 and Yki2 to activate transcription in luciferase assays in *Drosophila* S2 cells. Since Yki does not bind DNA directly, we fused Yki



isoforms to the DNA binding domain of GAL4 and tested their activity on a *UAS-luciferase* reporter. Our results confirm that Yki1 is a weak transcription activator compared to Yki2.

### **Lack of second WW domain reduces several interactions of Yki**

The only difference between Yki1 and Yki2 is the absence of the second WW domain in Yki1. Several studies have highlighted that the WW domain is indispensable for Yki's interaction with its specific partners that contain PPxY motifs (Hyangyee Oh & Irvine, 2010). It has been shown that mutation of these domains abolishes Yki activity (H. Oh & Irvine, 2009). It is thus apparent to assume that the lack of other WW domain in Yki1 can directly reduce its interaction with several above mentioned partners. Moreover, the lack of 2<sup>nd</sup> WW domain could hypothetically modify protein structure in such a way that partners of Yki that interact via its N terminal domain, i.e. WW-PPxY independent interactions could also be affected thus we decided to investigate this untested assumption.

In addition to kinase Wts/Hpo and transcription factor Sd, Yki has been shown to interact with Mad (Mothers against Dpp) which is an effector of Dpp signaling (Decapentaplegic) and Hth (Hemothorax) and Tsh (Teashirt) (Alarcón et al., 2009) (Peng et al., 2009) (Hyangyee Oh, Reddy, & Irvine, 2009). Yki also interacts with several chromatin-modifying proteins including GAGA factor (GAF) and a subunit of the Trithorax-related (Trr) histone H3 lysine 4 methyltransferase complex, Nuclear receptor coactivator 6 (Ncoa6) (Qing et al., 2014b) (Hyangyee Oh et al., 2013).

To ascertain the role of second WW domain and to understand the consequence of its alternative inclusion on Yki protein's function, we compared the interaction of each Yki isoform with several partners, by a semi-quantitative co-immunoprecipitation (Co-IP) after transfections of luciferase-tagged proteins in S2 cells (Refer to methods). We observed that indeed Yki 1 interacts weakly with Wts, Ex, NcoA6, Wbp2, and Tgi when compared to Yki2. All these partners contain PPxY motifs. However, both the isoforms interact similarly with partners that contain single PPxY motif such as Mad or Hpo. Our results are in agreement with the in vitro studies performed by (Nyarko, 2018) and (Webb et al., 2011) who have shown cooperative binding between multiple PPxY motifs and tandem WW domains of YAP and Yki.

We also observed similar interaction of both isoforms with transcription factors Sd, MAD, Cbt and Hth, and the chromatin-associated factors GAF Mor and 14-3-3 proteins. We were expecting such an observation for Sd, Mor and 14-3-3 which have been described to interact with Yki via its N terminal domain (Goulev et al., 2008) (Hyangyee Oh et al., 2013) (Dong et al., 2007). However, if Cbt associates with Yki directly or via other transcription factors, is

yet to be elucidated (Ruiz-Romero, Blanco, Paricio, Serras, & Corominas, 2015), the authors also showed the interaction of GAF but were unable to map the binding domain precisely. Same goes for Hth which is known to physically interacts with Yki but the domain of interaction is yet to be elucidated (Peng et al., 2009). However, this analysis should be extended to partners such as Dalao (another component of BRM complex), MED23, MED15, MED31, MED19, and MED1 (subunits of Mediator complex) which have been shown to associate with Yki via mass spectroscopy studies (Hyangyee Oh et al., 2013).

These results allowed us to verify our hypothesis that Yki1's lack of second WW domain reduces its interactions with several partners, but we saw similar interactions with partners containing single PPxY motif and transcription factors which bind to Yki via its N terminal domain. One curious point arose from the observation of these results is the fact that Yki1 isoform interacts weakly even with kinase Wts that functions to retain Yki in the cytoplasm. If weaker interaction of Yki1 results in lower phosphorylation then it should be accumulated in nucleus very easily even Yki1 binds weakly with Ex that has been shown to directly sequester Yki at the apical junction region to prevent it from entering the nucleus, thereby inhibiting its activation (Badouel et al., 2009). We did not yet realize experiments to determine the phosphorylation state of the two isoforms of Yki. One counter explanation to this observation could be the fact that in the normal state the expression level of Yki1 isoform is extremely low.

Further, we speculated that besides strengthening the binding of Yki2 with partners containing multiple PPxY motifs, the presence of the second domain would allow Yki2 to interact with another PPxY containing coactivator simultaneously. This would explain the lower activity of Yki1. Inside the nucleus, Yki2 binds to transcription factor Scalloped (Sd) via its N terminal domain and via WW domain to repressor Tondu domain-containing growth inhibitor (Tgi). Studies by (Koontz et al., 2013) and (Guo et al., 2013) have shown an interaction between Sd and Tgi. However, these two studies slightly differ from each other in terms of explaining the aftermath of Yki's recruitment. According to (Koontz et al., 2013) Yki displaces the complex between Tgi and Sd and converts Sd into a transcription activator while on the other hand, (Guo et al., 2013) reported that Tgi bind to Sd and Yki simultaneously to forms an inactive ternary complex that is transcriptionally inactive.

To this end, our Co-IP verified the interaction between Sd and Tgi. In addition, we observed that both Yki 1 and Yki2 isoforms could enhance this interaction between Sd and Tgi with Yki2 being more efficient, suggesting that both Yki isoforms can form a trimeric complex with Sd and Tgi in agreement with the study by (Guo et al., 2013).

Studies in mammalian Hippo pathway showed binding of a protein called Wbp2 to interact with YAP (Mammalian counterpart of Yki). In *Drosophila*, (X. Zhang et al., 2011) have described, WW-PPxY dependent association between Yki and Wbp2 and they further reported that this association enhances Yki's ability to drive transcription. Taking clues from this study, one way to explain the weaker activity of Yki1 could be due to the fact that it lacks the second WW domain and thus it cannot recruit other transcriptional activators such as Wbp2. So we extended our Co-IP experiments between Sd and Wbp2 in the presence of Yki1 or Yki2 and observed that both the isoforms could increase this Co-IP although Yki1 increases it weakly when compared to Yki2. However, in this experiment, only Wbp2 interacts via WW-PPxY motif but the other interaction was not dependent on WW-PPxY domain.

We suspected that our observations would not be the same if we check Co-IP between Tgi and Wbp2 in the presence of Yki2 and Yki1 as both of these partners realize interaction with Yki in WW-PPxY dependent manner. Indeed, we observed that Yki1 could not Co-IP Tgi and Wbp2 together whereas Yki2 does and point mutations in the two WW domains of Yki abolish these interactions.

In *Drosophila*, six isoforms of Wbp2 have been described (*source: FlyBase*), and indeed from our screening, we observed that AS of *wbp2* is affected by the depletion of B52. It will be interesting to extend our Co-IP to all these isoforms of Wbp2 and check the interaction of both isoforms of Yki with individual Wbp2 isoforms because B52 could co-regulate the AS of *yki* and *wbp2* and more effectively control the regulation of Yki activity.

### **Model of competition:**

Based on our observations so far, Yki isoforms interact similarly with transcription factors but differ by their capacity to bind and bridge PPxY-containing proteins. We propose that both the isoforms of Yki might compete with each other for binding to the transcription factors, when the Hippo pathway is OFF the canonical isoform Yki2 joins the transcriptionally repressed Tgi-Sd complex via one of its WW domain and could recruit transcription co-activators with the other WW domain such as PPxY containing Wbp2 or NcoA6 and finally may or may not displace Tgi. However, in B52 depleted state when there is more abundant Yki1, it successfully binds to the trimeric complex between Tgi and Sd but it cannot recruit Wbp2 or NcoA6 and might not displace Tgi so as to initiate the transcription effectively. This could reconcile with (Guo et al., 2013)'s observation that Tgi enhances Sd-

Yki interaction but decreases the distance between Sd and Yki as measured by FRET suggesting the remodeling of the complex.

To test this hypothesis of competition, we made use of activated forms of Yki which was first described by (Dong et al., 2007), the activated forms lack the necessary phosphorylation site that is phosphorylated by Wts, more specifically Ser168 is mutated preventing Yki's binding to 14-3-3 proteins and thus it cannot be retained in cytoplasm but accumulated in nucleus. Overexpression of activated Yki2<sup>S168A</sup> isoform led to a very strong overgrowth phenotype in the *Drosophila* eye and this was expected as it has been shown by (H. Oh & Irvine, 2009). In addition we observed a weaker overgrowth phenotype induced by activated Yki1<sup>S168A</sup> isoform too. To answer if there is a competition between the two isoforms, we co-overexpressed the Yki1 isoforms and remarkably only activated Yki1<sup>S168A</sup> could partially rescue this phenotype suggesting that in the nucleus there is a competition between two isoforms. This result is in perfect agreement of our proposed model of competition.

### **Role yki AS in development:**

Although the process of AS is vastly studied, and several isoforms that are switched at either development or in disease are reported but only a very few studies have focussed to dissect function of gene as isoform level, such is not only the case of yki/YAP AS but implies to vast number of genes with known isoforms. For example, it is recently reported that the presence or absence of exon7 in two splicing isoforms of MBNL1 conveys opposite phenotypical implications of cancer (Tabaglio et al., 2018).

Similarly, in AS is also regulated for development, for an example, ApoER2 which is a protein encoded by the *LRP8* gene also undergoes alternative splicing. The exon 19-containing domain of ApoER2 is essential for synapse formation while its inclusion is reduced in the brain of Alzheimer's patients (Hinrich et al., 2016). Thus it is now imperative to focus on the gene function at isoform level because it could be a key in understanding the complex regulation like AS and that is why we do not undermine the role of short isoform in the *yki* AS.

(Yeo, Holste, Kreiman, & Burge, 2004) highlighted that levels of AS usually differs from one tissue to another. So it is a possibility that AS of *yki* can differ from one tissue to another.

This could be checked by doing western on tissues at various stages of development. However, to ultimately, reveal the role of the *yki* AS, we aimed to abrogate this in vivo, i.e. to generate flies expressing exclusively Yki2 (*yki2-only*) or exclusively Yki1 (*yki1-only*), by editing the endogenous *yki* gene using the CRISPR/Cas9 system. We employed a different

strategy at first and failed (Please refer to Appendix Figure 37), but later we employed a second strategy and managed to create flies expressing Yki2 isoform only. We observed that flies were homozygous viable, but we noticed a phenotype in the population of these flies. We noticed an intra variation between the size of the right and left wings. This phenotype of Fluctuating asymmetry (FA) and it is the consequence of subtle deviation from normal development. The phenotype of asymmetry cannot be blamed on the environmental or genetic variations; it arises stochastically during the development. The fact that we observe a consistent and significant FA in our analysis of Yki2only population compared to control suggests that indeed the *yki* AS plays a role in development robustness. Further, we would like to generate heterozygous *yki2only* flies so as to check if we rescue the phenotype of FA. Recently, (Boone, Colombani, Andersen, & Leópold, 2016) have shown that Yki controls the expression of *dilp8* during normal development. They showed that a mutation Hpo-responsive element (HRE) within the *dilp8* promoter is enough to result in increased FA. One way to test our theory of *yki* AS a mean to maintain robust development would be via extending our analysis by testing for genetic interaction between *dilp8* mutant and our *yki<sup>2only</sup>* mutant. We expect that the FA will further rise in this population due to the lack of endogenous Yki1 isoform.

Yki has been implicated in the control of expression of hormone ecdysone (20-hydroxyecdysone) and couples insulin signaling with ecdysone production. (C. Zhang et al., 2015) have further shown that Yki interacts with Taiman, ecdysone pathway transcription factor to control the gene expression of some targets such as *piwi*, *nanos*, *dilp8*. We could further advance our isoform comparison studies on these targets of Yki2.

One key experiment that is currently underway with our *yki<sup>2only</sup>* flies is to investigate the effect of B52 depletion on the size of the posterior domain. We depleted B52 in the posterior domain of the wing in our *yki<sup>2only</sup>* flies and plan to measure the effect on growth. Upon comparing with it with the overgrowth induced by B52 depletion in the posterior domain of wild-type flies if we observe that the overgrowth phenotype exceeds in *yki<sup>2only</sup>* flies, then it would mean that indeed Yki isoform functions to buffer the activity of Yki2 isoform in development.

### Concluding Remarks:

Finally, it is worth noting that this AS is conserved, In mammals, *yki* homolog, *YAP*, is alternatively spliced and is expressed as eight protein isoforms that fall into two categories, YAP1 and YAP2, which respectively contain one or two WW domain(s)(Sudol, 2013).

Therefore alternative inclusion of the second WW domain is a conserved feature between Yki and YAP. Moreover (Komuro et al., 2003) reported that YAP2 is more efficient isoform than YAP1 in their studies where they co-precipitated ErbB-4 an epidermal growth receptor. This further supports the idea that Yki1 and YAP1 isoforms have important function *in vivo* and that alternative splicing of *Yki/YAP* is a conserved mechanism of control of the Hippo pathway.

There are a plethora of reports showing YAP is amplified and overexpressed in various tumors types where YAP protein relocalizes in the nucleus. YAP knockdown in many human cancer cell lines, such as prostate cancer or malignant mesothelioma cell lines, suppresses their growth and ability to trigger tumor formation. As of now, the focus of therapeutic research studies is towards targeting YAP, which indeed is a promising way to hamper tumor cell growth. Recently, molecules targeting YAP, such as verteporfin that inhibits the formation of the YAP-TEAD complex, have been described to block tumor growth in mice (Liu-Chittenden et al., 2012). Identification of such new modulators of this pathway can, therefore, open new therapeutic opportunities for cancer treatment. However, to this date, none of the studies differentiate between the various isoforms of YAP. We wonder if the AS of YAP in cancer cells allows the production of more YAP2 instead of YAP1 which could even be a hallmark of tumorigenesis. One amazing future perspective of this study would be to study the extent of YAP splicing and the ration of isoforms in various tumor samples.

Further investigation could be directed towards finding small molecules that manipulate the alternative splicing of YAP as a way to modulate YAP activity in cancer cells. Thus our research should open new perspectives for modulation of the Hippo pathway in cancer cells by altering *YAP* alternative splicing.

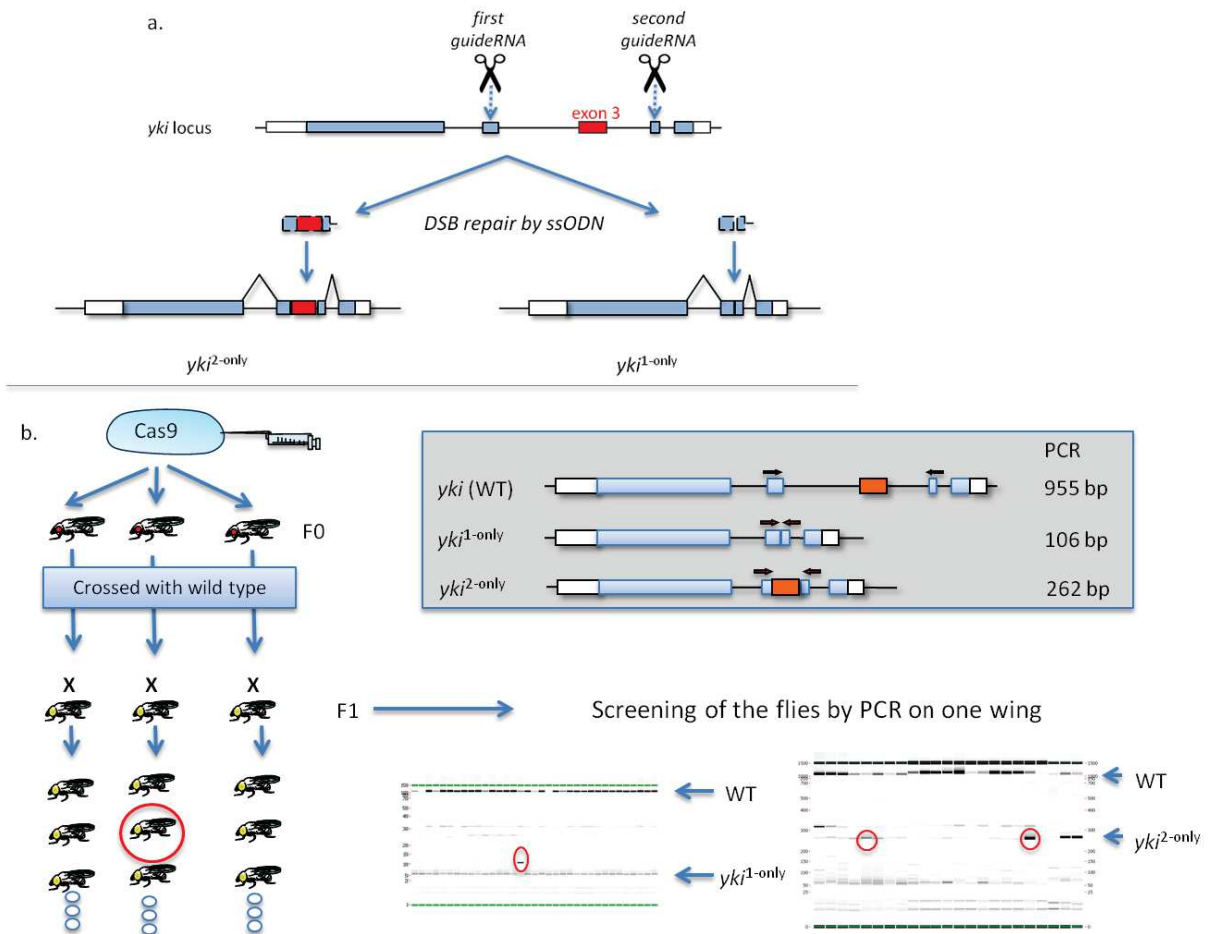
The regulation of the Hippo pathway has evolved into a complex network over the past two decades, with new players added at an unprecedented pace. Our study shall add even more complexity into this regulatory network.



# APPENDIX

## 4.0 APPENDIX

**first strategy employed to generate the *yki<sup>1only</sup>* & *yki<sup>2only</sup>* flies:** To be able to generate two versions of Yki gene that can encode only Yki1 or only Yki2, we targeted a Double-Stranded Break (DSB) in exon 2 and exon 4 of Yki with two guide RNAs and repair the gap with the portion of cDNAs encoding Yki1 or Yki2. The template to repair is provided as ssODN (single strand oligo donor nucleotide).

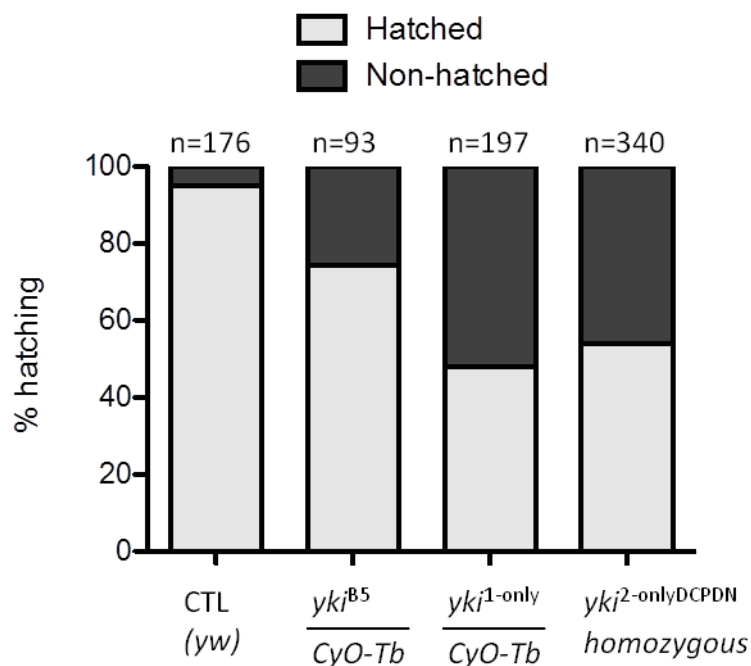


**Appendix Figure 37 : CRISPR and screening strategy:**  
 (a.) Our premier strategy to create *yki<sup>1-only</sup>* and *yki<sup>2-only</sup>* flies. (b.) screening

We injected the plasmid coding for two guide RNAs and repair template in the embryos expressing Cas9. Since the injections are done in the germline, it was necessary to cross the injected individuals with wild type flies before doing a non-lethal genetic screen on the progeny of this cross. The screening was done by performing a PCR on each wing of an adult progeny fly to determine the flies which are either *yki<sup>1only</sup>* & *yki<sup>2only</sup>* (Figure 37).

At first, we succeeded to establish Yki1 isoform expressing flies. We determined that the homozygous (*yki<sup>1only</sup> / yki<sup>1only</sup>*) were lethal at the embryonic stage and we even determined by using the null allele of *yki*, i.e. *yki<sup>B5</sup>* that the flies were lethal due to the mutation that we

created. However for an unknown reason we do not detect expression of neither Yki1 protein nor *yki1* mRNA in these flies. Therefore this allele is not a true *yki<sup>1-only</sup>* allele but a null. After another attempt we were able to generate Yki2 isoform expressing flies and here to we found out that it contains a small deletion of 4 amino acid residues at the repair site in exon 4, thus we named the allele as *yki<sup>2-only</sup>ΔCPDN* and nevertheless we decided to characterize these flies and in the meantime we worked on new strategy to create good *yki<sup>2-only</sup>* flies without. We reasoned that such a small mutation might not change the behavior of this isoform completely. We discovered that homozygous *yki<sup>2-only</sup>ΔCPDN / yki<sup>2-only</sup>ΔCPDN* flies were viable without any visible phenotype. What was surprising for us to find that *yki<sup>2-only</sup>ΔCPDN / yki<sup>2-only</sup>ΔCPDN* flies depicted 50% lethality which we did not expect given the flies had no visible phenotype (Figure 38). Could it be due to the lack of Yki1 which is absolutely required at the early stage of development? Alternatively, could it be an issue of parental fertility which is affected? Or is it merely due to the modification of the Yki2 peptide due to the minor mutation?

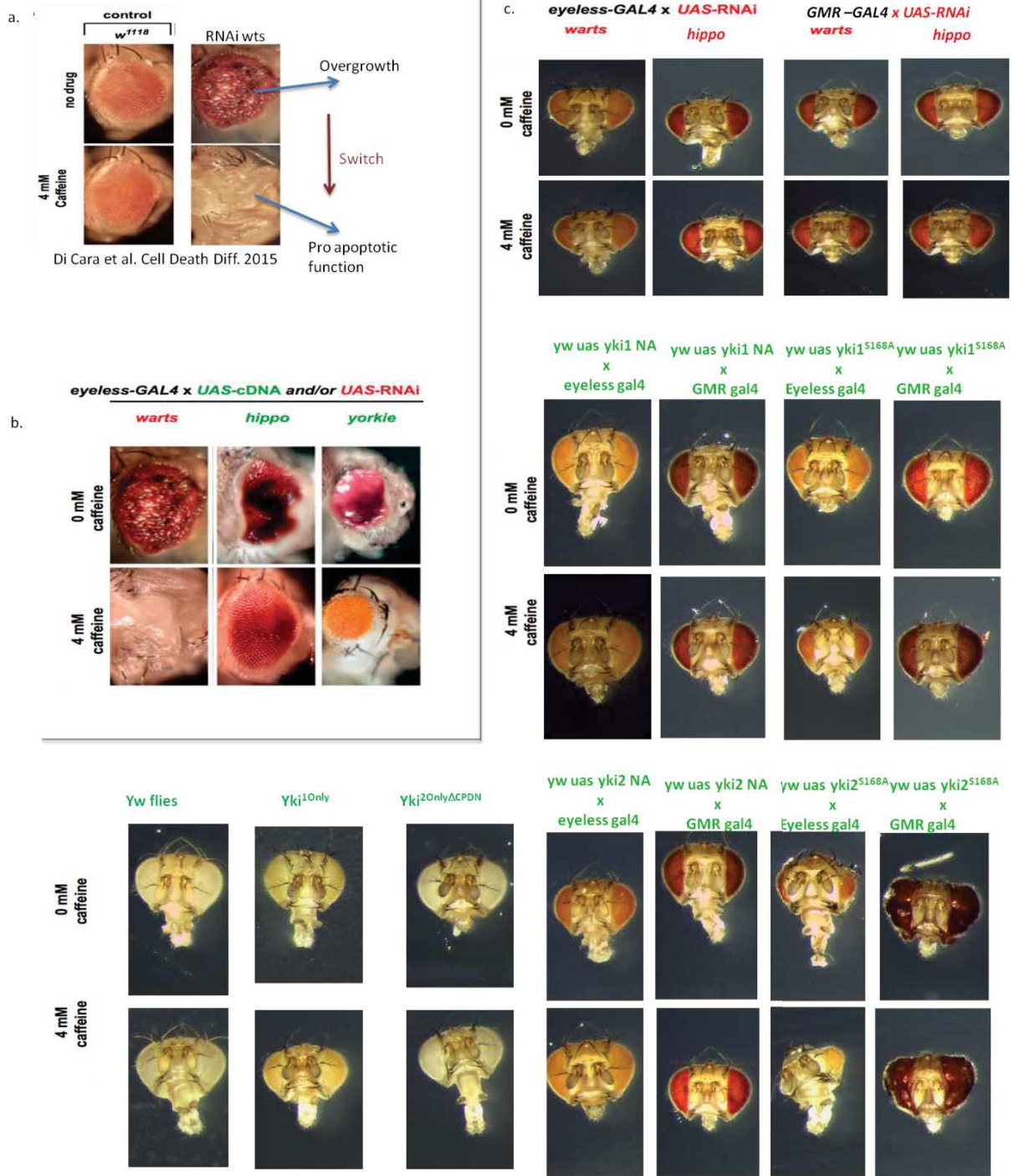


**Appendix Figure 38 Characterization of *yki<sup>1-only</sup>* and *yki<sup>2-only</sup>* flies:**

Schematic representing the embryo hatching. Parents were kept on medium at 25°C. Embryos were carefully counted. In general 5% lethality in wild-type flies is expected. 25% lethality in next two populations is expected because *CyO/CyO* is lethal. An additional 25% lethality in *yki<sup>1-only</sup>/CyO-Tb* suggests that homozygous is lethal. 50% lethality is observed in *yki<sup>2-only</sup>ΔCPDN* population. n= number of embryos counted.

Another argument that we tried to investigate was if the lack of phenotype in adult flies was is simply because in normal developmental conditions the Yki1 isoform has less activity, but in case of stress, it increases to counteract the severity of Yki2's transcriptional output. Indeed (Di Cara et al., 2015) showed the loss of Hippo pathway component during stress

induced by chemical causes the Hippo pathway to switch its role from being anti-apoptotic to apoptotic. They showed that upon stress stimulation by caffeine, Yki interacts with the stress-responsive transcription factor p53 in flies with loss of Hippo components such as Wts or Hpo (Figure 39 a & b). We asked how our heterozygous *yki<sup>1only</sup>* & homozygous *yki<sup>2only</sup>* would respond to caffeine treatment and what will be the effect of overexpression of Yki1 or Yki2 activated and non activated isoforms? Unfortunately, we were unable to reproduce the pro-apoptotic function in our experiments. It could be due to the technical problem with the medium. At this point, we decide to focus on generating the *yki<sup>2only</sup>* flies without any mutation and resume the characterization studies when we obtain it (Figure 39 c).



### Appendix Figure 39 is Yki AS involved in the pro-apoptotic function of Yki upon stress?

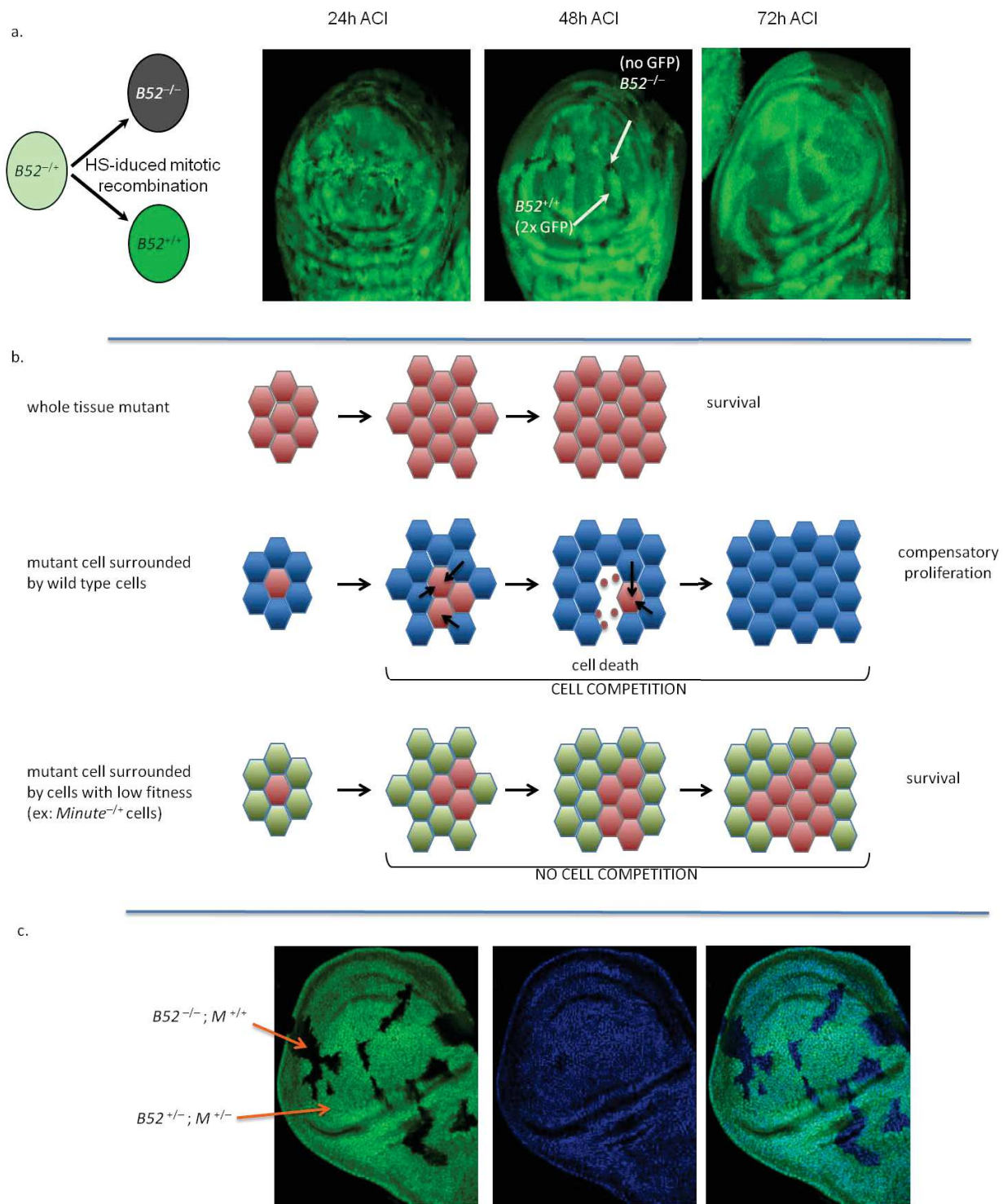
(a. & b.) (Di Cara et al., 2015) Genetic interactions between Hippo pathway components in no caffeine and caffeine medium. C. (Bottom right): We see no difference in the effect on the phenotype in wild type, heterozygous *yki1only* and *yki2only* flies raised on regular and caffeine medium. (Top right) we see no effect on the phenotype with *ey>UAS-wts-RNAi* or *ey>UAS-Hpo-RNAi* between flies raised on regular and caffeine medium. *GMR>UAS-wts-RNAi* or *GMR>UAS-Hpo-RNAi* too did not result in any visible difference in phenotype in eye or elsewhere in the two medium. (Right middle and bottom) Finally overexpression of activated and non activated Yki isoforms too *ey>UAS-Yki1*, *GMR>UAS-Yki1*, *ey>UAS-Yki1<sup>S168A</sup>*, *GMR>UAS-Yki1<sup>S168A</sup>*, *GMR>UAS-Yki2*, *ey>UAS-Yki2*, *GMR>UAS-Yki2*, *ey>UAS-Yki2<sup>S168A</sup>*, *GMR>UAS-Yki2<sup>S168A</sup>* did not yield any different phenotype.

### Role of B52 in cell competition:

To further explore the link between B52 and cell competition, we generated the *B52* mutant clones by mitotic recombination in wing imaginal disc. Interestingly we observed that *B52*<sup>-/-</sup> clones are eliminated over time via apoptosis. This could be due to the phenomenon of cell competition (Appendix-Figure 40 a). This phenomenon was first described by the analysis of a dominant mutation in a *Drosophila Minute (M)* gene that affects ribosomal biogenesis and therefore slows down translation and protein synthesis. While homozygous mutations (*M/M*) of these genes are cell lethal, heterozygous (*M/+*) adults are viable and fertile, although they suffer a developmental delay.

Interestingly, clones of heterozygous cells (*M/+*) growing in a wild type wing disc were not underrepresented in the adult wing, as expected, but completely eliminated (Morata & Ripoll, 1975). The eliminated *M/+* cells are referred to as “looser cells,” and surrounding wild type cells are referred to as “winner cells” (Appendix-Figure 40b). The elimination induces the compensatory proliferation of winner cells, and thus the organ retains the correct size. We induced, *B52*<sup>-/-</sup> mutant clones in disc where the surrounding cells have a mutation in the *minute* gene (*M/-*) and we observed no elimination suggesting that indeed the elimination was due to cell competition. Thus cell competition is a context-dependent phenomenon since the loser cells survive when surrounded by loser cells and gets eliminated when surround by winner cells (Appendix Figure 40c).

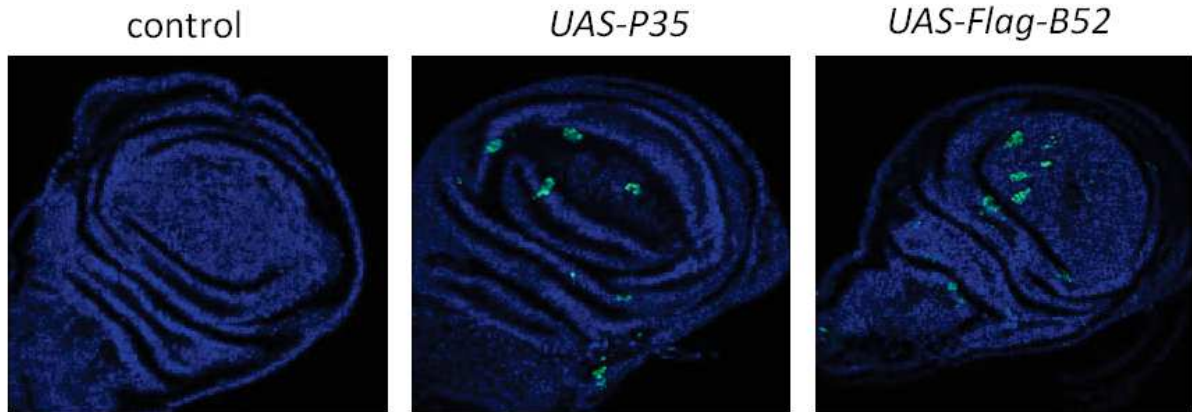




**Appendix Figure 40 B52 in cell competition:**

a.  $B52^{-/-}$  cells are eliminated from wing disc b. Phenomenon of cell competition c.  $B52^{-/-}$  cells survive when surrounded by disadvantaged cells (*Minute* background)

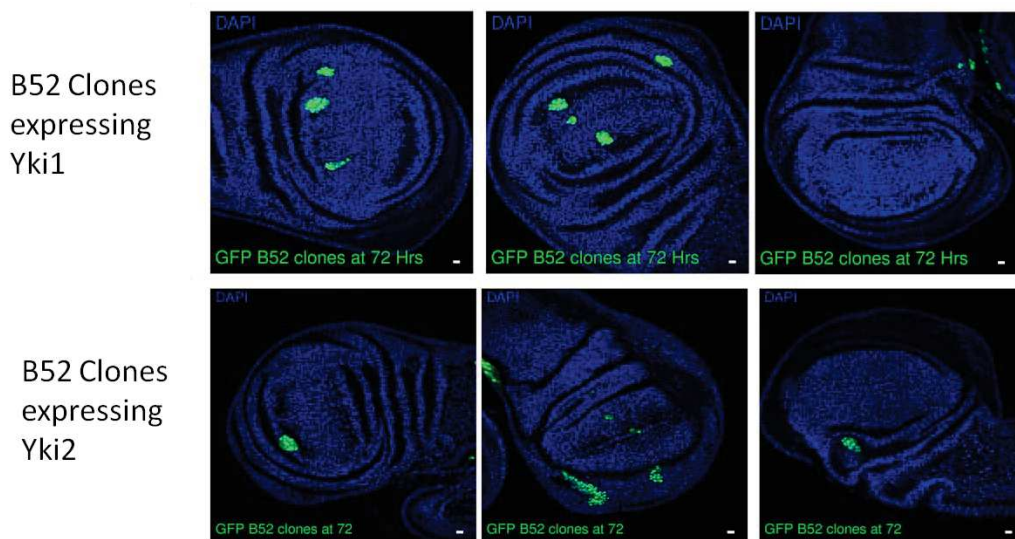
Since we observed the  $B52^{-/-}$  clones were eliminated via apoptosis, we wondered if we could rescue these clone by co-overexpression of *caspase* inhibitor. To realize this, we used MARCM technique(Luo & Wu, 2007). Indeed we observed partial rescue of B52 mutant clones via expression of P35, a *caspase* inhibitor and *Flag-B52* (Appendix Figure 41).



**Appendix Figure 41 *B52*<sup>-/-</sup> clones are partially rescued:**  
*B52*<sup>-/-</sup> clones are partially rescued by expression of the *caspase* inhibitor P35 in the clone (MARCM)

According to literature several pathways are involved in cell competition including Hippo pathway (Tyler, Li, Zhuo, Pellock, & Baker, 2007), cell polarity (Agrawal, Kango, Mishra, & Sinha, 1995) (Brumby & Richardson, 2003)(Woods & Bryant, 1991), JAK/STAT (Rodrigues et al., 2012) Dpp signaling (Moreno, Basler, & Morata, 2002). In one of our primary analysis of RNAseq data by (Bradley et al., 2015) (Brooks et al., 2015) we saw that indeed B52 alters the splicing of the *yki*, *dlg* and *STAT92E*.

First, we checked the overexpression of Yki2 in B52 mutant clones via MARCM technique, the idea was to be able to rescue the B52 mutant clones from getting eliminated because if B52 depletion leads to more production of Yki1 isoform the maybe overexpressing would prevent B52 mutant clones from elimination. However, to this end, we did not see any rescue (Appendix Figure 42).



**Appendix Figure 42 : Rescue of *B52* mutants by Yki1 and Yki2:**  
 Just by expressing Yki1 or Yki2 the elimination of *B52* mutant clones cannot be rescued efficiently.

As of now we have tools to analyze the expression of the two isoforms of *dlg* and *STAT92E* in *B52 mutant* clones hopefully we will be able to show if that it is B52 that affects the splicing of these genes, and thus it plays a role in cell competition.

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## Abstract:

The Hippo pathway is a conserved pathway involved in tissue growth and tumor suppression. Studies have demonstrated its significance in the development of human cancers. This cascade controls the activity of the transcription co-activator Yorkie (Yki) in flies and Yes-associated protein (YAP) in mammals. Due to Alternative Splicing (AS), both Yki and YAP proteins exist as two isoforms containing one (Yki1/YAP1) or two (Yki2/YAP2) WW domains. Since WW domains are essential for interaction with specific partners, the alternative inclusion of this domain in Yki/YAP protein may remodel their interaction network and therefore their activity. The regulation and functional consequences of AS of *yki/YAP* *in vivo* are unknown.

In this Ph.D. project, we identified that depletion of splicing factor B52 in *Drosophila* lowers inclusion of the alternative exon in *yki* mRNAs and favors the expression of Yki1 isoform at the expense of the Yki2 isoform. B52 depletion in the wing reduces growth and Yki activity. We demonstrate that Yki1 isoform is an attenuated version of Yki protein that can compete with Yki2 isoform in the nucleus. To ascertain the role of *yki* AS *in vivo* and the importance of short isoform Yki1, we abrogated this splicing by using CRISPR/Cas9 technology and created flies that can express Yki2 isoform only. *yki*<sup>2only</sup> flies are viable but display a random phenotype of asymmetric wing size. This rise in “fluctuating asymmetry” that is the consequence of subtle deviation from normal development, suggests that AS of *yki* is crucial for the development robustness. Taking together, these results highlight a new layer of modulation of Hippo pathway *via* AS of *yki*.

Alternative inclusion of the second WW domain is a conserved feature between Yki and YAP. This further supports the idea that Yki1 and YAP1 isoforms have an important function *in vivo* and that AS of *yki/YAP* is a conserved mechanism of control of the Hippo pathway. This study opens up new perspectives for modulation of the Hippo pathway in cancer cells by altering YAP AS.

## Résumé :

La voie Hippo est une voie conservée impliquée dans la croissance des tissus et la suppression de tumeurs. Des études ont démontré son implication dans le développement des cancers chez l'homme. Cette cascade contrôle l'activité du co-activateur transcriptionnel Yorkie (Yki) chez la drosophile et de la protéine YAP (Yes Associated Protein) chez les mammifères. En raison de l'épissage alternatif de leur transcrits, les protéines Yki et YAP existent sous deux isoformes contenant un domaine WW (Yki1/YAP1) ou deux (Yki2/YAP2). Puisque les domaines WW sont essentiels pour l'interaction avec des partenaires spécifiques, l'inclusion alternative de ce domaine dans la protéine Yki/YAP peut remodeler leur réseau d'interaction et donc leur activité. La régulation et les conséquences fonctionnelles de l'épissage alternatif de *yki / YAP in vivo* sont inconnues.

Dans le cadre de ce doctorat, nous avons constaté que la déplétion du facteur d'épissage B52 chez la drosophile réduit l'inclusion de l'exon alternatif dans l'ARNm de *yki* et favorise l'expression de l'isoforme Yki1 aux dépens de l'isoforme Yki2. La déplétion en B52 dans l'aile réduit la croissance et l'activité de Yki. Nous montrons que l'isoforme Yki1 est une version atténuée de la protéine Yki qui peut entrer en concurrence avec l'isoforme Yki2 dans le noyau. Pour déterminer le rôle de l'épissage alternatif de *yki in vivo* et l'importance de l'isoforme courte Yki1, nous avons abrogé cet épissage en utilisant la technologie CRISPR/Cas9 et avons créé des mouches capables d'exprimer uniquement l'isoforme Yki2. Ces mouches *yki*<sup>2only</sup> sont viables mais présentent un phénotype aléatoire d'ailes asymétriques. Cette augmentation de l'«asymétrie fluctuante», qui traduit une déviation par rapport au développement normal, suggère que l'épissage alternatif de *yki* est crucial pour la stabilité développementale. Ces résultats mettent en évidence un nouveau niveau de modulation de la voie Hippo *via* l'épissage alternatif de *yki*.

L'inclusion alternative du deuxième domaine WW est une caractéristique conservée entre Yki et YAP. Cela conforte l'idée que les isoformes Yki1 et YAP1 ont une fonction importante *in vivo* et que l'épissage alternatif de *yki/YAP* est un mécanisme conservé de contrôle de la voie Hippo. Cette étude ouvre de nouvelles perspectives pour la modulation de la voie Hippo dans les cellules cancéreuses en modifiant l'épissage alternatif de YAP.