# UNIVERSITÀ DEGLI STUDI DI TRIESTE

## XXIX CICLO DEL DOTTORATO DI RICERCA IN

## BIOMEDICINA MOLECOLARE

# The Pin1 orthologue Dodo regulates heterochromatin formation and activity of transposable elements in *Drosophila* germline and neural tissue.

Settore scientifico disciplinare BIO/13

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# Abstract

All eukaryotic genomes contain a substantial proportion of repetitive DNA sequences known as transposable elements (TEs), some of which retain the ability to mobilize in the genome. In different organisms, some TEs have been shown to transpose in the germline and in somatic tissues, in particular in the Central Nervous System, leading to genome heterogeneity. Mechanisms restricting TE activity both in the germline and in somatic tissues have evolved in all organisms, because excessive transposition of TEs may cause mutagenesis and genomic instability. Indeed, unscheduled activation of TEs has been associated with ageing and age-related pathological conditions, including neurodegenerative diseases, in different animal models.

Pin1 is an evolutionarily conserved enzyme with the unique feature of promoting phosphorylation-dependent isomerization of S/T-P motifs. Its activity is essential to link phosphorylation signalling to modulation of cellular processes. Pin1 widely impacts on chromatin state and transcription, cell proliferation, DNA repair and stress response pathways. Additionally, Pin1 activity appears to be required for healthy ageing and prevention of age-related diseases, such as neurodegeneration of Alzheimer's Disease type.

*Drosophila* is a widely used model organism that offers specific advantages for studying the regulation and the impact of TEs. Importantly, the *Drosophila* Pin1 orthologue Dodo protein shares a high degree of sequence similarity with mammalian Pin1

In this thesis, we provide evidence that Dodo acts as a negative regulator of TE expression and integration in both the germline and Central Nervous System. We showed that Dodo depletion licenses the expression of some TEs that are normally repressed by heterochromatinization through HP1a. Mechanistically, we observed that loss of Dodo leads to reduction of HP1a expression at the post-transcriptional level, with reduced formation of HP1a-containing heterochromatin foci and loss of HP1a occupancy at TE regulatory sequences. Moreover, we observed that loss of Dodo impaired the formation of HP1a/B-type Lamin complex at the intranuclear periphery. These observations suggest that Dodo negatively regulates TE expression through heterochromatin-mediated transcriptional gene silencing.

Moreover, we observed that *dodo* mutant brains display increased *de novo* TE insertions especially in coding and regulatory sequences involved in neuronal function. Consistently, we report that both Dodo and HP1a exert a neuroprotective function in ageing flies and that, upon loss of either Dodo or HP1a, TE mobilisation impairs maintenance of neuronal survival.

In conclusion, Dodo may exert its neuroprotective function by restricting TE activity through heterochromatin maintenance.

# Introduction

## Transposable elements

Transposable Elements (TEs) are DNA sequences with the peculiar ability to move from one chromosomal location to another. Barbara McClintock first described their ability to "appear at new locations and disappear from previously determined locations", unveiling their potential to shape the genome in maize. She realised that TEs could influence the action of nearby genes, behaving as "controlling elements" rather than simply mutators, as stated by the majority of scientists (McClintock 1956). At the time, the actual abundance of mobile sequences in eukaryotic genomes was ignored. Only recently the advent of whole-genome sequencing revealed that TEs are amazingly numerous (**Figure 1A**) in almost all organisms. TEs constitute 12% of *Drosophila* genome (Pimpinelli et al. 1995), 50% (up to 70% accordingly to recent algorithm calculation) of the human genome (de Koning et al. 2011; Lander et al. 2001) and more than 80% of the genome of some plants (Mascagni et al. 2015). In fact, the dynamic nature of TEs has allowed them to increase in copy number at numerous genomic loci, affecting the evolution of genomes. However, it has to be noted that only few elements retain the ability to move along the genome, while the others represent molecular fossils (Brouha et al. 2003; Wildschutte et al. 2016). Moreover, only some TEs are competent for autonomous transposition, while the most depend on other elements to complete a transposition cycle. Depending on the mechanism of transposition, TEs can be classified into two major groups: i) DNA transposons and ii) retrotransposons (RTE). The former insert into the genome by a cut-and-paste mechanism, the latter by an RNA mediated copy-and-paste process (Slotkin and Martienssen 2007). TEs are further classified in 40 subfamilies or clades, which consists of numerous families, according to enzymology, structural similarities and sequence relationships (Kapitonov and Jurka 2008).

*i) DNA transposons*

DNA transposons contain Terminal Inverted Repeat (TIR) sequences which flank a gene that encodes a transposase protein with DNA binding and integrase activities. Upon translation and nuclear import the transposase binds either within or near TIR sequences of an autonomous or non-autonomous transposon to "cut" and "paste" it into a new genomic region. The cut leaves staggered ends, resulting in target-site duplication (TSD) of typically 4–8 bp (**Figure 3**, Levin & Moran 2011). DNA transposons have contributed to the formation of new functional genes in human genomes. An example is the RAG-1 endonuclease, responsible for V(D)J recombination and immune system development (Kapitonov and Jurka 2005). This enzyme shares high similarity to the transposase

encoded by DNA transposons of the *Transib* superfamily. Nowadays, virtually all human DNA transposons are mutated and incapable of transposition, and can be considered as molecular fossils. Conversely, they may be still active in other organisms, examples being P-elements in *Drosophila*, Ac/Ds elements in maize (ones discovered by Barbara McClintock) and piggyBat element in the little brown bat (Mitra et al. 2013).

*ii) Retrotransposons*

RNA transposons (or retrotransposons) still retain the ability to move throughout the human genome. Depending on the presence of Long-Terminal Repeats (LTR) at the ends of the element, retrotransposons are divided in LTR, also named ERVs and non-LTR or LINE-like elements (**Figure 1B**).

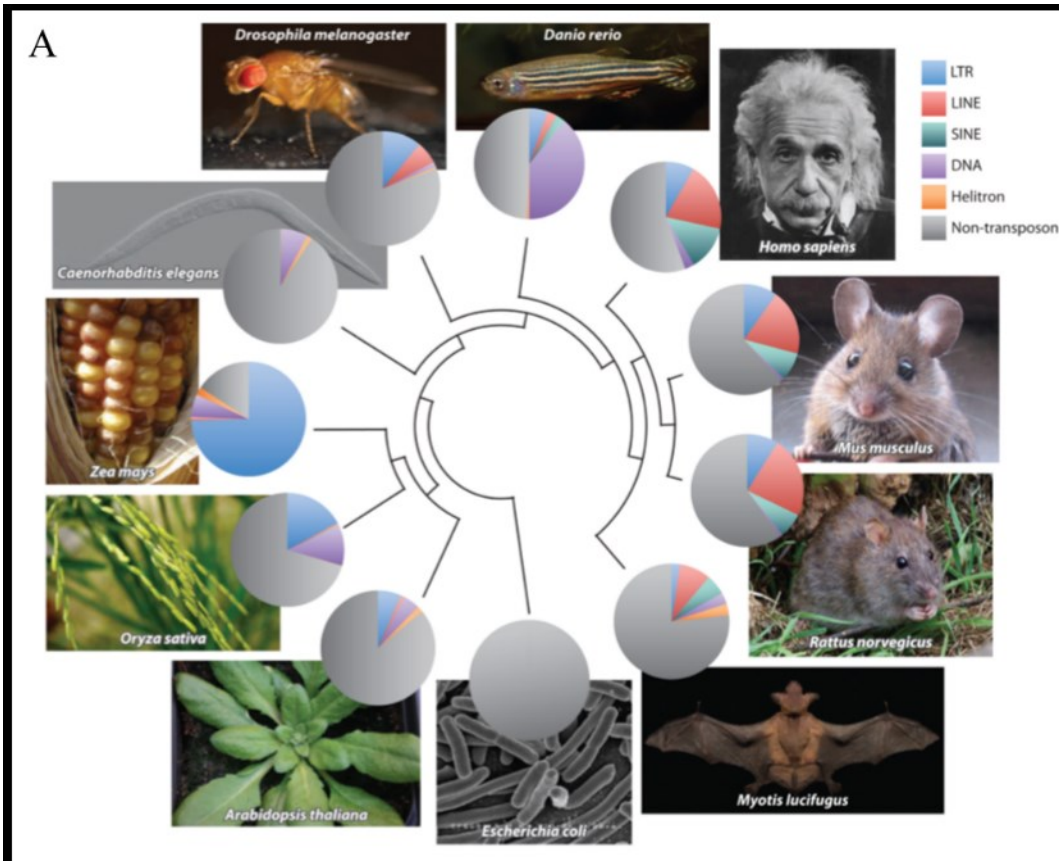
LINE retrotransposons lack LTRs, can reach several kilobases in length and are found in all eukaryotic kingdoms. LINEs predominate over LTR retrotransposons in many mammals. Human LINEs in particular reach 20% of the genome, however only about 100 of them are full-length and retrotransposition competent (Wicker et al. 2007). The autonomous LINEs encode two proteins: ORF1p, which binds LINE-1 RNA in the cytoplasm and ORF2p, which has Reverse Transcriptase (RT) and nuclease activity that copies and reinserts the retrotransposon in a new genomic site. The proteins encoded by autonomous LINE1s also may promote the retrotransposition of non-autonomous elements and non-coding RNAs (Garcia-perez et al. 2007). A *gag*-like ORF is sometimes found 5' to ORF2, but its role remains unclear (Wicker et al. 2007).

The active LINE-1 elements have the potential to cause genomic alterations by retrotransposing their flanking genomic sequences to new chromosomal locations and by serving as substrates for non-allelic homologous recombination (Moran et al. 1999). LINE-1 insertions can exert diverse effects on gene expression. In particular, the long polyA tail and the strong internal 5' and 3' promoters of LINE-1 can dramatically alter the expression of a host gene in case of intronic integration, while the epigenetic marks associated with L1 can modify the chromatin state at integration sites and thereby drive rapid shifts in gene expression (Faulkner et al. 2009; Han, et al. 2004; Swergold 1990).

In addition, insertion of L1 sequences can lead to translation of previously untranslated intron sequences, generating new protein isoforms as observed for the human *ATRN* gene, which codes for two differentially regulated isoforms and contains a short L1 insertion near the 3' end (Tang et al. 2000).

SINEs are non-autonomous TEs, that derive from accidental retrotransposition of various polymerase III transcripts, such as tRNA, 7SLRNA or 5S RNA. They rely on LINEs for trans-acting transposition function, such as retrotranscription (Kramerov and Vassetzky 2005).

The best known SINE is the *Alu* element, which is present at least in 500000 copies in the human genome (Rowold and Herrera 2000).



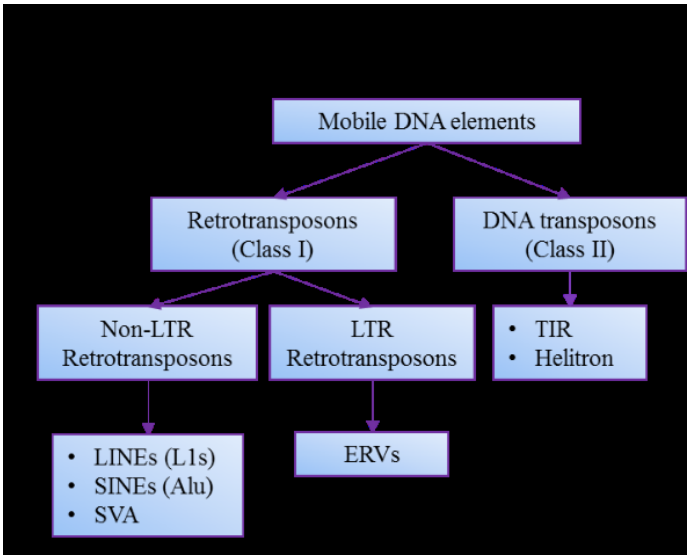




**Figure 1. Distribution and classification of transposable elements. A)** Composition of transposon population in the genome of different species. The phylogenetic tree in the centre describes the evolutionary relationships among them. The pie charts illustrate the fraction of the genome accounted for by different transposon classes (Huang, Burns, and Boeke 2012). **B)** Classification of TEs. Examples of most studied transposons are indicated. LTR, Long Terminal Repeats; LINE, long interspersed nuclear element; SINE, short interspersed nuclear element; SVA, SINE VNTR (Variable Number of GC-rich Tandem Repeats) Alu; ERV, Human endogenous retrovirus.

**Transposable Elements in *Drosophila melanogaster.*** *Drosophila melanogaster* is an ideal model organism for the study of eukaryotic TEs since 30% of transposons in the *Drosophila* genome are full-length and believed to be active (Petrov et al. 2011). Among retrotransposons, LTR retrotransposons are abundant in *Drosophila melanogaster* and belong to 3 groups, namely Gypsy, Copia, and BEL/Pao, consisting of 8 clades and at least 35 families. The Gypsy group is the largest, consisting of 27 families, separated into 5 subgroups: *gypsy, ZAM, Idefix, 412,* and *blastopia* (Bowen and Mcdonald 2001). A detailed classification is shown in **Figure 2**. Of note, the *ZAM* element localises exclusively at constitutive heterochromatin (Baldrich et al. 1997). Among non-LTR retrotransposons are *IVK, Rt1b, TAHRE, G6* elements. SINEs have been previously reported as being rare or absent in most *Drosophila* species, with the exception of the non-autonomous *Drosophila* interspersed element 1 (DINE-1) which consists of repeats randomly distributed (not clustered) similar to mammalian SINEs. As for DNA transposons, these are flanked by relatively short terminally inverted repeat structures (TIR elements) and at least 16% of them are full-length and potentially active in *Drosophila melanogaster*, including *1360, hobo, Bari1, pogo*, and *P*-elements (Kaminker et al. 2002). The DNA transposon *NOF* has a non-repeated sequence of approximately 4 kb with one or two potential ORFs that suggests that it could be, or could have been, an autonomous transposable element. At least one of its ORFs codes for a protein, possibly a transposase (Badal et al. 2006). Interestingly, *NOF* is always associated with Foldback (FB) elements. FB elements are quite distinct from typical class I or II elements and are characterized by great heterogeneity in size and structure. They resemble satellite DNA for the presence of imperfect tandem repeats of short sequences (Truett, et al. 1981). The contribution of FB and FB-NOF elements to genome plasticity is well recognized since they are able to promote genomic rearrangements including inversions, duplications and translocations (Moschetti et al. 2004).

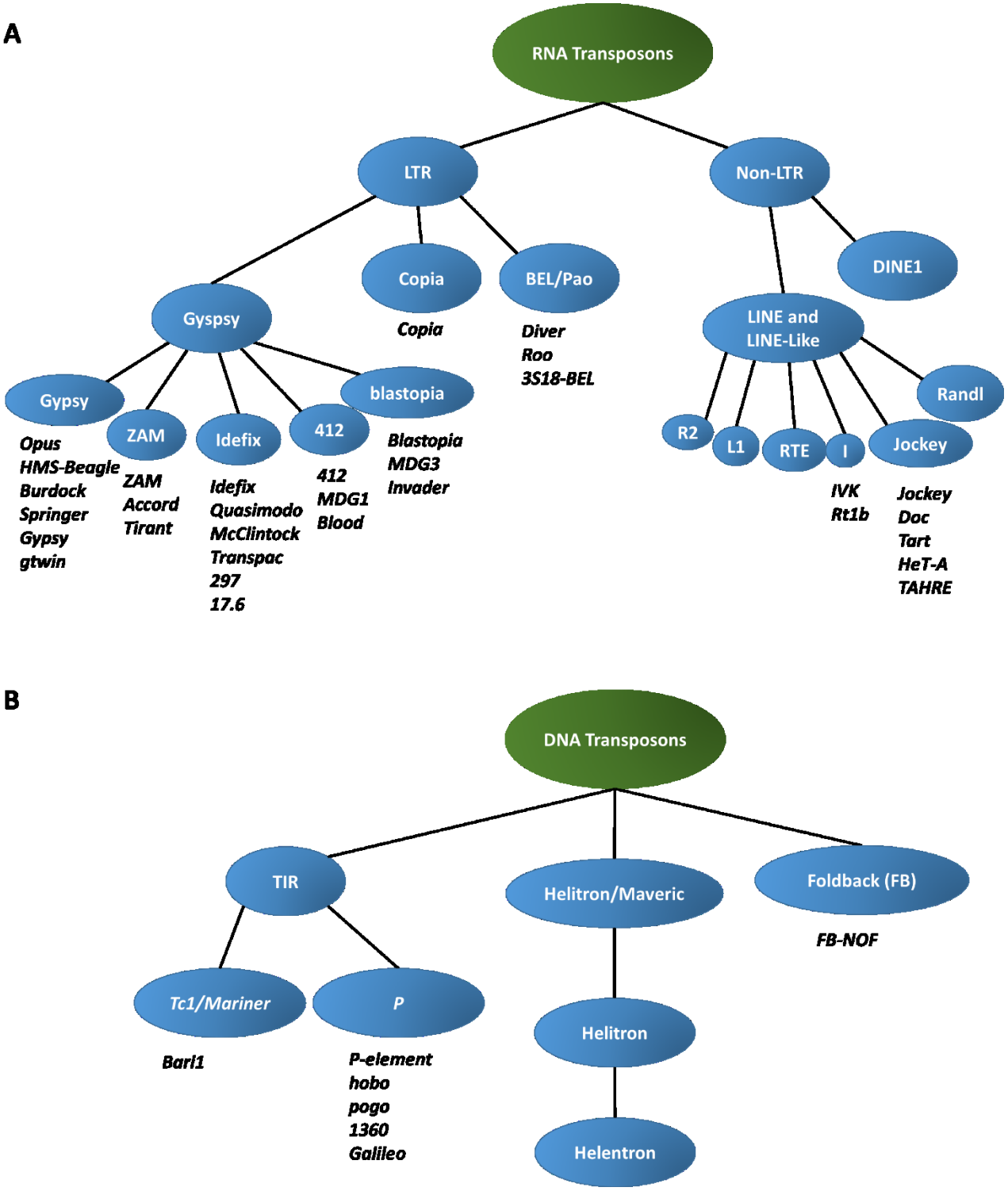



**Figure 2. Classification of Transposable Element in *Drosophila Melanogaster.*** Modified from Mccullers and Steiniger 2017.

**Mechanisms of transposon mobilisation**. The transposition process of the LTR-RTE class requires, as first step, the transcription of a genomic RTE locus from an internal promoter within LTR sequences (Mager and Stoye 2015) or at the 5′ boundary of non-LTR elements, mediated by RNA polymerase II (Richardson et al. 2015). The newly transcribed RNA is exported to the cytoplasm where it is translated. LTR-RTEs encode Gag, protease, reverse transcriptase and integrase proteins; non-LTR-RTEs encode an RNA-binding protein, endonuclease and reverse-transcriptase enzymes. These proteins form ribonucleoprotein particles (RNP) preferentially including the transcripts they are derived from (Kulpa and Moran 2006). For LTR retrotransposons, Gag proteins assemble into virus-like particles that contain TE mRNA, reverse transcriptase and integrase. Then, the reverse transcriptase copies the TE mRNA into a full-length cDNA. The particles are imported to the nucleus and, finally, the integrase inserts the cDNA into a new genomic site (Bannert and Kurth 2006). The non-LTR insertion mechanism is different. The newly formed RNP moves to the nucleus and the integration of the RTE occurs via a process termed Target Primed Reverse Transcription (TPRT) generating a single-strand 'nick' in the genomic DNA, unleashing a 3′-OH that is used to prime reverse transcription of the RNA (**Figure 3**) (Levin and Moran 2011). The final step of integration has not been yet elucidated, but for sure the process inactivates the newly inserted TE by producing 5’ truncations that are probably due to incomplete reverse transcription (Szak et al. 2002).

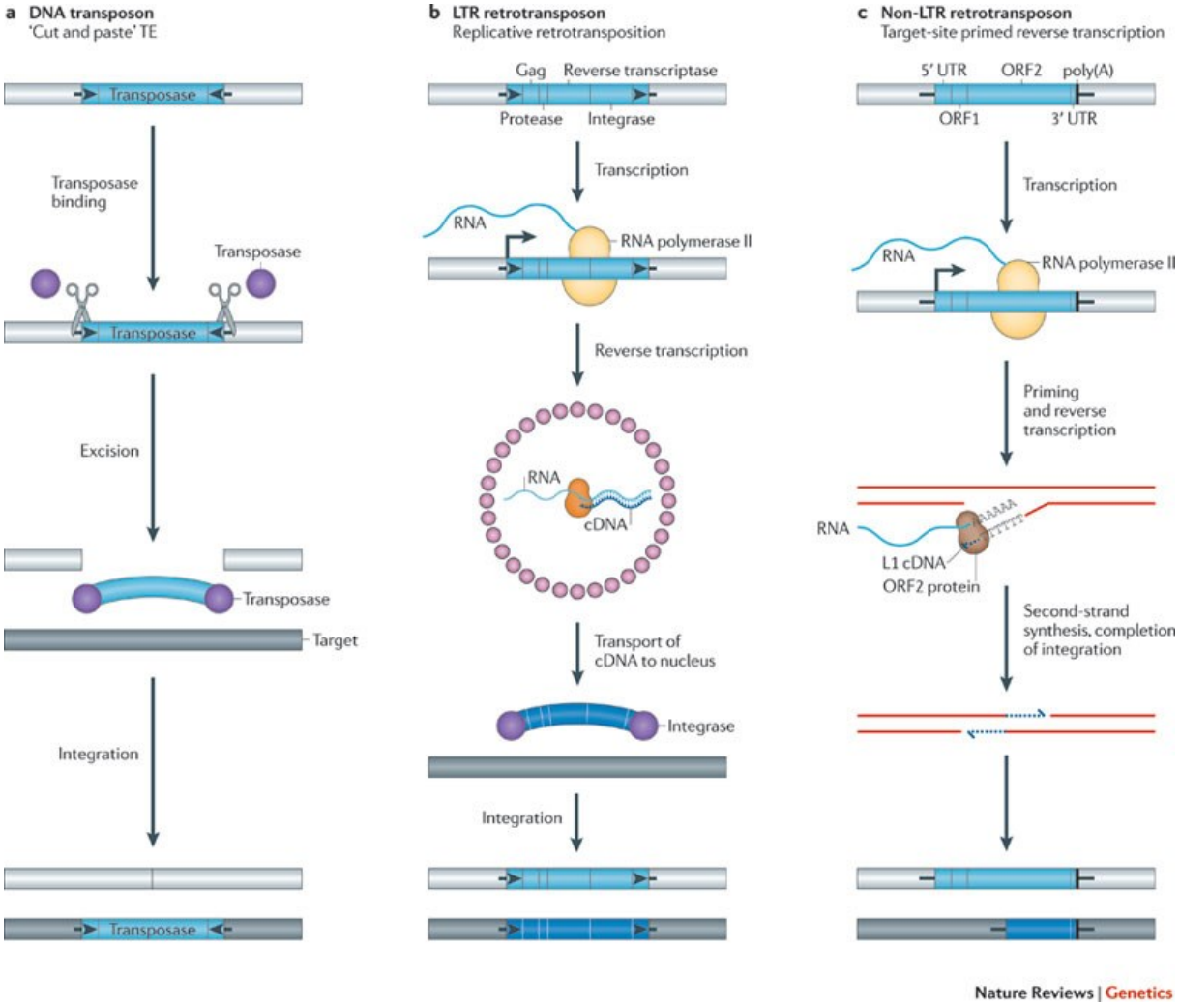



**Figure 3. The diverse mechanisms of transposon mobilisation.** See text for details. (Levin & Moran 2011)

## Cellular mechanisms regulating Transposable Elements

The ability of TEs to mobilise and change location in the genome may cause gene mutations, chromosome breaks and chromosome recombination, and possibly transcriptional and epigenetic interference. All these events may have potentially devastating consequences for the host, and indeed many studies have associated unscheduled activation of TEs with both cancer and neurodegenerative conditions (Reilly et al. 2013). Accordingly, complex mechanisms restricting TE activity have evolved in all organisms and TEs are highly suppressed in most cell types (Friedli and Trono 2015). Restriction of TE activity by the host can occur at two main levels:

- blocking new TE insertions;
- limiting TE expression.

The integration can be inhibited by the host DNA damage response (DDR) pathways: in fact, DNA breaks generated during the integration process are recognized as DNA lesions, recruiting the appropriate repair system. Indeed, proteins involved in the DDR signal transduction, such as ATM

(Matsuoka et al. 2007) as well as in the non-homologous end joining and DNA excision repair pathways appear to restrict retrotransposition (Coufal et al. 2011).

The control of TE expression is based on mechanisms that repress TE RNA expression, through both Transcriptional and Post-Transcriptional Gene Silencing mechanisms (TGS and PTGS respectively) (**Figure 4)**.

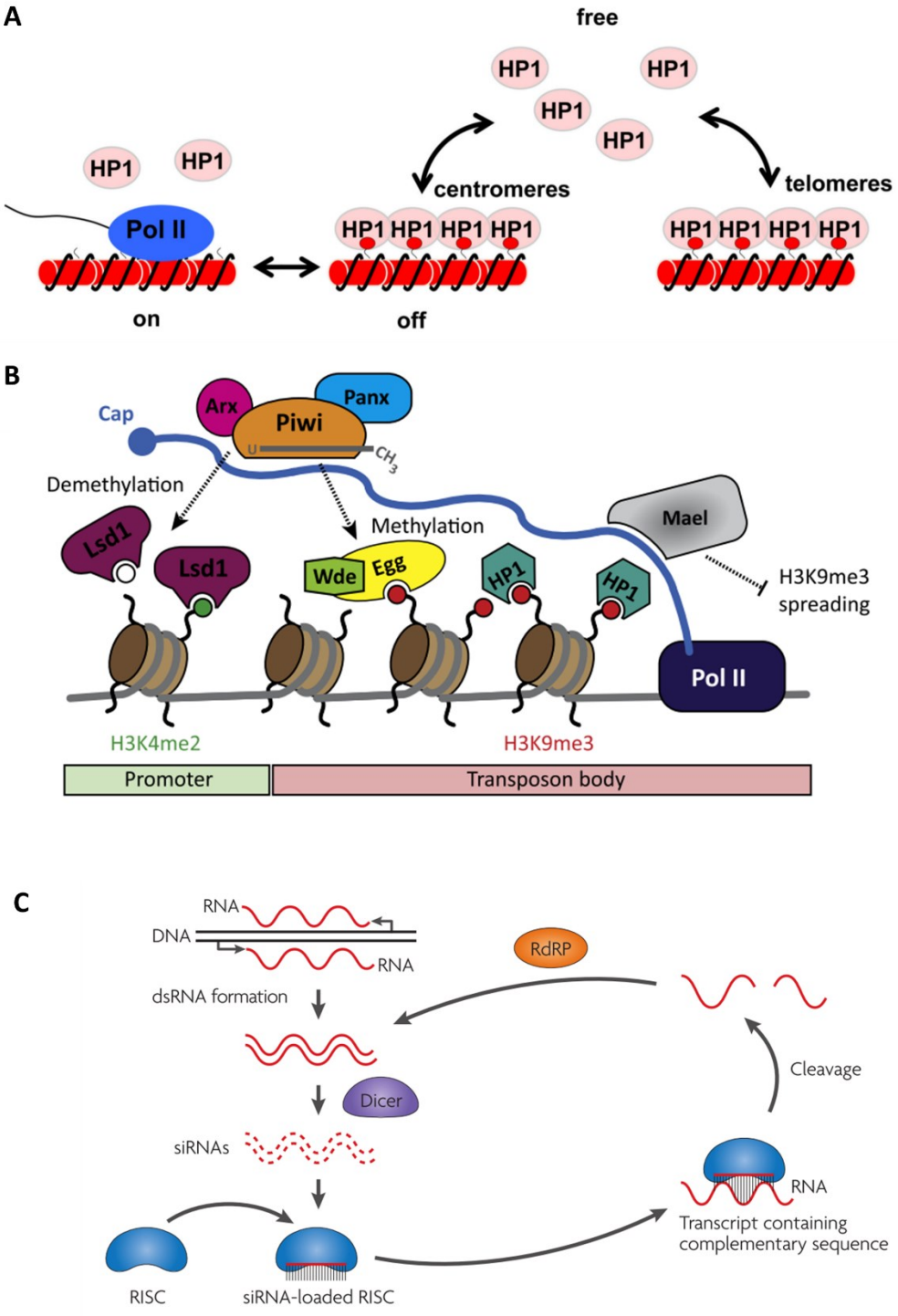



**Figure 4. Regulation of TEs. A-B)** Transcriptional gene silencing mediated by HP1 (**A**) and PIWI-interacting RNA (piRNA) (**B**). In the *Drosophila* ovary, piRNA-Piwi/Asterix (Arx) complexes scan for, and detect, nascent transposon transcription. Upon target engagement, Piwi likely undergoes conformational changes that

lead to the recruitment of Panoramix (Panx). This piRNA-protein (comprising Piwi, Arx, and Panx,) complex induces co-transcriptional repression through recruitment of general silencing machinery components. As a consequence, transposon bodies receive repressive histone 3 lysine 9 trimethylation (H3K9me3) marks, a modification produced by Eggless (Egg) and its cofactor Windei (Wde). Subsequent recruitment of HP1 to H3K9me3 leads to heterochromatin formation. In addition, Lysine-specific demethylase 1 (Lsd1) likely removes active histone 3 lysine 4 dimethylation (H3K4me2) marks from transposon promoter regions, leading to efficient suppression of transposons at the transcriptional level. Maelstrom (Mael), a putative single-stranded RNA-binding protein, is required for transcriptional silencing and blocks H3K9me3 spread. **C)** In the post-transcriptional RNAi pathway, dsRNA are cleaved into small interfering RNAs (siRNAs) by a dicer-family protein. These siRNAs are incorporated into the RISC complex, which contains an argonaute-family protein. The siRNA-loaded RISC complex then cleaves transcripts that are complementary to the siRNA sequence. Adapted from Czech and Hannon 2016; Slotkin and Martienssen 2007; J. Wang, Jia, and Jia 2016.

Regulation of chromatin structure by deposition of repressive histone marks is a mechanism of TE repression conserved from flies to mammals. In particular the trimethylation of histone-3 on lysine 9 (H3K9me3) is found in the majority of TEs (Bulut-Karslioglu et al. 2014; Pezic et al. 2014). In mammals, H3K9 methylation is catalysed by a group of histone methyl transferases, including ESET (Matsui et al. 2010) and SU(VAR)3-9 (Bulut-Karslioglu et al. 2014). In *Drosophila,* Su(var)3-9 is responsible for H3K9 methylation. In both *Drosophila* and mammals, this modification is recognized by HP1 protein (Heterochromatin Protein-1) (Allis et al. 2007).

HP1 proteins are characterized by two conserved domains (**Figure 5**):

- the chromodomain (CD) at the N-terminus, which binds to H3K9me3;
- the chromoshadow domain (CSD) at the C-terminus, which is important for protein homo- and hetero-dimerization and for the interaction with other protein partners containing either the PxxVxL or the PxVxL motif (Liu et al. 2017; Zeng, Ball, and Yocomory 2010).

These domains are connected by a variable hinge region (HR) or linker, which harbors a nuclear localisation signal and is involved in the interaction with DNA and RNA (Liu et al. 2017) (**Figure 5**).

In mammals, there are three main HP1 paralogs: alpha (α), beta (β), and gamma (γ), encoded by the CBX5, CBX1 and CBX3 genes, respectively. In addition to the three main HP1-coding genes, numerous HP1 pseudogenes have been discovered in vertebrates. For example, there is one CBX5 pseudogene, at least five CBX1 pseudogenes and eleven CBX3 pseudogenes in humans, suggesting that HP1-like sequences have been duplicated multiple times during evolution. *Drosophila* possesses at least five paralogs (a, b, c, d, and e), while the fission yeast *S. pombe* has two paralogs (Swi6 and Chp2). Human HP1α (hHP1α) and hHP1β primarily associate with heterochromatic regions of the genome, such as centromeres and telomeres, and help mediate transcriptional gene silencing. In contrast, hHP1γ largely localises to euchromatic regions and plays roles in transcriptional elongation and RNA processing (Espinosa et al. 2012; Hayakawa et al. 2003). Similarly, *Drosophila* HP1a is

mainly associated with heterochromatin, while *Drosophila* HP1c helps regulate the gene transcription in euchromatin. Surprisingly, it has been reported that HP1a is present at many euchromatic sites and is required for positive regulation of specific genes (Piacentini et al. 2009). The genomic structure of HP1-coding genes is conserved from *Drosophila* to humans, and expression of human HP1α can rescue the lethality of homozygous mutants in the *Drosophila* HP1-coding gene *Su(var)2-5* (Norwood et al. 2004) suggesting a high conservation of HP1 function among species.

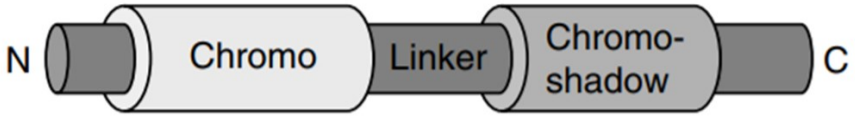



**Figure 5. Structure of HP1 proteins.** The scheme depicts the conserved linear structure of HP1 proteins. N, amino terminus; C, carboxy terminus. Modified from Lomberk et al. 2006.

The regulatory action of HP1a on transposons has been studied in several organisms. In *D. melanogaster*, it has been shown that both HP1a and HP1b interact with the histone methyltransferase Su(var)3-9, which is responsible for deposition of H3K9me3 modification recognized by HP1 chromodomain. This three-component complex forms a specialised higher order chromatin state that defines heterochromatin and represses gene activity (Fanti and Pimpinelli 2008). Consistently, loss of either *HP1* or *su(var)3-9* function in *Drosophila* ovary leads to the up-regulation of a subset of transposons (Minervini et al. 2007). In *Drosophila*, HP1a also plays a central role in the regulation of telomere length. In this organism telomere homeostasis is regulated by the presence of the transposons *Het-A* and *TART*; HP1 prevents their over-expansion and also protects chromosome ends by capping their sequences (Perrini et al. 2004).

Protein complexes responsible for chromatin packaging and condensation are also involved in TE silencing. For instance, the linker histone dH1 plays a crucial role in stabilising higher order chromatin structure, and appears to be required for transposon silencing in *Drosophila* (Vujatovic et al. 2012).

Genomic regions that contain mostly inactive genes and transposon-rich regions tend to be clustered together at the nuclear periphery whereas active genes are preferentially found in the nuclear interior. In mammalian cells, large-scale repression of chromatin domains is achieved through the interaction of HP1α with the nuclear lamin complex (Ye et al. 1997). A nuclear envelope-binding site within the chromodomain of HP1 appears to interact with Lamina-associated polypeptide 2 (LAP2β), Lamin B Receptor (LBR) and B-type lamins, suggesting that HP1 may tether peripheral heterochromatin to the inner nuclear lamina (Kourmouli et al. 2000).

Lamins are long and stringy intermediate filament proteins that represent the major architectural proteins of the nucleus, forming a mesh-like structure called the nuclear lamina. These filaments provide a platform for the binding of proteins and chromatin, and confer mechanical stability to the nucleus. There are two main types of lamins: "A-type" lamins, expressed in a controlled manner during development, and "B-type" lamins, ubiquitously expressed and essential for cellular life. *D. melanogaster* has two *lamin* genes, *lamin Dm0* and *lamin C*, equivalent to the B-type and A-type genes of vertebrates, respectively. In *Drosophila*, Lamin Dm0 has been shown to act as a negative regulator of transposable elements by promoting deposition of H3K9me3 repressive histone modification (Chen et al. 2016).

An important means of TE regulation is represented by RNA-based mechanisms in which small RNA molecules guide repressor protein complexes to target TEs in a sequence-specific manner. In this context, piRNAs (PIWI-interacting RNAs) have been extensively studied, especially in the *Drosophila* germline. piRNAs are single stranded RNAs of 24-30 nt that are processed independently of DICER; they are loaded onto specific members of the Argonautes proteins (Zamudio and Bourc'his 2010). PIWI proteins have been extensively studied in the germline, and are well conserved from flies to mammals. piRNAs can be generated from either RNA transcripts of active TE copies or transcripts originating from specialised loci in the genome, called piRNA clusters. These loci harbour dysfunctional remnants of TEs and form the basis of immunity against TE propagation (Brennecke et al. 2008). The piRNAs that are generated from piRNA clusters are mostly antisense to TE mRNA sequences and serve as guides for PIWI proteins to find TE transcripts by complementary base pairing and for destruction of the TE mRNA and the concomitant amplification of defensive sequences targeting active TEs. In fact, the cleavage product itself is processed into piRNAs, generating a potent mechanism to block TE transcripts. In particular, Aub and Ago3 proteins participate to post-transcriptional gene silencing (PTGS) in the "Ping-Pong cycle" where they cooperate to amplify piRNA populations (Yang and Xi 2016) targeting the most active transposon transcripts.

The piRNA pathway can promote TE repression also acting at the transcriptional level. In *Drosophila,* Piwi and HP1 were reported to interact directly (Brower-Toland et al. 2007). Combined analyses of RNA Pol II occupancy at mobile elements, nascent transcription, steady-state mRNA levels, and H3K9me3 marks in fly gonads upon HP1 knockdown in germ cells results in transposon derepression similar to PIWI downregulation (Sienski, Dönertas, and Brennecke 2012; Wang and Elgin 2011). As for the mechanism by which PIWI interacts with HP1 in order to repress transposons, a possible simplified model is shown in Figure 3B: PIWI can detect nascent transposon transcripts and complex with other proteins, inducing co-transcriptional repression through recruitment of general silencing machinery components. As a consequence, transposon bodies receive repressive H3K9me3 marks,

which in turn recruit HP1 (Czech and Hannon 2016). It has been recently shown that the association of dH1 to target TE loci is also promoted by complex formation with PIWI, independent of its action on HP1 (Yuka W. Iwasaki et al. 2016).In addition to the germline, a role of PIWI proteins in TE regulation has been documented also in somatic tissues. This evidence came from a study in which the *Drosophila* PIWIs Argonaute-3 and Aubergine, once thought to be germline-specific, were found expressed in specific regions of the brain, and their mutation was found to lead to transposon upregulation in fly heads (Perrat et al. 2013).

### Physiological roles of TEs

Changes in genomes occur constantly in nature, and very often as a response to changing environments. Genetic and epigenetic plasticity is considered as the main means to avoid population extinction under increasing ecological stress (Lindsey et al. 2013). In these terms, by their ability to transpose, TEs may generate mutations, alter gene expression networks and promote chromosomal alterations. All these events can be positively selected by evolution. Indeed, bursts of TEs have been connected with significant events in evolution: for example, a mass insertion of SINE elements occurred during the formation of Primates (Belyayev 2014). The importance of TEs in evolution is also supported by the fact that tens of thousands of non-coding TE fragments in the human genome have orthologous conserved across species, showing clear signatures of purifying selection (Feschotte 2008). TEs have indeed provided a rich source of non-coding sequence material, fuelling regulatory innovation during vertebrate evolution (Chuong, Elde, and Feschotte 2016).

Several host genes have a high degree of homology to one or more transposable elements (Reilly et al. 2013). In addition, examples of host genes driven by TE promoters have been documented in diverse species; further, TE-derived sequences show biochemical hallmarks of active regulatory elements (Chuong et al. 2016), including enhancers, insulators and repressive elements (Sundaram et al. 2014). LINE-1 elements contain both a sense and antisense Pol II promoter in their 5′UTR and ORF1 sequence, respectively, as well as a recently discovered Pol II promoter in their untranslated 3′UTR. Bidirectional transcription from the sense and antisense promoter can produce chimeric transcripts, non-coding RNA, antisense mRNA or double stranded RNA (dsRNA), which can affect gene expression.

In **Figure 6** different types of regulatory activities exerted by TEs are schematically shown.

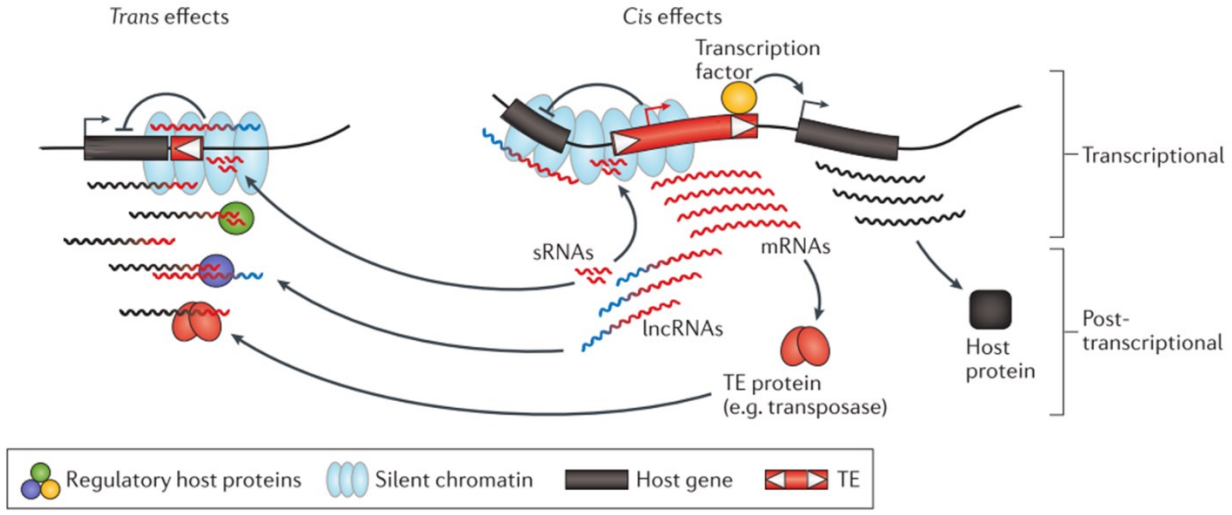



**Figure 6. Regulatory activities exerted by TEs on host genome**. Diagram depicting different types of regulatory activities exerted by TEs. These include effects mediated by cis-regulatory DNA and RNA elements as well as trans effects mediated by TE-produced non-coding RNAs and proteins. Adapted from Chuong et al. 2016.

Transposable elements seem to exert particularly relevant functions in the Central Nervous System: several lines of evidence proved that LINE-like elements are expressed and are actively retrotransposed in the brain of many species, from *D. melanogaster* to humans (Muotri et al. 2005; Perrat et al. 2013). Several studies have reported somatic insertions of TEs in the brain of mammals and flies (Evrony et al. 2012). It has been estimated that the new insertions occur at the rate of 1 new insertion per 300 cells in the human brain, with a total amount of more than 100 million unique somatic insertions (Reilly et al. 2013). Mobile element insertions thus generate cells with unique genomes, leading to neuronal mosaicism.

There is currently a huge effort to decipher the roles of physiological transposition in neuronal tissue. Brain-specific insertion of TEs into genes that are important for neuronal function, including those encoding dopamine receptors and neurotransmitters, have been identified in both humans and *D. melanogaster* (Perrat et al. 2013). Somatic retrotransposition acts as a stochastic generator of neuronal diversity and broadens the variance of cellular phenotypes. At the single neuron level, somatic retrotransposition could alter synaptic activity, response to stimuli or the competitive innervation of neuronal circuitry, depending on which genes are affected (Erwin, Marchetto, and Gage 2014). Hence, this mechanism could sustain the complexity of the neuronal network.

## Pathological impact of TE activity

In line with their parasitic origin and selfish behaviour, TEs have long been associated with disease, due to their ability to induce insertional mutagenesis and chromosomal rearrangements (Chuong et al. 2016). For instance, germline TE insertions disrupting normal gene function have been implicated in more than one hundred inherited diseases in humans (Hancks and Kazazian 2016). Also somatic TE mobilisation has been causally linked to several types of cancer and to some diseases of the CNS (Chuong et al. 2016; Krug et al. 2017).

Despite the increasing interest in the field, the mechanisms underlying TE upregulation in pathological conditions are not fully understood. Recent reports suggest that environmental stimuli, including infections and cellular stress, may destabilise epigenetic mechanisms that normally silence the bulk of TEs in the genome, thereby triggering their transcriptional activation (Chuong et al. 2016). If TE de-repression plays a major role in disease or rather represents a side effect, is however difficult to understand. The presence of high amounts of TE transcripts can have several pathogenic consequences:

- increase the probability of transposition, with mutagenic or gene-regulatory outcomes;
- direct the production of TE-encoded pathogenic RNAs or proteins;
- induce nucleic acid toxicity (caused by massive presence of RNAs or cDNAs)
- cause DNA damage (DNA breaks) and genomic instability.

There are evidences supporting all these possibilities.

TE insertions can contribute to disease risk modulating the expression of adjacent genes (**Figure 6**). Recent studies have shown that *de novo* LINE-1 insertions can activate oncogenic pathways in hepatocellular carcinoma and colon cancer (Scott et al. 2016). Overexpression of ERV envelope proteins, as seen in the brain of patients with neurodegenerative and autoimmune responses, can induce a wide range of cellular processes and abnormalities associated with these pathologies (Li et al. 2015). Furthermore, the cytoplasmic accumulation of nucleic acids derived from activated TEs, including double-stranded RNA, reverse transcribed cDNA or RNA-DNA hybrids, are increasingly regarded as potent immunological adjuvants that may trigger autoimmune responses (Yu 2016).

Activation of the LINE1 transposition machinery increases double-strand breaks (DSBs) in the genome through LINE1-encoded endonuclease activity. The extent of these DSBs could contribute to the genome instability observed during ageing and cancer development (Gasior et al. 2008).

Although controlled retrotransposition may be beneficial for neuronal plasticity, upregulated elements could also have deleterious effects on cognitive functions. Two aspects of TEs misregulation

have been linked with neurological diseases: altered levels of TE RNAs and increased somatic insertions.

Individuals affected by Schizophrenia display increased rate of somatic LINE1 retrotransposition in neurons (Bundo et al. 2014). In particular, insertions are identified more frequently within genes associated to synaptic activity. In the neurodevelopmental disorder Rett syndrome, a higher somatic retrotransposition rate has been observed in patient-derived cells (Muotri et al. 2010). The disease is caused by mutation of Methyl CpG binding Protein 2 (MeCP2), that binds methylated DNA at LINE1 promoter, repressing this element in neuronal progenitor cells.

Similarly, TEs deregulation has been found in TAR DNA binding protein 43 (TDP43)-related neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS) and frontetemporal lobar degeneration (FTLD) (Erwin et al. 2014). One of the physiological roles of TDP43 indeed consists in binding many transposon sequences, including LINEs, SINEs and LTRs, counteracting their activity in the brain cortex (Li et al. 2012). Hence, when TDP43 is mutated, TE regulation is compromised.

Ataxia telangiectasia (AT) is a neurodegenerative disorder characterized by the mutation of ATM, the apical kinase of the DNA damage response. In AT-derived cell lines and patients' post-mortem brains it has been observed an increase in LINE1 retrotransposition (Coufal et al. 2011). These results suggested that factors involved in the DNA damage response play also a role in repressing TE transposition.

It is believed that DNA damage and genomic instability contribute to ageing. Studies in mice have proven that during normal ageing several families of retrotransposons start being transcribed in different tissues. In advanced age, this expression culminates in active transposition. In human fibroblasts in culture, RTEs become derepressed and start actively transposing during replicative senescence (De Cecco et al. 2013). In *Drosophila*, the LINE-like elements *R1* and *R2*, and *gypsy* elements are highly expressed in an age-dependent manner in fly heads (Li et al. 2013). Moreover, the deletion of Ago2, one of the major components of the piRNA pathway, correlates with accelerated age-dependent memory decline and decreased lifespan (Li et al. 2013). Chen and colleagues recently reported that expression of many retrotransposons is increased in aged *Drosophila* fat body, an organ equivalent to the mammalian liver and adipose tissue (Chen et al. 2016). This derepression correlates with an increased number of DNA damage foci and decreased level of *Drosophila* Lamin-B in the old fat body cells, suggesting that TEs become activated upon ageing as a result of age-associated deregulation of Lamin-dependent heterochromatin. This hypothesis is also supported by a recent publication in which it was demonstrated that loss of repressive heterochromatin integrity and thus of TE control correlate with ageing (Wood et al. 2016). Interestingly, this work also suggested that

genetic interventions promoting heterochromatin maintenance or administration of reverse transcriptase inhibitors (thus blocking TE transposition) could maintain longevity of fruit flies (Wood et al. 2016). These findings suggest that an age-related failure of TE silencing may be a contributing factor to ageing, a previously proposed concept known as “retrotransposon theory of ageing”.

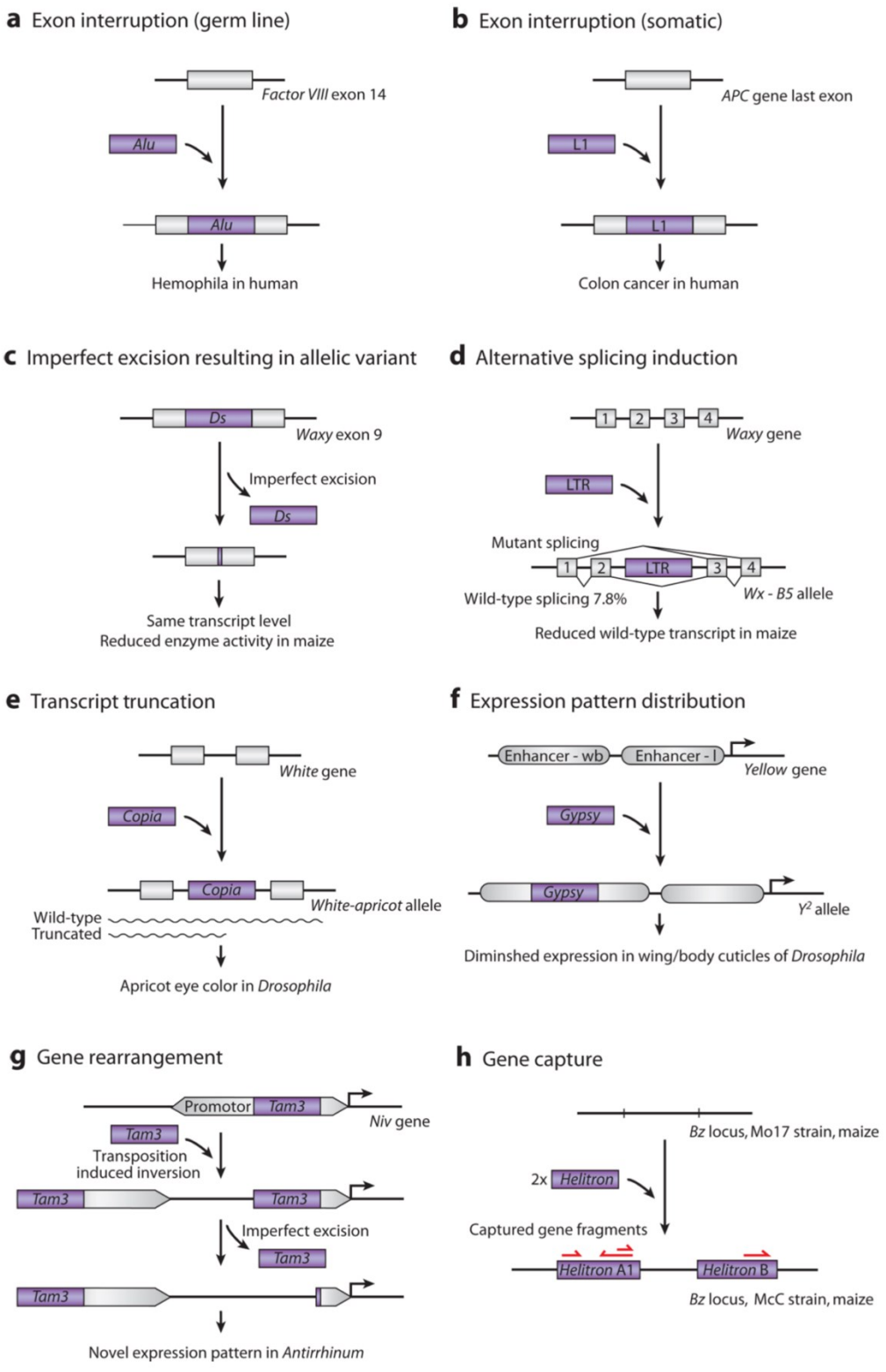



**Figure 7. Functional impact of TE *de novo* insertions. (a)** Exon interruption (germ line). A heritable human *Alu SINE* retrotransposon insertion interrupting exon 14 of human factor *VIII* gene causes haemophilia. **(b)** Exon interruption (somatic). A somatic insertion of a human *L1* retrotransposon leads to loss of function of the *APC* gene, thereby promoting colon cancer development. **(c)** Long terminal repeat (LTR) recombination resulting in allelic variant. Recombination between the two LTRs of a *Gret* retrotransposon at the *VvmybA1* locus rescued its gene expression. This recombined allele, *VvmybA1b*, reconstituted the expression of skin pigment in red grapes. **(d)** Alternative splicing induction. Insertion of an LTR retrotransposon in the *waxy*

locus leads to exon skipping and kernel color changes in maize due to reduced transcript level. **(e)** Transcript truncation. A *copia* LTR retrotransposon insertion in *Drosophila* causes a hypomorphic white allele (*white-apricot, w[a]*). The intronic insertion causes truncated and nonfunction transcripts, with some readthrough transcripts remaining functional; the result is the apricot phenotype. **(f)** Gene silencing. In the Melon genome, a *hAT* family DNA transposon inserted into the second intron of *CmWIP1* gene leads to the spread of DNA methylation to the promoter region and subsequent gene silencing. Organisms with this insertion develop female flowers because of repressed *CmWIP1* gene expression. **(g)** Gene rearrangement. A *Tam3* DNA transposon–related inversion at the niv locus in *Antirrhinum*. The inversion results from DNA breaks on opposite ends of replicated copies of *Tam3* rather than on opposite ends of a single copy. Their recombination with the upstream sequence leads to an inversion with altered *niv* promoter sequences and reduced expression. Excision of the proximal *Tam3* causes another allele with increased and novel patterning of anthocyanin pigment. **(h)** Gene capture. *Helitron* DNA transposons inserted at the *bronze (bz)* locus in maize have also captured several neighboring genes, leading to their duplication and significant noncolinearity at this locus in different strains. Functional consequences have not been shown. (Huang et al. 2012).

*The Stellate repetitive sequences*

The *Drosophila X* chromosome contains sets of tandem repeats of a gene called *Stellate (Ste)*. The *Ste* gene encodes a putative β–subunit of casein kinase II and *Ste* repeat loci reside in distinct hetero- and eu-chromatic regions in numbers ranging from 10 to 400 (Bozzetti et al. 1995; Shevelyov 1992). The *Stellate* transcript is testis-specific and the encoded Stellate proteins, when expressed, aggregate causing partial or total male sterility. Stellate expression is normally repressed by heterochromatinization and by piRNAs produced from antisense transcripts of *Suppressor of Stellate [Su(Ste)]* or *crystal (cry)* and located on Y chromosome (**Figure 8**). (Bozzetti et al. 2011).

Thus, detection of Stellate protein aggregates (Stellate phenotype) is indicative of derepression of repeated sequences and is therefore widely employed as a readout to identify mutations in TE regulators, examples of which being *Aubergine*, *Ago3*, *Zucchini* and *Hsp83* (Bozzetti et al. 2011, 2015).

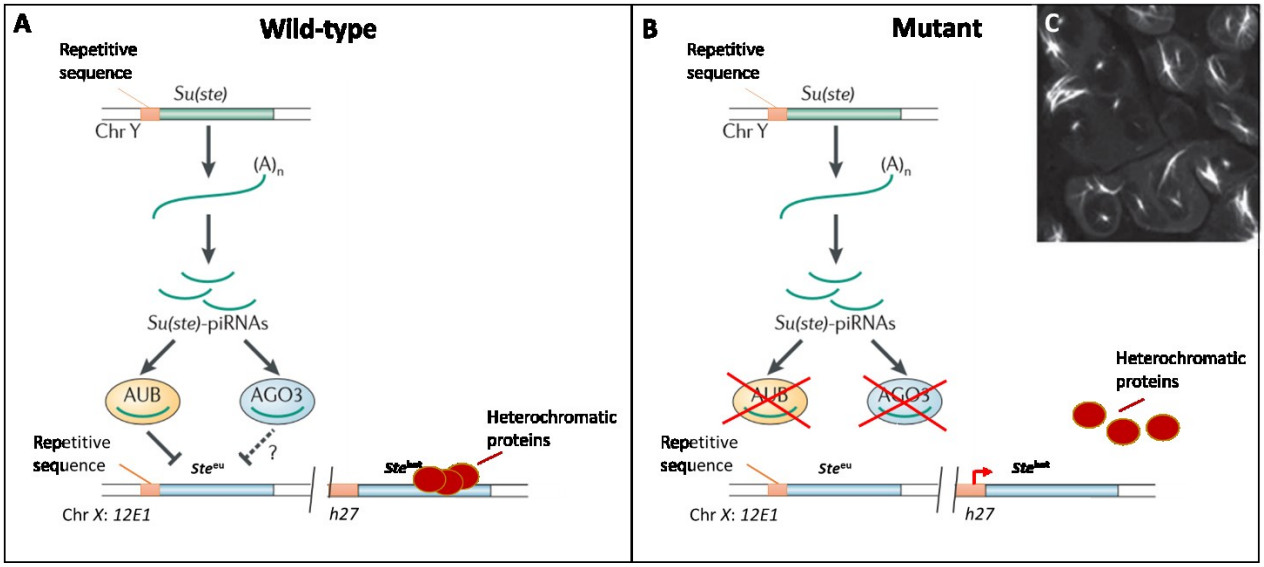



**Figure 8. Mechanisms of repression of the *Stellate* sequences in *Drosophila* testis. A)** *Su(ste)-piRNAs* are derived from the *Su(ste) locus* on the *Y* chromosome. *Su(ste)* shows a strong sequence homology to *Ste* on the *X* chromosome. *Su(ste)-piRNAs* bind both Aubergine (AUB) and Argonaute 3 (AGO3) and have a role in euchromatic *Ste (12E1)* silencing. In addition, Stellate repeats located at heterochromatic regions of *X* chromosome (*h27*), are trancriptionally repressed by epigenetic mechanisms. In the absence of *Su(ste)-piRNAs* or upon chromatin relaxation, *Ste* is derepressed (**B**), which results in the aggregation of Stellate, leading to formation of Stellate crystals. Modified from Siomi et al. 2011 (**C,** testes immunostained with anti-Stellate in a $hsp^{83scratch}$ mutant, modified from Specchia et al. 2010).

## The Pin1 phosphorylation-dependent peptidyl–prolyl isomerase

Protein structure is intimately linked to function. Among all amino acids that compose the primary structure, prolines provide conformation-restrained peptide bonds. Indeed, while most amino acids show preference for the trans peptide bond conformation, the cyclic structure of proline stabilises the cis conformer so that both isomers are represented under biologically relevant conditions (Fanghanel and Fischer 2004). Although cis-trans isomerisation of proline can occur spontaneously, cells can use specific enzymes to catalyse this conversion, making it faster. These enzymes are collectively called prolyl isomerase (PPIases). Significantly, the activity of several PPIases has been associated with signal transduction, cell differentiation, regulation of metabolism, apoptosis, and many other physiological and pathological processes. To date, three families of highly conserved PPIases have been identified based on binding to three different compounds namely, cyclophilins (Cyp), FK506 binding proteins (FKBPs) and parvulins. One of the most widely studied members of the Parvulin family is the prolyl-isomerase Pin1, due to its unique feature of acting as a phosphorylation-depedent PPIase; in fact, it can catalyse *cis/trans* isomerisation of a specific motif, formed by serine or threonine preceding proline (Ser/Thr-Pro), only after phosphorylation.

### Structure and activity of Pin1

The human *PIN1* gene encodes a protein of 163 amino acids with a mass of 18kDa. The Pin1 protein is structurally divided into two domains connected by a flexible linker: the N-terminal WW domain, characterized by two invariant tryptophans, mediates protein-protein interaction, while the C-terminal PPIase domain catalyses prolyl isomerisation. Although Pin1 exhibits a very high specificity for pS/T-P motifs, it can catalyse isomerisation of aspartic/glutamic-proline motifs as well, thus suggesting an involvement in canonical protein folding (M B Yaffe et al. 1997). As shown in **Figure 9**, Pin1 residues involved in substrate recognition and catalytic activity are highly conserved from yeast to humans (Ranganathan et al. 1997). High homology is detected in residues serving as structural links between the WW domain and the PPIase and also in amino acids involved in WW domain folding (Ranganathan et al. 1997).

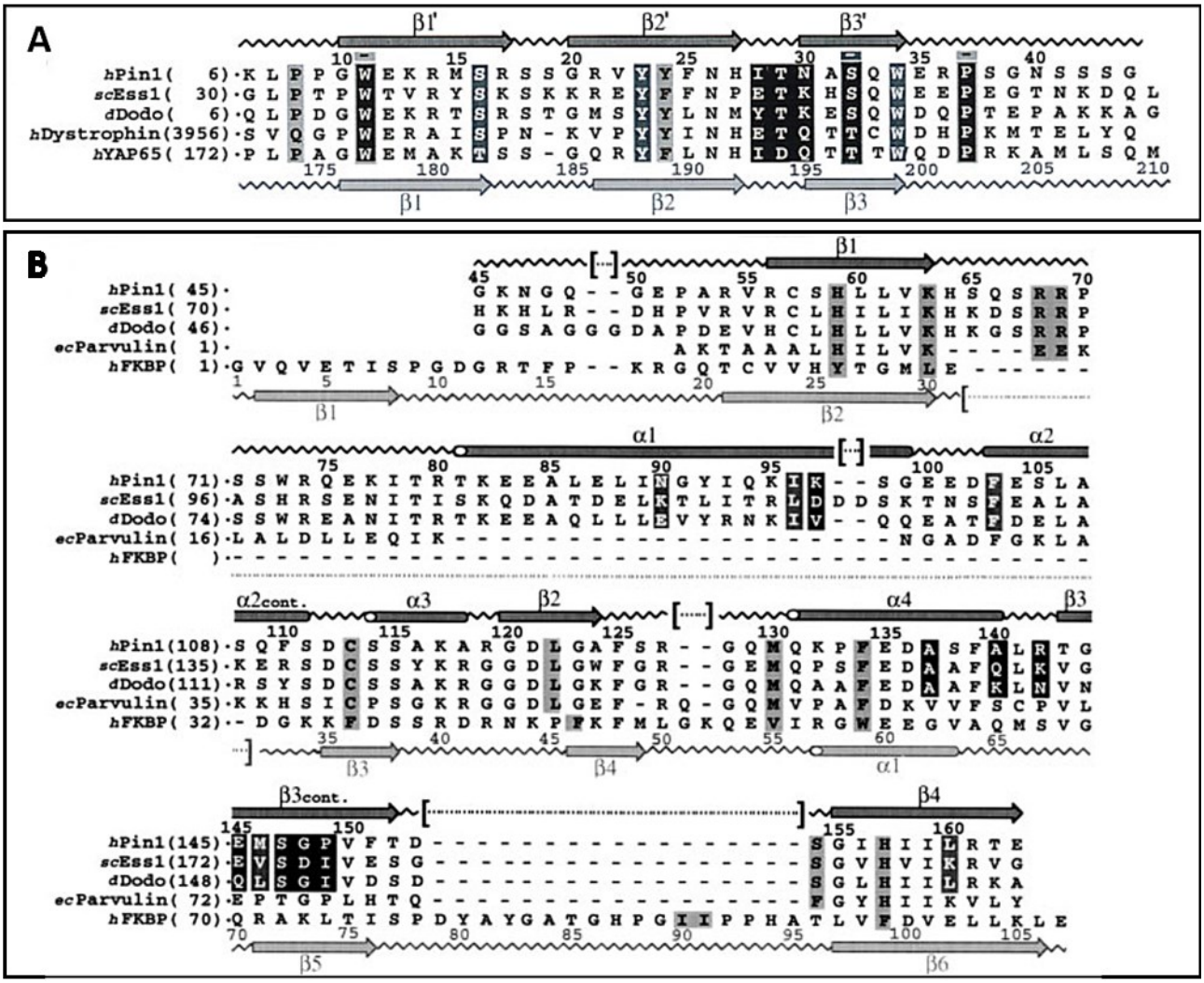



**Figure 9. Conservation of Pin1 WW and PPIase domains across species**. (**A**) Sequence alignment of the WW domain in Pin1 orthologues and other non-prolyl isomerase proteins. The numbers in parentheses correspond to the first residue on each line for each of the five WW domains. Gray boxes with white letters delineate residues in direct contact with PEG400 in the case of Pin1. Black boxes with white letters define residues serving as structural links between the WW domain and the PPIase domain of Pin1. Black letters in gray boxes highlight residues that contribute to the WW domain fold. ([h] is human, [sc] is Saccharomyces cerevisiae, and [d] is Drosophila. (**B**) Sequence alignment of the PPIase domain among prolyl isomerases. The top and bottom lines illustrate the structural elements observed in Pin1 and FKBP, respectively. The top and bottom numbering schemes refer to Pin1 and FKBP, respectively. Numbers in parentheses correspond to the first residue on each line for each of the five PPIases. Dashed lines indicate gaps. Gray boxes with black letters delineate the active site residues. White letters in gray boxes highlight residues contributing to Pin1's PEG binding sites. Black boxes with white letters define residues serving as structural links between the PPIase domain and the WW domain. ([h] is human, [sc] is Saccharomyces cerevisiae, [d] is Dro- sophila, and [ec] is E. coli). Adapted from Ranganathan et al. 1997.

The conformational changes that Pin1 induces on its protein substrates as a consequence of prolyl-isomerisation can have profound effects on their stability, catalytic activity, protein–protein interactions and subcellular localisation, thus finely regulating the functions of the substrates in response to cellular signalling pathways (Liou, Zhou, and Lu 2011) (**Figure 10-11**).

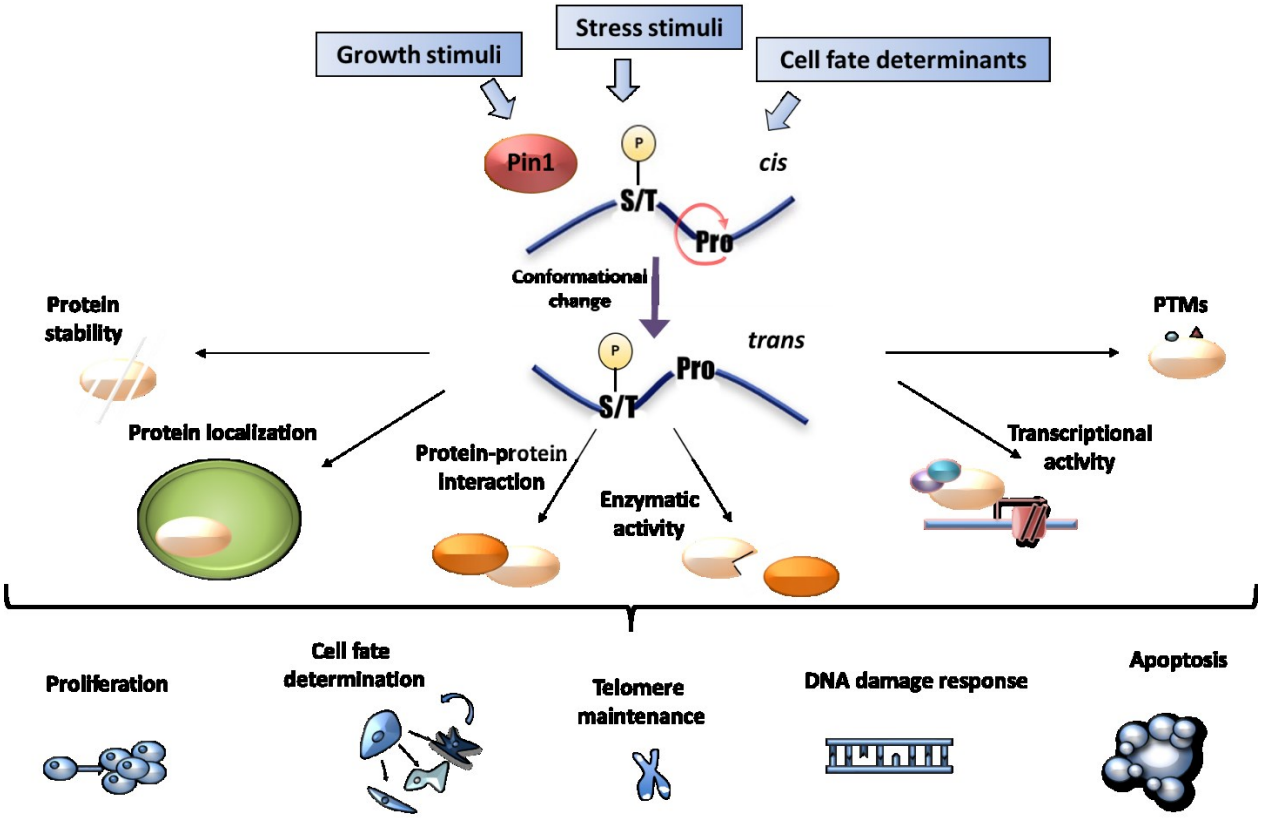



**Figure 10. Pin1 transduces phosphorylation signalling to regulate numerous cellular processes.** (Courtesy of A. Zannini)

In particular, a common consequence of Pin1-dependent isomerisation is a change in protein stability, since phosphorylation of S/T-P motifs constitutes a major regulatory mechanism controlling ubiquitin-mediated proteolysis (Orlicky et al. 2003). Pin1 has been reported to regulate the stability of many key cellular proteins such as p53, NF-kB, Oct4, cyclinD1 and BRD4 by affecting their conformation and interactions with regulators (Hanes 2015; Hu et al. 2017; Raghuram et al. 2013). Moreover, Pin1-dependent isomerisation can promote protein de-phosphorylation mediated by the trans-specific phospho-S/T-P phosphatase PP2A (Rudrabhatla, Albers, and Pant 2009) or affect Post Translational Modifications (PTMs) (e.g. phosphorylation and acetylation) at other sites.

Ser/Thr phosphorylation represents the most frequent post-translational modification and a key mechanism of signal transduction (Olsen et al. 2006). Pin1 works conjointly with proline-directed protein kinases such as all cyclin-dependent kinases (CDKs), most of the mitogen-activated protein kinases (MAPKs) and glycogen synthase kinase 3β (GSK-3β). Pin1 governs a variety of cellular processes including cell cycle, germ cell development, stem cell self renewal/expansion, cell survival, transcription and splicing, DNA damage and oxidative stress responses and cell death (Brenkman et al. 2008; Pinton et al. 2007; Rustighi et al. 2014).

Among the major Pin1 functions is the control of cell proliferation (Lu, Hanes, and Hunter 1996).

Pin1 has been shown to be a regulator of mitotic events by acting on several substrates, such as the CDC25C phosphatase and the WEE1 kinase (Okamoto and Sagata 2007; Zhou et al. 2000). Pin1 also stabilises EMI1 (early mitotic inhibitor-1), which prevents the anaphase-promoting complex (APC) from acting on cyclin A and B during S and G2 phases, allowing the coordination of S and M phases (Bernis et al. 2007) . Pin1 has also an important function in chromosome condensation during mitosis. It was demonstrated that the interaction of Pin1 with chromatin is greatly elevated in G2/M phase and that this correlates with the presence on chromosomes of several mitotic phosphoproteins, especially topoisomerase(Topo)IIα (Xu and Manley 2007). Pin1 can control chromatin organization also by the interaction with the histone H1, which plays a crucial role in stabilising higher order chromatin structure. In fact, Pin1 can recognize and bind phosphorylated pS173 and pS187 residues on histone H1, modulating the conformation of its C-terminal domain. This action contributes to regulate chromatin accessibility to transcription (Raghuram et al. 2013). In addition to epigenetic modification, Pin1 can promote gene expression through the induction of dephosphorylation of RNA Pol II CTD (Zhang et al. 2012) or mRNA stability by preventing AUF1-mediated RNA degradation (Esnault et al. 2006).

Pin1 has also been implicated in the cell response to DNA damage. As a consequence of DNA double-strand breaks (DSBs), cells initiate an elaborate signalling cascade known as the DNA damage response (DDR) to maintain genomic integrity (Jackson and Bartek 2010). The DDR coordinates cell-cycle checkpoints and DNA repair or, if the damage cannot be repaired, triggers specialised programs such as apoptosis and senescence (Ciccia and Elledge 2011). Numerous factors lead to the activation of the DDR, including ionizing radiation, chemotherapeutic drug treatment, and strong hyper-proliferative signals as induced by oncogene expression. A major mechanism underlying the cellular response to DNA damage is protein phosphorylation. In fact, DSBs activate ATM/ATR kinases which trigger a signaling cascade that arrests cell cycle and triggers repair of DNA lesions (Matsuoka et al. 2007). Two main pathways orchestrate the repair of DNA DSBs, namely homologous recombination (HR) and non-homologous end-joining (NHEJ). HR represents a highly accurate, error-free repair mechanism, whereas NHEJ ligates together the two DNA ends with little or no processing, thus it is more error-prone. NHEJ occurs with faster kinetics and functions throughout the cell cycle. In contrast, HR is a rather slow, multistep repair process restricted to S/G2 phase when the intact sister chromatid is available. Briefly, HR requires 5’ to 3’ nucleolytic degradation of DSB ends to generate long stretches of single-stranded DNA (ssDNA) - a mechanism generally described as DNA end resection. Besides cell-cycle stage and DSB complexity, the division of labor between the two DSB repair pathways was shown to depend on the chromatin state around the lesion (Goodarzi et al., 2010). Mechanistically, DNA end resection is the key determinant of DSB

repair pathway choice, because it prevents repair by NHEJ and commits cells to HR (Chapman, Taylor, and Boulton 2012). Pin1 plays a critical role in coordinating DNA repair pathway choice by suppressing HR and promoting the NHEJ pathway. It was demonstrated that Pin1 overexpression attenuates HR, while Pin1 depletion reduces NHEJ as a result of increased DNA end resection (Steger et al. 2013) This is due the fact that Pin1 interacts with prominent DSB repair factors. In particular, Pin1 mediates isomerisation of the repair factor CtIP after its phosphorylation at two conserved S/T-P motifs ($S^{276}$ and $T^{315}$) by cyclin-kinase CDK2. This conformational change results in negative regulation of CtIP stability by Pin1-dependent promotion of CtIP polyubiquitylation and subsequent proteasomal degradation (Steger et al. 2013).

Pin1 has been shown to play a central role in transducing genotoxic stimuli and oncogenic stress into full activation of wild-type p53 functions (Mantovani et al., 2015). While almost undetectable in normal conditions, the interaction between p53 and Pin1, mediated by the N-terminal WW domain of the isomerase, can be promoted by several stimuli activating p53, such as γ- and UV irradiation, treatment with chemotherapeutic drugs and overexpression of activated oncogenes. These stimuli lead to the phosphorylation of different Ser/Thr-Pro motifs in p53, making them sites for Pin1 binding. Pin1 is indispensable for transducing stress-induced phosphorylation of p53 into conformational changes that affect its stability and function. The structural change mediated by Pin1 can trigger various functional outcomes mediating either cell cycle arrest or apoptosis, depending on the specific phosphorylation events. In particular, while phosphorylation of p53 on Thr81 triggers its Pin1-dependent dissociation from the MDM2 ubiquitin ligase, phosphorylation on Ser46 promotes its Pin1-dependent dissociation from the apoptosis inhibitor iASPP, thus unleashing activation of apoptotic target genes by p53, as well as its monoubiquitination and mitochondrial trafficking, thus directly triggering apoptosis (Mantovani et al., 2015). Moreover Pin1 assists also stress-induced activation of the p53 family member p73 since it promotes p73 acetylation and subsequent protein stabilisation and transcriptional activity; this circuitry has an important role for the response to chemotherapy in tumors lacking p53 (Mantovani et al. 2004).

Pin1 has an important role in the orchestration of several cell signalling pathways, such as the Wnt/β-catenin and cytokine/NFκB pathways, since it can bind to phosphorylated β-catenin and NF-κB, increasing their protein levels and transcriptional activity, with the consequence of promoting cyclin D1 gene expression (Ryo et al. 2001, 2003).

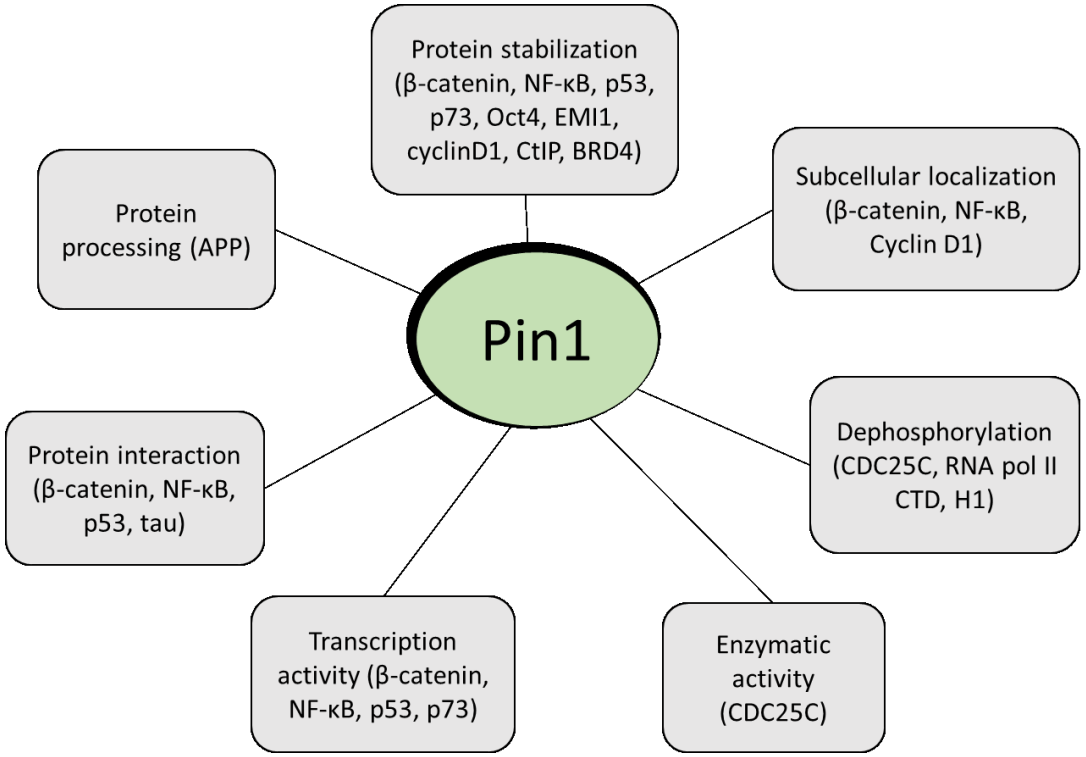



**Figure 11. Pin1-catalysed prolyl isomerisation regulates a variety of protein substrates.** See the text for explanation.

## Regulation of Pin1

Pin1 expression and catalytic activity are tightly regulated by several mechanisms (Liou et al. 2011; Lu and Zhou 2007). The transcription factor E2F has been shown to regulate Pin1 expression transcriptionally in response to growth factor stimuli, Ras, and Her2/Neu (Pulikkan et al. 2010; Ryo et al. 2002). In breast cancer cells, Pin1 mRNA levels are also upregulated by the Notch signalling pathway (Rustighi et al. 2009) and the Insulin-like growth factor (You et al. 2002). The stability and activity of Pin1 are regulated by phosphorylation at several residues. Protein kinase A (PKA) or Aurora A-mediated phosphorylation of the human Pin1 Ser16 residue within the WW domain inhibits Pin1 substrate binding and nuclear localisation (Lee et al. 2013; Lu et al. 2002), while phosphorylation by death-associated protein kinase 1 (DAPK1) at Ser71 within the catalytic domain inactivates Pin1 PPIase activity (Lee, et al. 2011). On the contrary, phosphorylation of Ser65 by Polo-like kinase 1 (PLK1) increases the stability of Pin1 by reducing its ubiquitination (Eckerdt et al. 2005). In addition, Mixed Lineage Kinase 3 (MLK3) phosphorylates Pin1 on S138 increasing its activity and nuclear translocation (Rangasamy et al. 2012). Recent evidences have reported that specific mechanisms are operated in different cell types to regulate Pin1 function and that Pin1 expression is highly regulated during embryogenesis at least in the Zebrafish *Danio rerio* (Ibarra et

al. 2017). On the contrary, nothing is known about the regulation of Dodo protein, even if Ser16 and Ser71 residues conservation may suggest a phosphorylation-dependent regulation of Dodo function as demonstrated in mammalian models.

## Pin1 in health and disease

Normal Pin1 activity serves to control the dynamics of physiological cell behavior downstream to phosphorylation signaling. However, perturbation of signaling pathways and/or deregulated Pin1 expression or activity may amplify pathologic conditions such as age-related neurodegeneration, cancer, neurological disorders, autoimmune and inflammatory diseases (Lee et al. 2011; Nath and Isakov 2014).

Analysis of Pin1 KO mice and other studies emphasized the role of Pin1 in protecting against age-dependent neurodegeneration. Alzheimer's disease (AD) is the most common cause of dementia, characterized by the formation of insoluble aggregates at the extracellular (amyloid plaques) and intracellular (neurofibrillary tangles) level in the brain. These aggregates are formed from overproduction and/or reduced clearance of Aβ peptides, derived from amyloid precursor protein APP, and from hyper-phosphorylation or dysfunction of the microtubule-binding protein tau (Tanzi and Bertram 2005), respectively. As a result of hyperphosphorylation, tau detaches from microtubules leading to destabilisation of cytoskeletal structure and cell death (Iqbal et al. 2009). Further, it precipitates into insoluble aggregates, later forming larger neurofibrillary tangles (Andorfer et al. 2003). Pin1 has been shown to exert a protective function against both Aβ and tau pathogenic mechanisms. Indeed, Pin1 can promote non-amyloidogenic APP processing, thereby producing neurotrophic αAPPs and reducing neurotoxic Aβ peptides (Pastorino et al. 2006). In addition, Pin1 is able to bind pThr231-tau and restores its ability to bind to microtubules and promote microtubule assembly (The et al. 1999).

In normal human brains, Pin1 is present in the neuronal nucleus and cytoplasm. In AD brains, its expression is relatively lower in subregions of the hippocampus that are more susceptible to neurofibrillary degeneration (Liou et al. 2003). Importantly, Pin1 is downregulated or inactivated by various mechanisms and colocalises with neurofibrillary tangles in AD brains. In neurons of AD patients, Pin1 has been reported to be delocalised (The et al. 1999) inactivated by oxidation (Butterfield et al. 2006; Sultana et al. 2005) or down-regulated (The et al. 1999). In addition, Pin1 promoter polymorphisms appear to associate with reduced Pin1 levels and late-onset AD (Ma et al. 2012; Segat et al. 2007).

Pin1 is also involved in the immune response and in the regulation of infection processes of exogenous viruses, such as HIV-1 and Hepatite B Virus (HBV) by stabilisation of the integrase and the HBx-oncogenic peptide respectively (Manganaro et al. 2010; Pang et al. 2007). In particular, it has been shown that in activated T lymphocytes, viral integrase, which mediates HIV-1 cDNA integration into the host cell genome, is phosphorylated by JNK on a highly conserved serine residue in its core domain. Pin1, in turn, binds the viral integrase and stabilises it by catalysing conformational changes of the viral integrase. Eventually, the stabilised viral integrase allows efficient HIV-1 integration and infection (Manganaro et al. 2010).

Pin1 has been shown to play a critical role during oncogenesis. It is overexpressed in the majority of cancers and acts as a modulator of several cancer-driving signalling pathways, including c-MYC, NOTCH1, WNT/b-catenin and RAS/MEK/ ERK pathways, while it simultaneously curbs several tumour suppressors (Liou et al. 2011; Lu and Hunter 2014). PIN1 enables a mutant p53 pro-metastatic transcriptional program (Girardini et al. 2011) and boosts breast cancer stem cells (CSCs) expansion through activation of the NOTCH pathway (Rustighi et al. 2009). Genetic ablation of PIN1 reduces tumour growth and metastasis in several oncogene-induced mouse models of tumorigenesis, indicating the requirement for PIN1 for the development and progression of some tumours (Lu and Hunter 2014). In addition, PIN1 inhibition sensitizes breast cancer cells to different targeted- and chemotherapies or overcomes drug resistance (Rustighi et al. 2016). For detailed description of the role of Pin1 in cancer, see Rustighi et al., 2016 and Zhou and Lu 2016.

### The *Drosophila melanogaster* Pin1 orthologue Dodo

*Drosophila* Dodo is a 166 amminoacids long protein encoded by the *dod* gene located, on the X chromosome. Dodo protein shares 57% of identity with human Pin1 and 47% of identity with yeast *ESS1* and it has probably a conserved function, given that its expression rescues vitality in *ESS1* KO yeast (Maleszka et al. 1996). However, little is known about the function of Dodo in the fruit fly. *dodo* null fly embryos exhibit ventralised eggshell phenotype (Hsu et al. 2001), a developmental defect that has been ascribed to the ability of Dodo to promote dephosphorylation and subsequent degradation of the CF2 transcription factor (Hsu et al. 2001), which regulates the dorsal–ventral polarities in the egg and in the developing embryo (Mantrova and Hsu 1998). Recently, Dodo has been shown to regulate circadian clock machinery by stabilisation of phosphorylated period (PER) protein, altering the locomotor behaviour following dark-to-light transition (Kang et al. 2015).

# Aim of the thesis

Transposable Elements (TEs) positively contribute to genetic heterogeneity in both germline and somatic tissues, offering the potential for the organism to adapt to stress. However, aberrant TE activation may cause gene mutations, epigenetic interference and genomic instability and it has indeed been associated to organismal ageing and age-dependent neurodegeneration in flies. Moreover, excessive TE insertion rates have been found in human neurological diseases including Schizophrenia, Rett syndrome and Parkinson's Disease. Interestingly, both ageing and neurodegenerative conditions are characterized at the cellular level by heterochromatin loss and increased DNA damage.

The prolyl-isomerase Pin1 is essential to link phosphorylation signalling to modulation of crucial cellular processes. Pin1 activity appears to be necessary for healthy ageing and prevention of age-related diseases. Pin1 knock-out mice anticipate ageing phenotypes such as reduced body size, atrophy of testis and retina, locomotor and behavioural defects and progressive age-dependent neurodegeneration. Moreover, deregulated Pin1 expression and/or activity have been observed in Alzheimer's Disease patients. Interestingly, Pin1 has been shown to broadly impact on chromatin state and transcription and to act as a key modulator of DNA repair, all processes that regulate TE activity.

Based on all the above evidence, we hypothesized that Dodo may be involved in regulation of TE activity and that this function may be essential to preserve genomic integrity in both germline and somatic tissues, particularly in the Central Nervous System.

We chose *Drosophila melanogaster* as model organism for this study since it combines a detailed knowledge of TE regulation, which is still incomplete in mammalian models, with a proven value to study neurodegenerative processes. Importantly, the *Drosophila* Pin1 orthologue Dodo protein shares a high degree of sequence similarity with the evolutionarily conserved Pin1 enzymes, and it has been reported that *dodo* null flies display developmental defects common to mutants of known components of TE surveillance mechanisms.

The major aims of this thesis are:

- to analyse the role of the *Drosophila* Pin1 orthologue Dodo in controlling TE expression and mobility in both germline and somatic tissues;
- to investigate whether loss of Dodo function and the related TE hyperactivation may lead to pathologic consequences for neuronal homeostasis in *Drosophila,* in particular age-dependent neurodegeneration.

# Results

## Dodo regulates the expression of mobile genetic elements in the germline and brain tissues

### Dodo regulates *Stellate* repetitive sequences in the *Drosophila* testis

To study the role of Dodo in the regulation of transposons, we took advantage of the *dod*$^{EY03779}$ mutant fly strain, previously obtained by P-element insertional mutagenesis (Bellen et al. 2004) (**Figure 12**) and hereafter referred to as "*dodo* mutant". First, we characterized this mutant for Dodo protein levels in whole adult flies, ovaries and heads, observing a strong reduction of Dodo protein levels compared to an isogenic control fly strain (**Figure 12B-C**).

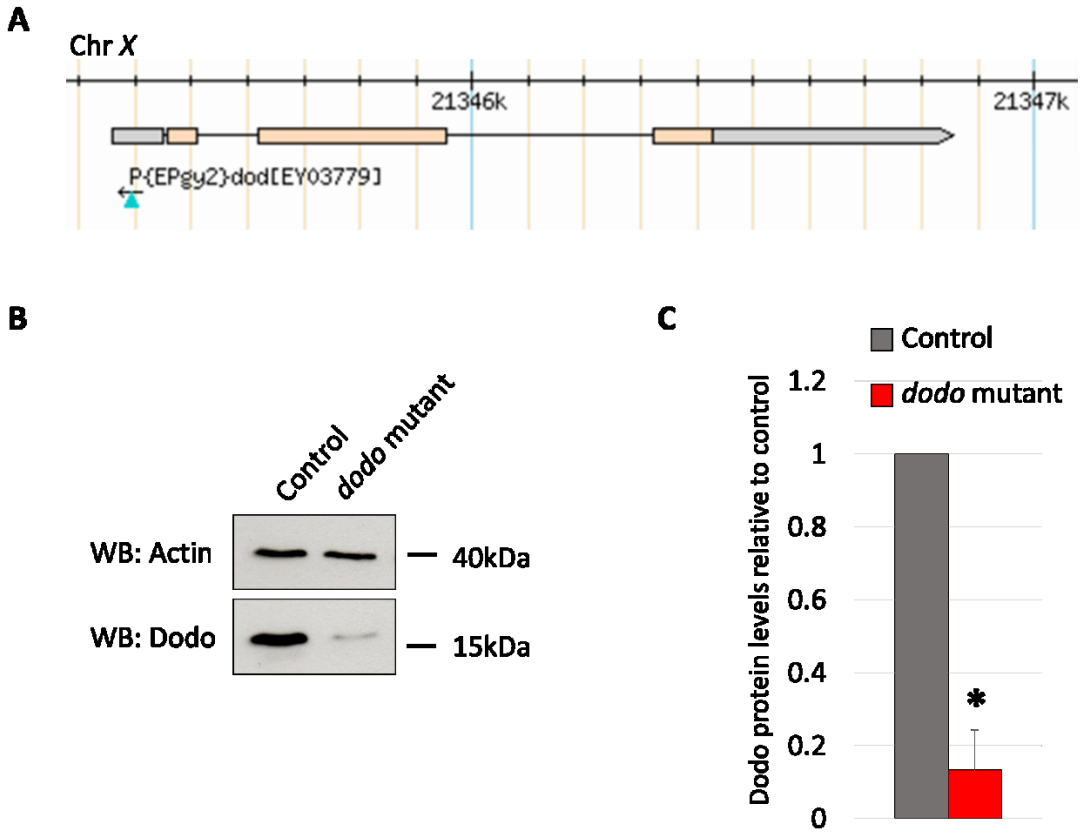



**Figure 12. Analysis of Dodo protein expression in *dod*$^{EY03779}$ *dodo* mutant strain. A)** Annotation of *Dosophila melanogaster dodo* transcript according to Flybase_2.0 (released R6.17; http://flybase.org/). The *dod*$^{EY03779}$ mutant strain harbours an insertion of a P{EPgy2} element in the 5'UTR region (grey) of the *dodo* gene, localized on the *X* chromosome (Chr X), as indicated by light blue arrowhead. Exons are indicated in orange and introns by thin lines. Untranslated regions are indicated in grey. **B)** Western blot analysis of Dodo protein from lysates of *w*$^{1118}$ (control) and *dod*$^{EY03779}$ (*dodo* mutant) whole adult flies. Actin was used as loading control. The image is representative of n=3 biological replicates. **C**) Quantification of Western blot analysis (**B**) of Dodo protein in *dod*$^{EY03779}$ (*dodo* mutant) relative to *w*$^{1118}$ (control) whole adult flies. Actin was used as reference for quantification. Values represent mean ± s.e. of n=3 biological replicates. *P value < 0.05 by two-tailed unpaired Student's t-test.

Regulation of TEs is most critical in germline tissues where genome instability and detrimental mutations caused by TE mobilisation directly threaten the reproductive capacity of an organism and the viability of its offspring. For this reason, the germline compartment has fine mechanisms to restrict the expression of TEs, making the germline an ideal tissue to study TE regulation.

Immunostaining in *Drosophila* testis showed that Dodo is ubiquitously expressed in the nuclei of *Drosophila* male germ cells, including the spermatocytes (**Figure 13B-B''''**). Thus, as a convenient method to inspect for the regulation of repeated sequences in *dodo* mutant flies, we analysed the expression of the *Stellate* repeated DNA sequences in the spermatocytes, a phenotype that has proven instrumental to dissect conserved pathways regulating transposons (Bozzetti et al. 2011). The *Stellate* sequences, although not mobile, are normally silenced by the same transcriptional and post-trancriptional mechanisms that silence DNA transposable elements (see Introduction). The expression of the *Stellate* sequences leads to a testis-specific 750bp long transcript whose product is the Stellate protein. Failure of silencing and degradation of the *Stellate* transcript leads to the formation of Stellate crystalline needle-shaped aggregates in the spermatocytes, causing male infertility. In collaboration with Dr. V. Specchia (University of Salento), we found that *dodo* mutant spermatocytes exhibited Stellate crystalline aggregates (**Figure 13D)**, suggesting that Dodo may be involved in the mechanisms that regulate the expression of repeated sequences. Dr. V. Specchia took advantage of the Gal4/UAS system to drive the expression of an interfering RNA (RNAi) targeting the *dodo* mRNA with a testis-specific enhancer expressed in the spermatocytes (using the *c-135-Gal4* driver), which lead to the formation of Stellate aggregates, similar to the *dodo* mutant (**Figure 13E**). Strikingly, co-expression of human Pin1 fully suppressed the accumulation of Stellate crystalline aggregates induced by RNAi-mediated knock-down of *dodo* in the fly spermatocytes (using a double transgenic flies co-expressing *UAS-dodo* RNAi and *UAS-hPin1* with the *c-135-Gal4* driver) **(Figure 13F**). This suggests that the regulation of repeated sequences may represent an evolutionarily conserved function of Pin1.

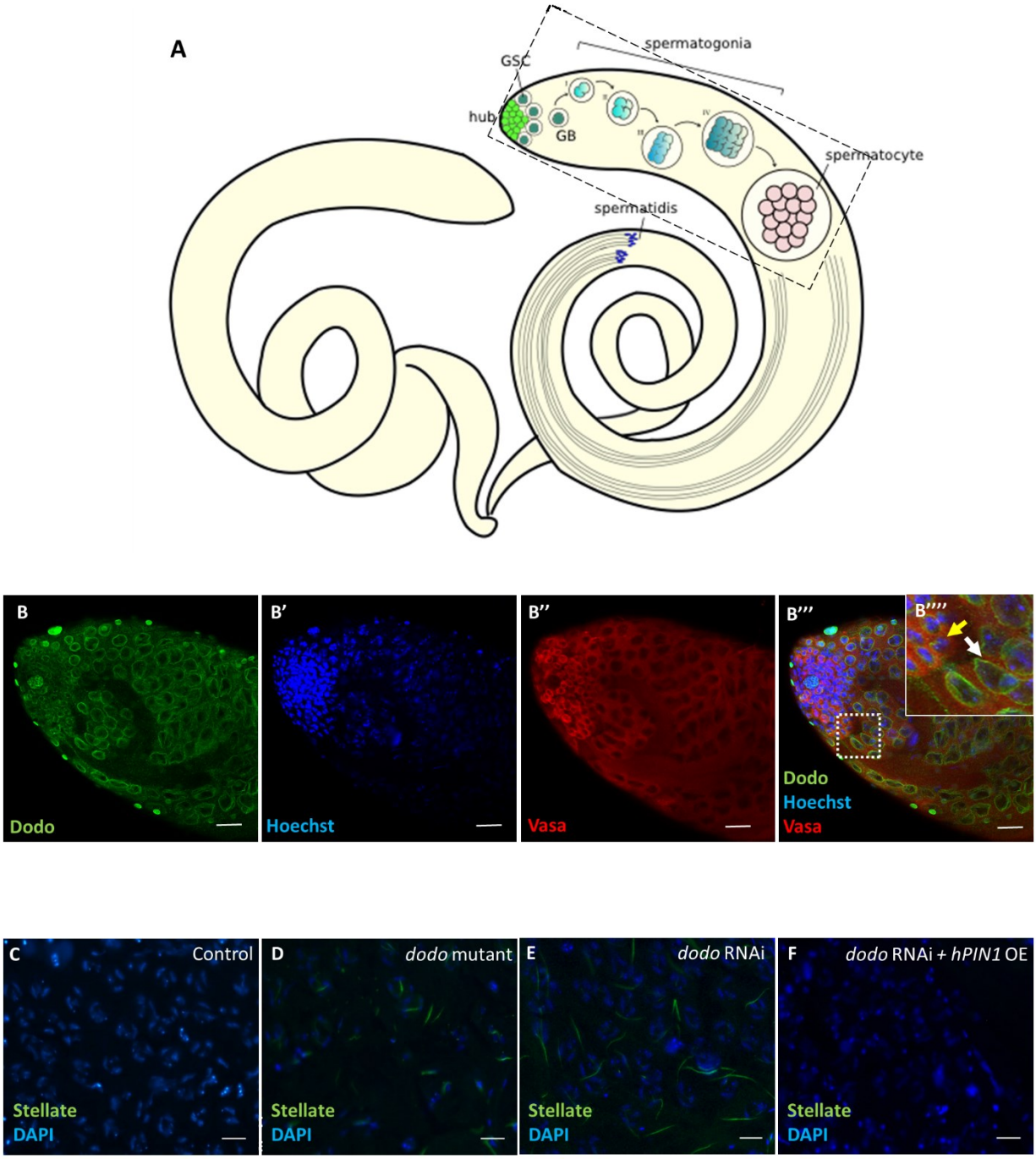



**Figure 13. *dodo* mutant testes display Stellate overexpression, which could be rescued by overexpression of the Dodo human orthologue Pin1. A)** A schematic representation of *Drosophila* testes shows germ stem cells (GSCs) located in the "hub" region and gonioblasts (GB) that mature in spermatogonia and than in spermatocytes before meiosis occurs and generates spermatids (Perna, MSc Thesis 2017). **B-B''')** Single confocal section immunofluorescence images of Dodo (green, **B**) and Vasa germline cytoplasmatic marker (red, **B''**), in the region highlighted in **A,** of testis of young wild type ($w^{1118}$) flies (4 days old). The inset (**B''''**) shows spermatogonia (yellow arrow) and spermatocytes (white arrow). Scale bar, 20µm. **C-F)** Stellate protein immunostaining (green strings) in *Drosophila* spermatocytes of the following genotypes: **C,** Control: $w^{1118}$;*UAS-dod*$^{KK108535}$/+; **D,** *dodo* mutant: *dod*$^{EY03779}$;; **E,** *dodo* RNAi: $w^{1118}$;*UAS-dod*$^{KK108535}$/+;*c135-Gal4*/+. **F,** *dodo RNAi + hPIN1* overexpression (OE): $w^{1118}$;*UAS-dod*$^{KK108535}$/+;*c135-Gal4/UAS-Pin1*;. For mRNA expression levels of *dodo* or h*Pin1* see **Figure S. 1**. Nuclei are stained with DAPI (in blue). Scale bar, 15µm.

## Dodo negatively regulates TE expression in the *Drosophila* ovary

To further investigate the role of Dodo in the regulation of Transposable Elements, we sought to monitor the expression of TEs in the *dodo* mutant female germline. Co-immunostaining of Dodo and the germ cell cytoplasmatic marker Vasa showed that Dodo is ubiquitously expressed in both germ cells (nurse cells and oocyte) and somatic cells (follicle cells) in the fly ovary. In nurse cells, Dodo appeared to localise in both nucleus and cytoplasm, with significant enrichment at the intranuclear periphery (**Figure 14B**). We analysed the expression of representative LTR-retrotransposons (*MDG1, Idefix, ZAM, ROO, GYPSY, 412, Quasimodo, Invader4 and Gypsy6*), non-LTR or LINE-like retrotransposons (*G6, R1, TAHRE, IVK*) and one DNA transposon (NOF) by RT-qPCR, in the ovaries of isogenic wild-type and *dodo* mutant flies. Dodo depletion led to increased mRNA levels of LTR-elements *412, Quasimodo, MDG1* and *ZAM*, and of the LINE-like element *IVK* (**Figure 14C**). These results suggest that Dodo is a negative regulator of the expression of at least some LTR-retrotransposons and LINE-like elements.

Interestingly, our collaborator Dr. V. Specchia observed an increase in TE expression also in the ovaries of flies expressing an RNAi targeting the *dodo* mRNA under the the promoter of *nanos*, a germline-specific gene (data not shown). This indicates that Dodo function may be required in the germ cells to protect against TE aberrant activation. Moreover, Dr. V. Specchia observed overexpression of TEs also in both *dodo* mutant and *dodo* RNAi expressing fly testis (data not shown), suggesting that Dodo may repress TE expression in both female and male gonads.

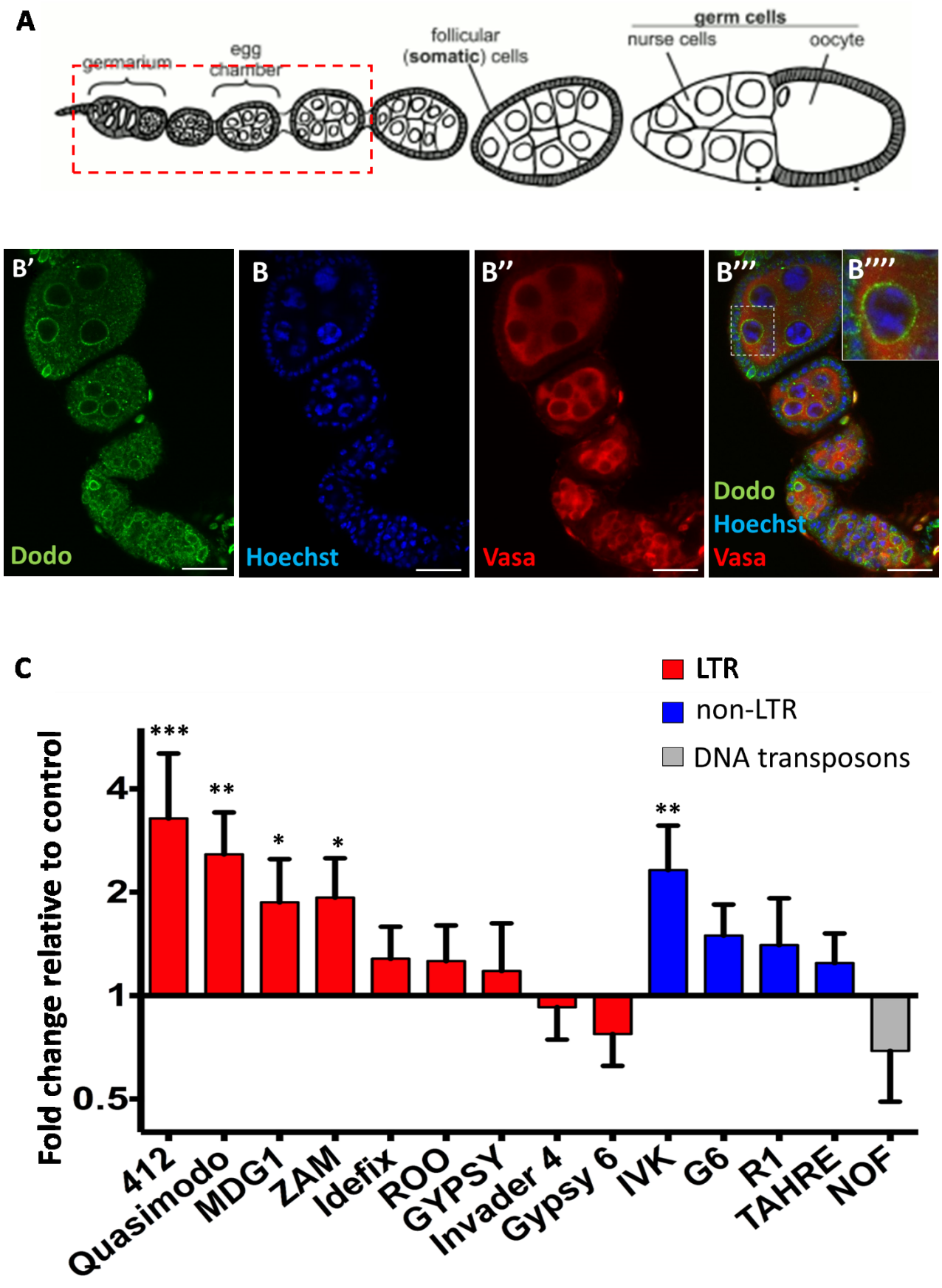



**Figure 14. *dodo* mutant flies exhibit upregulation of TE expression in the ovary. A)** Schematic representation of developing egg chambers in the fly ovary, showing follicular (somatic) cells and germ cells. In each chamber, germ cells include 15 nurse cells interconnected with one oocyte (modified from Olovnikov

& Kalmykova 2013). **B-B'''')** Single confocal section immunofluorescence images of Dodo in the egg chambers of $w^{1118}$ flies. Nuclei are stained with Hoechst (in blue). The inset **(B'''')** shows a single nurse cell. Scale bar, 20µm. **C)** RT-qPCR analysis of the expression of the indicated TEs in the ovary of *dodo* mutant flies relative to control flies ($w^{1118}$); *actin* was used as reference for quantification. Values represent mean ± s.e. of n=6 biological replicates, calculated with the ΔΔCt method. *P value <0,05, **P value <0,01; ***P value <0,001 by two tailed unpaired Student's t-test.

## Dodo negatively regulates TE expression in the *Drosophila* brain

Several lines of evidence from both human patients and animal models indicate that TEs are actively transcribed in the Central Nervous System (CNS), where their uncontrolled expression and excessive mobilisation is associated to impaired cognitive functions and neuronal death (Krug et al. 2017; Li et al. 2013). Moreover, aberrant expression of TEs was observed in the CNS of ageing organisms in both flies and mice, suggesting that TE overexpression with age may also contribute to age-related pathologies of the nervous system (Li et al. 2013; Van Meter et al. 2014). Of note, Pin1 function is required for healthy ageing and neuronal survival in the mouse (Liou et al. 2003). Hence, we investigated the role of Pin1 on TE regulation in the CNS, by using *dodo* mutant flies. First, we characterized the expression pattern of Dodo in the adult *Drosophila* brain by immunofluorescence analysis. We observed that Dodo is ubiquitously expressed in the nuclei of adult brain neurons, as highlighted by co-immunostaining of the nuclear pan-neuronal marker Elav. Similar to what observed in germ cells, Dodo is preferentially localised at the nuclear periphery (**Figure 15D**). Dodo perinuclear enrichment appears to be a general feature, as it was observed also in the nuclei of glial cells (Elav-negative, **Figure 15D'**).

Next, we monitored the expression of representative transposable elements belonging to LTR, LINE-like and DNA transposons families by RT-qPCR analysis in the brain of *dodo* mutant flies (**Figure 15E**). The expression of both LTR (*QUASIMODO, MDG1, ZAM,*) and LINE-like (*IVK, TAHRE*) TEs was increased in the brain of *dodo* mutant compared to isogenic wild-type flies, suggesting that Dodo normally represses these TEs.

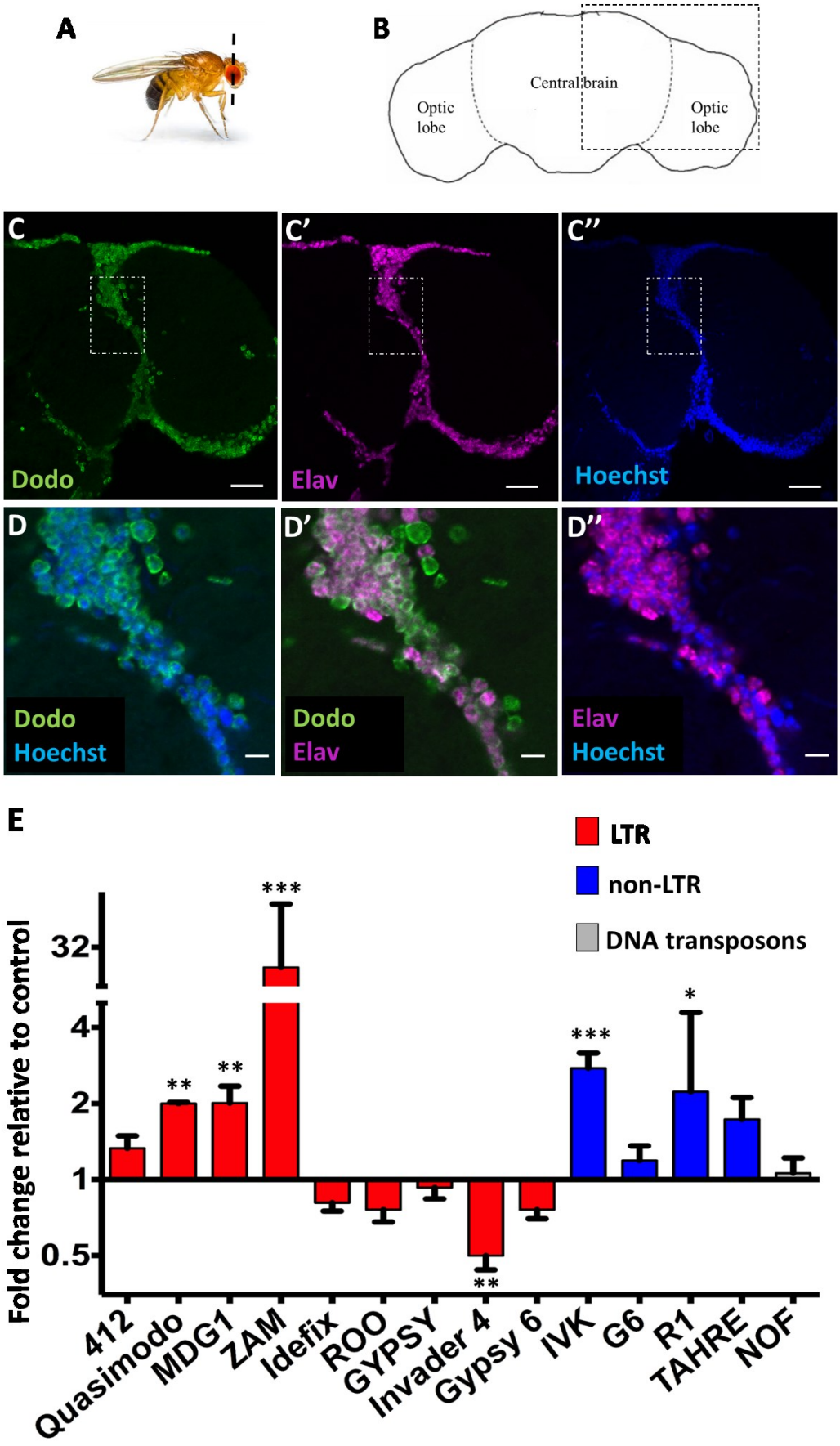



**Figure 15.** ***dodo*** **mutant flies exhibit upregulation of TE expression in the brain. A-B)** Transversal section of fly head (**A**) shows brain anatomy (**B**). **C-D'')** Single confocal section immunofluorescence images of Dodo

(green) and Elav pan-neuronal marker (magenta) in head transversal cryosection of wild type flies. Magnifications (**D-D''**, indicated by white dashed boxes in **C-C''**) show pan-neuronal expression of Dodo and few Elav-negative Dodo positive cells (**D'**). Nuclei are stained with Hoechst (in blue). **E)** RT-qPCR analysis of the expression of the indicated TEs in the ovary of *dodo* mutant flies relative to control flies ($w^{1118}$); *actin* was used as reference for quantification. Values represent mean ± s.e. of n=3 biological replicates, calculated with the ΔΔCt method. *P value <0,05, **P value <0,01; ***P value <0,001 by two tailed unpaired Student's t-test.

## Dodo maintains HP1a protein occupancy at transposon genomic sequences

The heterochromatin protein 1a (HP1a) has a fundamental role in the establishment and maintenance of heterochromatin (Elgin and Reuter 2013), representing an important negative regulator of TE expression in *Drosophila* (Minervini et al. 2007; Moshkovich and Lei 2010; Slotkin and Martienssen 2007). Interestingly, TEs whose expression we found up-regulated upon loss of Dodo in both germline and brain tissue were previously reported to be negatively regulated by HP1a in *Drosophila* cell lines and larvae (Colmenares et al. 2017; Lundberg, Stenberg, and Larsson 2013).

Therefore, we decided to verify if HP1a repressed TE expression in the ovaries and in the brain of adult flies, similar to Dodo. To this aim, we analysed the mRNA level of the *ZAM* retrotransposon as a representative transposon known to be an HP1a target (Minervini et al. 2007) and that we found regulated by Dodo (**Figure 14, 15**). RT-qPCR analysis showed higher *ZAM* mRNA levels in the ovary and brain of flies heterozygous for the *HP1a* loss of function mutation $Su(var)205^{04}$ (Eissenberg et al. 1992), compared to the wild-type control (**Figure 16**).

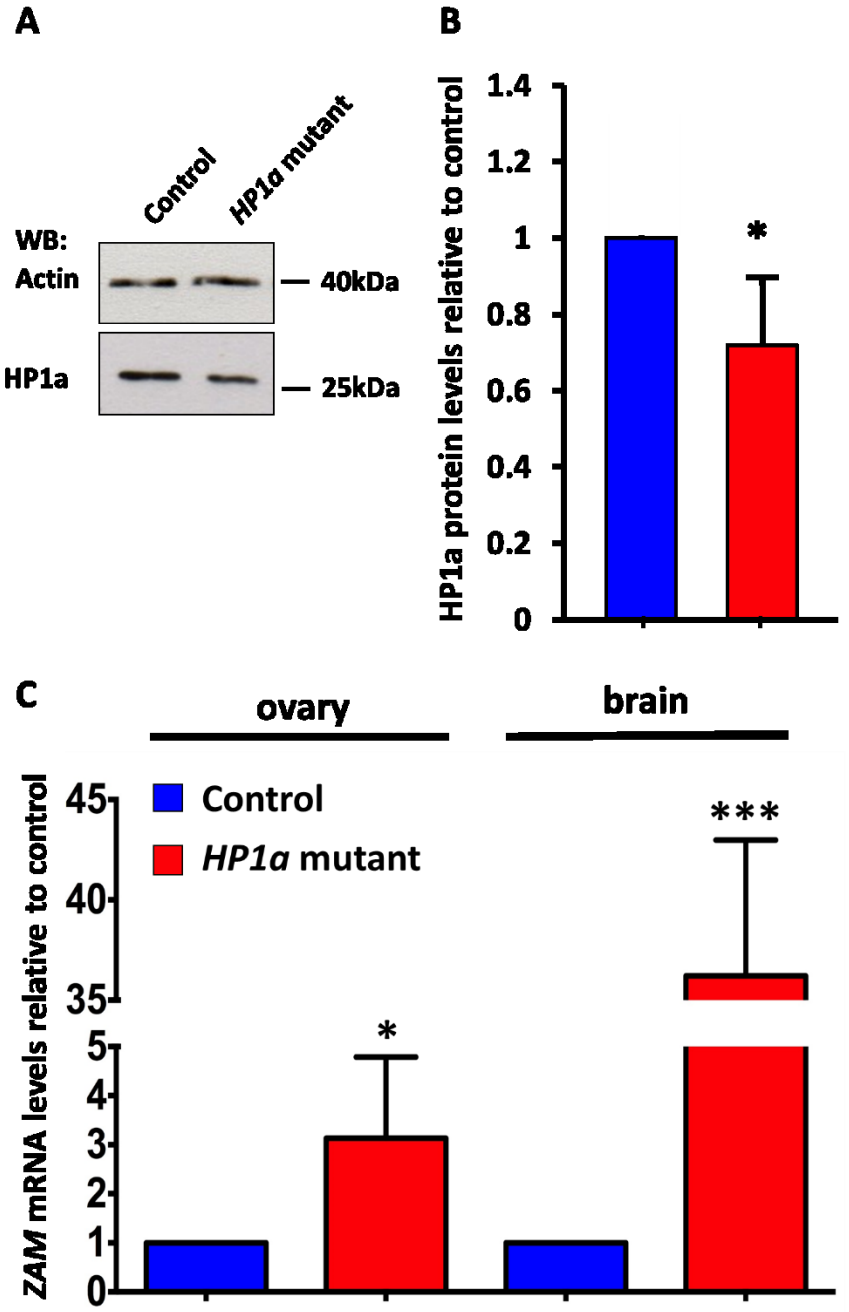



**Figure 16. *HP1a* mutant flies exhibit upregulation of the *ZAM* retrotransposon. A)** Western blot analysis of HP1a protein from lysates of $w^{1118}$ (control) and $w^{-};Su(var)205^{04}/+;$ (*HP1a* mutant) *Drosophila* heads. Actin was used as loading control. **B**) Quantification of Western blot analysis (**A**) of HP1a protein in $dod^{EY03779}$ (*dodo* mutant) relative to $w^{1118}$ (control) *Drosophila* whole adult flies. Actin was used as reference for quantification. Values represent mean ± s.e. of n=3 biological replicates. **C**) RT-qPCR analysis of *ZAM* in the ovaries and brains of flies of the following genotypes: wild-type $w^{1118}$ (control) and *HP1a* mutant $w^{-};Su(var)205^{04}/+;$ (*HP1a* mutant). *rp49* was used as reference for quantification. Values represent mean ± s.e. of n=3 biological replicates, calculated with the ΔΔCt method. *P value <0,05; ***P value <0,001 by two tailed unpaired Student's t-test.

We hypothesized that Dodo may repress TE expression by affecting HP1a deposition on chromatin at TE genomic sequences. To test this hypothesis, we evaluated HP1a binding to the genomic

regulatory region (5'UTR) of the *ZAM* retrotransposon (Minervini et al. 2007) in wild-type and *dodo* mutant flies, by chromatin immunoprecipitation (ChIP) experiments. As shown in **Figure 17**, we observed a significant decrease of HP1a occupancy at the *ZAM 5'UTR* genomic sequence in *dodo* mutant compared to wild-type fly ovaries. This result suggests that Dodo regulates HP1a deposition onto *ZAM* regulatory genomic sequences, thus favouring heterochromatinization to silence the expression of the *ZAM* retrotransposon. Interestingly, this experiment highlighted that deposition of HP1a at the *H23* pericentromeric heterochromatin region of chromosome 2 (Lin et al. 2011) was also reduced in *dodo* mutant compared to wild-type fly ovaries, suggesting that the function of Dodo as positive regulator of heterochromatinization by modulation of HP1a deposition on genomic sequences may not be restricted to TE sequences (**Figure 17**).

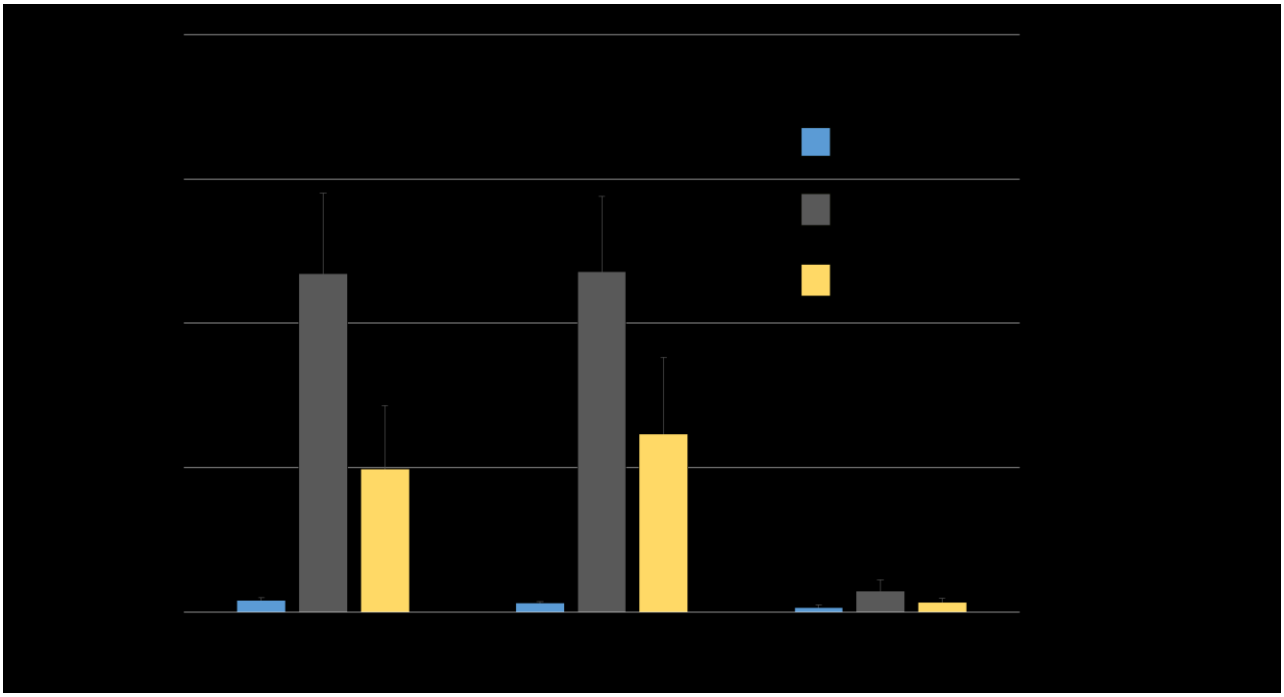


**Figure 17. *dodo* mutant flies exhibit reduced deposition of HP1a on specific genomic regions.** $w^{1118}$ (control) and $dod^{EY03779}$ (*dodo* mutant) *Drosophila* ovaries were subjected to chromatin immunoprecipitation analysis with either anti-HP1a (C1A9) antibody or anti-HA antibody as control. The percentage of HP1a binding on the indicated genomic regions was quantified by calculating the percentage of input chromatin bound by real-time PCR. A region of the *nanos* locus that is not bound by HP1a was amplified as negative control. Values represent mean ± s.e. of n=3 biological replicates.*P value <0,05 by two tailed unpaired Student's t-test.

## Dodo stabilises heterochromatin formation at the intraperinuclear region

### Dodo maintains heterochromatin foci and HP1a protein levels in brain neurons

The above results prompted us to assess if loss of Dodo might affect the formation of HP1a-containing heterochromatin foci. We then performed immunofluorescence analysis of HP1a in brains of wild-type and *dodo* mutant flies. As shown in **Figure 20,** we observed a global reduction in HP1a-containing heterochromatin foci in *dodo* mutant adult fly brains compared to wild-type control. To assess if this reduction was due to a decrease in HP1a total protein levels, we performed HP1a Western Blot (WB) analysis in brain lysates of wild-type and *dodo* mutant flies. Strikingly, we observed that HP1a protein levels were greatly decreased in the brain tissue of *dodo* mutant compared to wild-type control flies (**Figure 20C**). A similar decrease of both heterochromatic foci and HP1a total protein levels were also observed in the ovaries of *dodo* mutant compared to wild-type control flies (**Figure S. 2**). Interestingly, this decrease did not depend on reduced *HP1a* mRNA levels, as shown by RT-qPCR analysis (**Figure S. 3**). These observations suggest that Dodo may play an important role in maintaining proper levels of HP1a protein through post-transcriptional regulation. We then investigated whether this effect required Dodo isomerase activity. Given the high degree of sequence similarity between Dodo and human Pin1 proteins in both the WW and catalytic domains, we decided to employ a specific inhibitor of Pin1 activity, i.e. the small molecule PiB (Uchida et al. 2003). We evaluated total HP1a protein levels in the ovaries of wild-type flies fed with different doses of PiB. Strikingly, we observed a significant decrease of HP1a protein levels upon PiB treatment (**Figure 19**). HP1a, in complex with the histone methyl-transferase SU(VAR)3-9, promotes the spread of H3K9 methylation in heterochromatic regions (Al-Sady et al. 2013). Consistently with HP1a decrease, we observed reduced H3K9me3 protein levels in the ovary and in the brain of *dodo* mutant flies by Western Blot analysis (**Figure S. 4**).

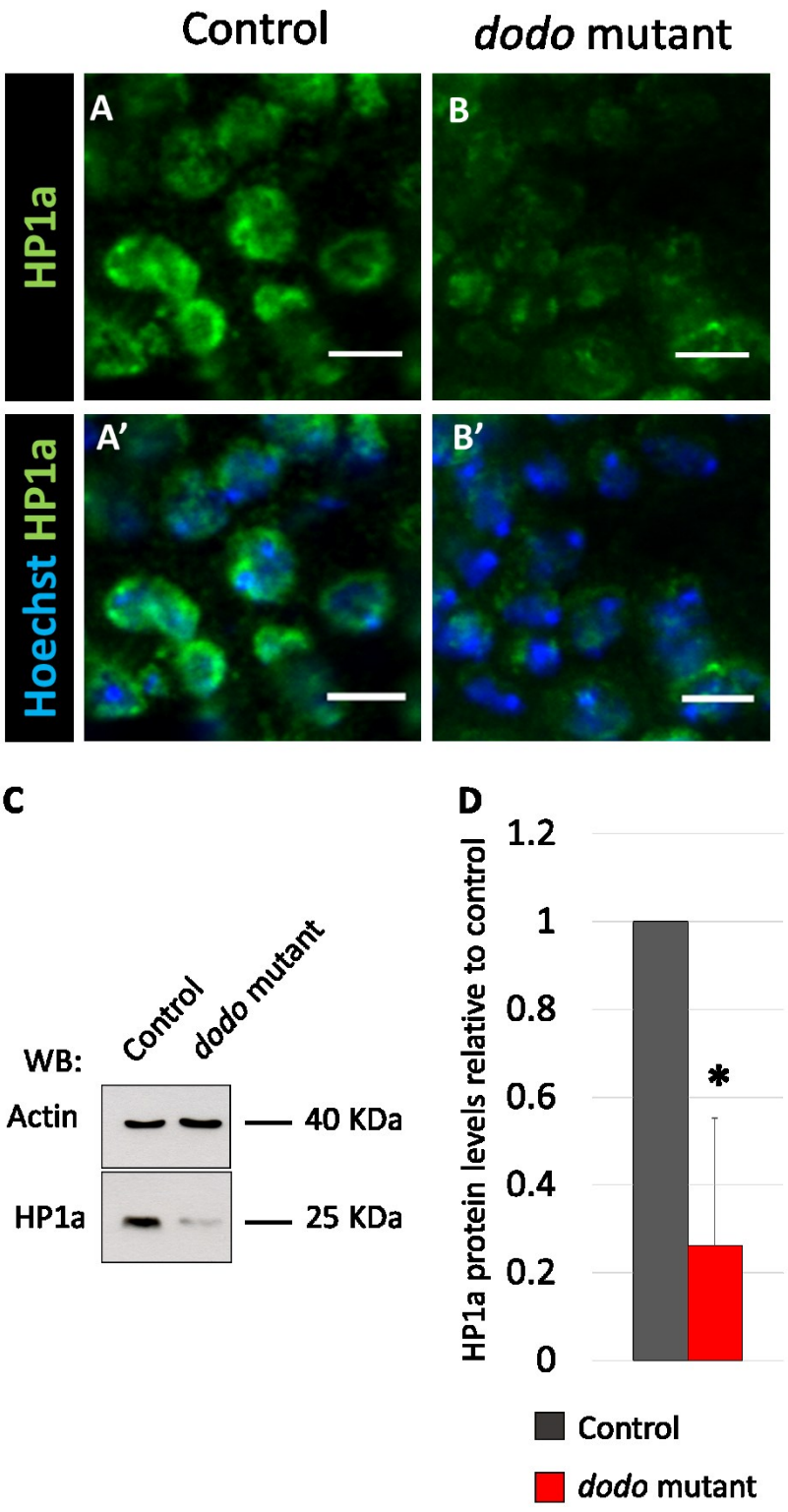



**Figure 18. HP1a protein levels and heterochromatin foci are reduced in the brain of *dodo* mutants flies. A-B'**) Single confocal section immunofluorescence images of HP1a in the brain of $w^{1118}$ (control, **A**) and $dod^{EY03779}$ (dodo mutant, **C**). Nuclei are stained with Hoechst (in blue in **A', B'**). Scale bar 4µm. **C**) Western blot analysis of HP1a protein from brains of $w^{1118}$ (control) and $dod^{EY03779}$ (*dodo* mutant) young adult flies (4days old). Actin was used as loading control. The image is representative of n=3 biological replicates. **D**) Quantification of Western blot analysis (**C**) of HP1a protein in $dod^{EY03779}$ (*dodo* mutant) relative to $w^{1118}$ (control) *Drosophila* brains. Actin was used as reference for quantification. Values represent mean ± s.e. of n=3 biological replicates. *P value < 0.05 by two-tailed unpaired Student's t-test.

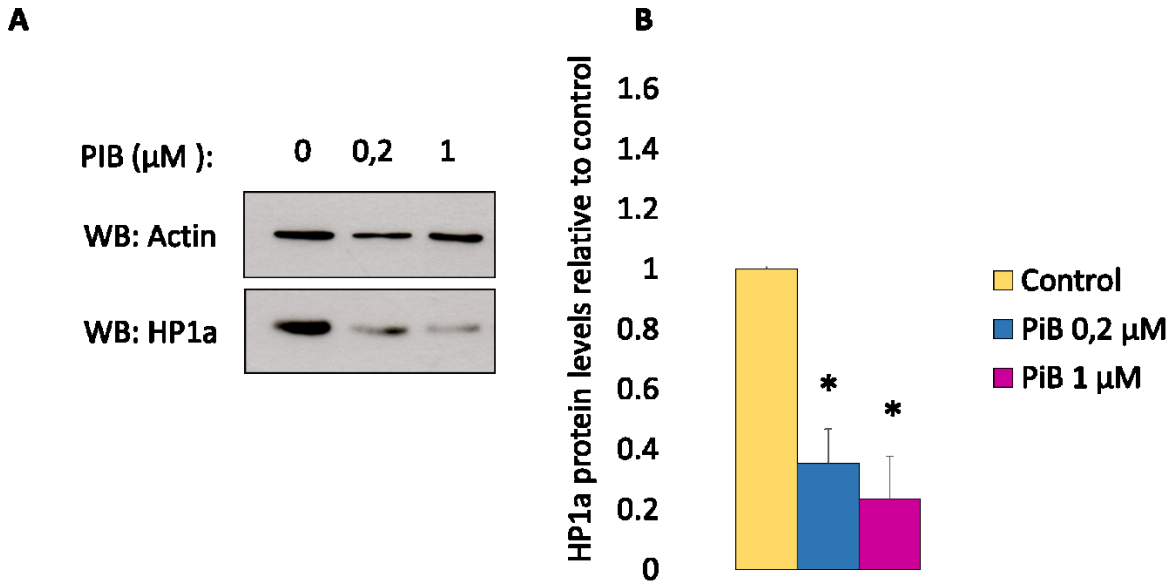



**Figure 19. Dodo catalytic activity is required to maintain proper levels of HP1a protein. A**) Western blot analysis of HP1a protein from lysates of ovaries of $w^{1118}$ young flies (4 days old, n=5 individuals per group) fed with the indicated doses of PiB. DMSO was used as mock treatment (control). Actin was used as loading control. The image is representative of n=3 biological replicates. **B**) Quantification of Western blot analysis (**A**) of HP1a protein in the indicated conditions. Actin was used as reference for quantification. Values represent mean ± s.e. of n=3 biological replicates. *P value < 0.05 by two-tailed unpaired Student's t-test.

## Dodo promotes the formation of a protein complex containing HP1a and Lamin Dm0 at the nuclear periphery in brain neurons

Pin1 appears to be broadly associated with chromatin in mammalian cells and to widely impact on chromatin state and transcription (see Introduction). In flies, we observed that Dodo protein is localised at the intraperinuclear periphery, a compartment associated to transcriptionally repressed heterochromatin in flies and mammals (Pickersgill et al. 2006; Reddy et al. 2008; Van Steensel and Belmont 2017). In mammalian cells, it has been reported that heterochromatin is targeted to the nuclear periphery by tethering of HP1a to Lamin B via the Lamin B receptor (LBR) (Padeken and Heun 2014; Ye et al. 1997). These evidences, together with the observed effect of Dodo on HP1a, prompted us to verify if Dodo may physically associate with HP1a and with the nuclear lamina. Co-immunoprecipitation (co-IP) experiments performed in the adult fly head demonstrated that Dodo formed complexes with both Lamin Dm0 and HP1a proteins (**Figure 20A**). To confirm these findings *in vivo* we then performed *in situ* Proximity Ligation Assay (PLA) of these proteins in *Drosophila* adult brain neurons. The PLA technique detects epitopes that are within 40 nm far from each other, thus indicating *bona fide* direct protein-protein interactions and their subcellular localisation. As

shown in **Figure 20B, C**, we observed positive PLA signals of Dodo with both HP1a and Lamin Dm0 at the edges of perinuclear heterochromatin *foci*. We then asked whether Dodo might regulate the formation of a complex between HP1a and Lamin Dm0 proteins. By co-immunoprecipitation assays we observed a strong reduction of the amount of Lamin Dm0 in complex with HP1a in *dodo* mutant fly heads as compared to isogenic wild-type controls (**Figure 21**), suggesting that Dodo positively regulates the interaction between these two proteins.

Taken together, the above results provide hints into the molecular mechanism whereby Dodo acts to maintain heterochromatin at the nuclear periphery.

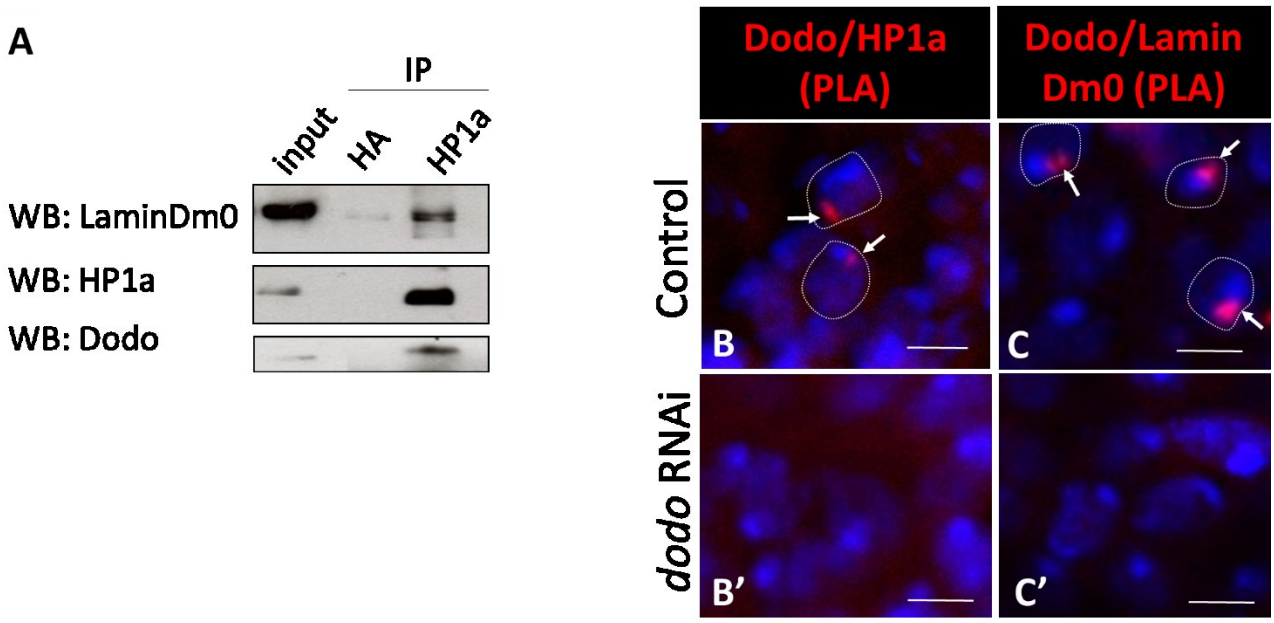



**Figure 20. Dodo is found in complex with HP1a and with Lamin Dm0 in brain neurons. A**) Co-immunoprecipitation of HP1a, Lamin Dm0 and Dodo in $w^{1118}$ fly head. Anti-HP1a (C1A9) antibody was used for immunoprecipitation (IP) reaction in total protein lysates. Anti-HA antibody was used as negative control. Maximal projection of 8 confocal section images of PLA with anti-Dodo and anti-HP1a antibodies (**B, B'**) or anti-Dodo and anti-Lamin Dm0 antibodies (**C, C'**) performed on transversal cryosections of central brain. Nuclei are stained with Hoechst (in blue). Genotypes: control *FM7/+;UAS-dod/+; dodo* RNAi *elav-Gal4/+;UAS-dod*$^{KK108535}$*/+;*. *FM7* is a wild-type *X* chromosome expressing selectable markers.

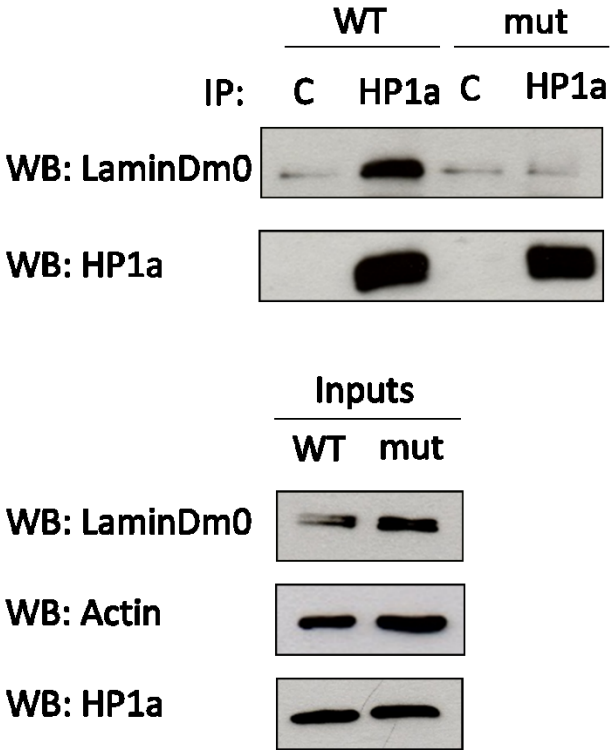



**Figure 21. Dodo regulates HP1a-LaminDm0 complex formation. A**) Co-immunoprecipitation of HP1a and Lamin Dm0 in $w^{1118}$ wild-type (WT) and $dod^{EY03779}$ (mut) *Drosophila* heads. Equal amounts of HP1a immunoprecipitated from total protein lysates with anti-HP1a (C1A9) antibody were used for SDS-PAGE and the amount of co-immunoprecipitated Lamin Dm0 protein was then analysed by western blot. Anti-HA antibody was used as negative control for IP reactions. The image is representative of n=3 biological replicates.

## Dodo negatively regulates TE insertions in the *Drosophila* brain

Increased TE expression may result in *de novo* TE genomic insertions. Thus, we sought to investigate whether loss of Dodo leads to an increase of TE insertional events in the genome of adult brain neurons. In collaboration with Dr. V. Specchia, we took advantage of the Gal4/UAS system to drive the expression of an RNAi targeting the *dodo* mRNA with the promoter of the *elav* gene, which is expressed in all postmitotic neurons (using the *elav-Gal4* driver) (**Figure 22A**). This system allowed the analysis of the effects of neuron-specific silencing of *dodo*. Paired-end deep sequencing of brain genomic DNA was performed comparing flies expressing *dodo* RNAi with the *elav-Gal4* driver (*elav-Gal4;UAS-dod*$^{KK108535}$*;*) to their parental strains (either expressing *elav-Gal4* alone or harbouring the *UAS-dod*$^{KK108535}$ transgene). With this strategy, about 200 *de novo* TE insertions were found to occur upon silencing of *dodo* in adult brain neurons, suggesting that Dodo inhibits *de novo* TE insertions in the brain (**Figure 22B**). Mapping of new insertion sites highlighted that these occurred predominantly in intronic and intergenic regions, whereas a small percentage was located in exons (**Figure 22C**).

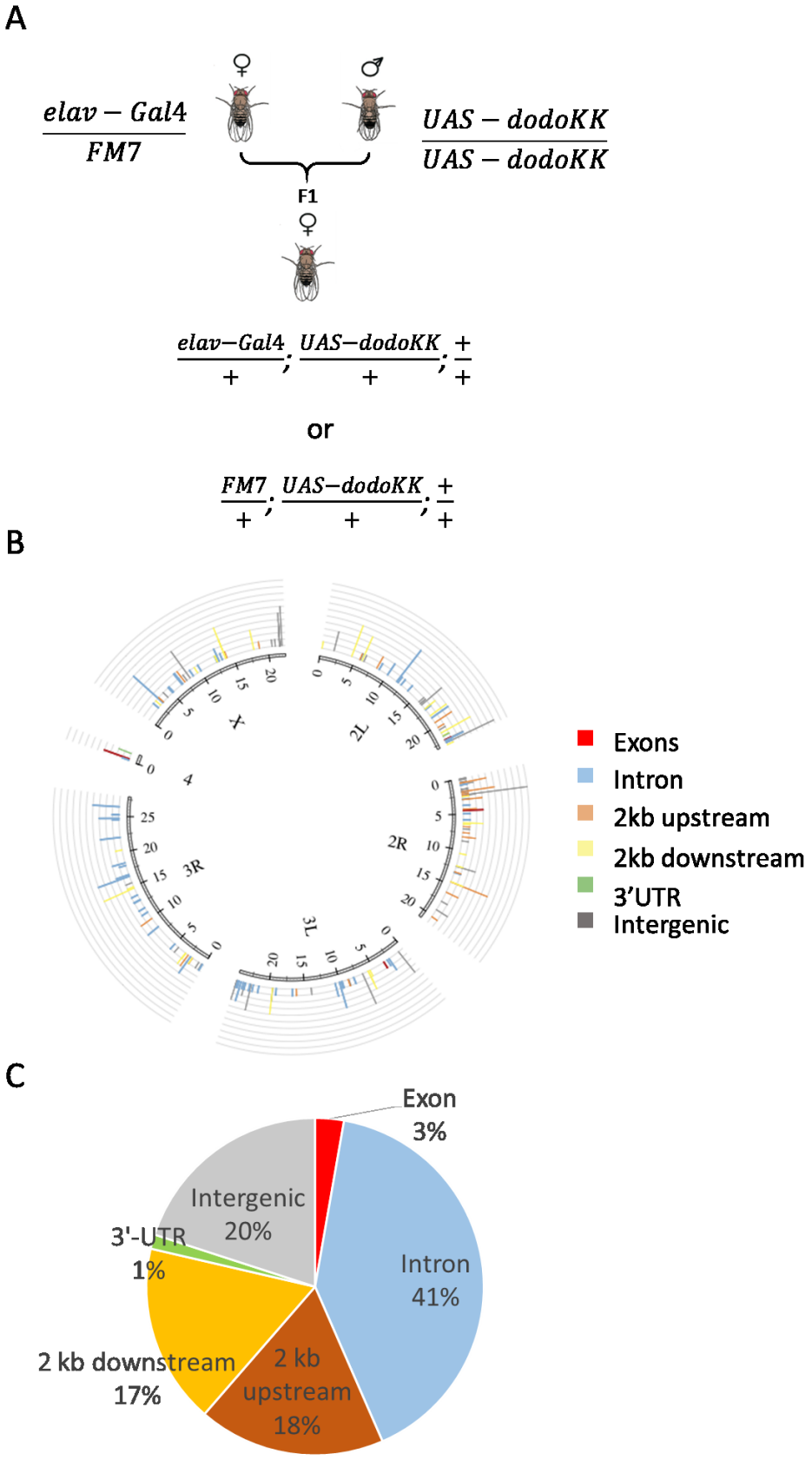



**Figure 22. Silencing of *dodo* increases TE insertions in the *Drosophila* brain A)** Mating scheme with the *elav-Gal4* driver line shows the genotypes of both parental strains and progeny that were used in DNA-seq analysis. **B)** Graphical representation of genomic distribution of 199 new TE insertions in the *Drosophila* brain

of *dodo* RNAi flies *(elav-Gal4/+;UAS- dod*$^{KK108535}$*/+;). FM7* is a wild-type *X* chromosome expressing selectable markers. The bars represent the frequency of insertional events at different positions of indicated chromosomes. The color code indicates genomic regions of insertional events, as summarized in the pie chart in **C**.

Interestingly, analysis of functional annotation of genes perturbed by new insertion sites in their intronic or exonic regions, according to Gene Ontology (http://www.geneontology.org/) highlighted that many of the genes potentially affected by *de novo* TE insertions were involved in neuronal physiology and homeostasis (**Figure 23**). This evidence raised the question whether TE integration may perturb the expression of these genes, affecting neuronal homeostasis. We are currently analysing the expression levels of candidate genes in the brain of *dodo* mutant flies. Inspection of the list of transposable elements found to be mobilized in the brain of *dodo-RNAi* expressing flies, highlighted that the majority belonged to LTR familily (**Figure 24**).

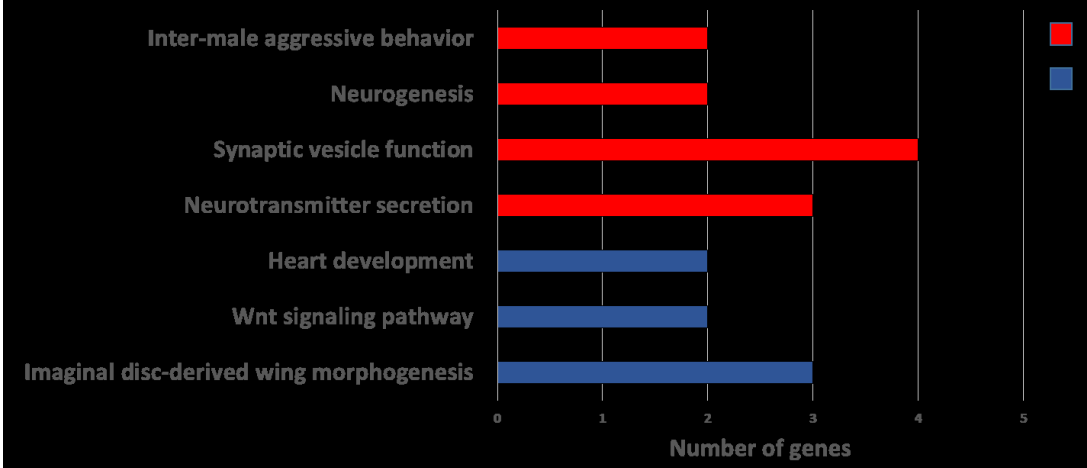



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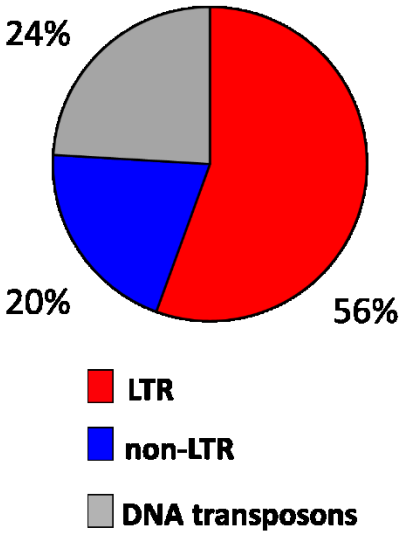
**Figure 23. Genes putatively perturbed by *de novo* TE insertions in the brain of *dodo* RNAi expressing flies**. **A)** Gene Ontology analysis of genes located proximal to *de novo* TE insertions in the brain of *dodo* RNAi expressing flies (*elav-Gal4/+;UAS-dod*$^{KK108535}$*/+);* The number of potentially perturbed genes is reported on the X axis and their functional classification is indicated on the Y axis. **B)** List of genes perturbed by *de novo* TE insertions that occurred in exons, introns or regulatory region.

**A**




**B**

| LTR RTE | non-LTR RTE | DNA-Transposons |
|---|---|---|
| 412 | BAGGINS | 1360 |
| ACCORD | DME278684 | AF541951 |
| AF222049 | DMIFACA | DDBARI1 |
| Beagle | DMW1DOC | DM33463 |
| Beagle2 | DMW1DOC,F | DMBARI1 |
| BLOOD | DV26847 | DMCR1A |
| DM_ROO | F | DMHFL1 |
| DM23420 | G6_DM | DMPOGOR11 |
| DMBLPP | IVK | DMTNFB |
| DMCOPIA | JUAN | DMTRDNA |
| DMDM11 | RT1B | PPI251 |
| DME487856 | | TC3 |
| DMGYPF1A | | TRANSIB2 |
| DMIS176 | | |
| DMIS297 | | |
| DMMDG3 | | |
| DMRTMGD1 | | |
| DMTN1731 | | |
| DMU89994 | | |
| GTWIN | | |
| INVADER2 | | |
| McCLINTOCK | | |
| OPUS | | |
| QUASIMODO | | |
| ROVER | | |
| SPRINGER | | |
| STALKER | | |
| STALKER2 | | |
| TABOR | | |
| Tinker | | |

**Figure 24.** Family distribution (**A**) and list (**B**) of transposons mobilized in the brain of *dodo-RNAi* expressing flies.

We also investigated the epigenetic signature of the genetic loci hit by *de novo* TE insertions, based on the available modENCODE ChIP-seq dataset of chromatin marks (http://www.modencode.org/). Interestingly, more than 70% of *de novo* TE insertions in the brain of *dodo* RNAi flies occurred in genomic regions with heterochromatin features. In particular, these regions were characterized by high levels of the heterochromatin protein HP1a (**Figure 25**).

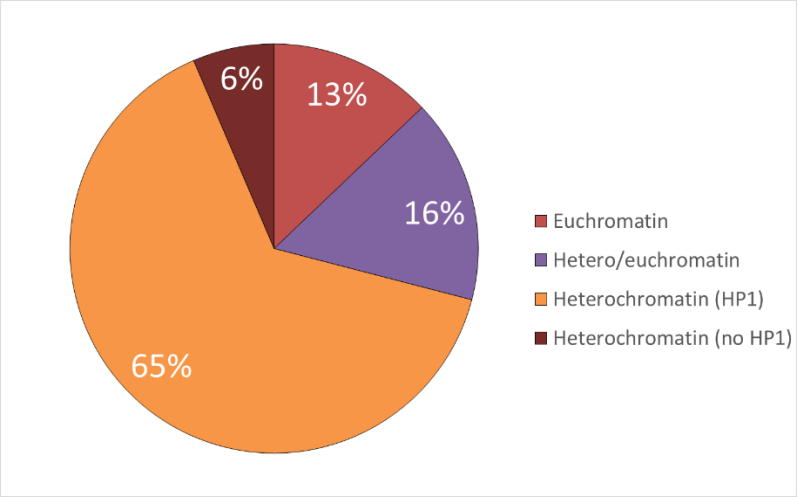



**Figure 25. Epigenetic characterization of genomic loci interrupted by loss-of-Dodo dependent *de novo* TE insertions in the fly brain**. Pie chart showing the percentage of *de novo* insertional genomic loci characterized by the chromatin signatures listed in the legend.

## Dodo and HP1 protect CNS neurons from TE toxicity

### Dodo-dependent inhibition of TE activity has a neuroprotective function in *Drosophila*

Excessive TE insertion rates have been associated to neurological diseases, including Schizophrenia, Rett syndrome and Parkinson's Disease (Reilly et al. 2013), as well as age-dependent neurodegeneration in flies (Krug et al. 2017; Li et al. 2013; Perrat et al. 2013). Moreover, Pin1 has been shown to protect mice against age-associated neurodegeneration. We thus sought to investigate if Pin1/Dodo neuroprotective function was conserved in *Drosophila melanogaster*, and whether this may involve its ability to restrain TE activity.

To this aim, we assessed the impact of Dodo loss on the viability of post-mitotic neurons in the Central Nervous System (CNS) of ageing flies, by monitoring the survival of the two main neuronal populations of the adult *Drosophila* CNS, the sensory photoreceptors in the retina and the central brain neurons. The *Drosophila* adult retina is composed of 800 units (ommatidia), each including 6 outer photoreceptor neurons (R1-6), which express the Rhodopsin-1 (Rh1) sensor at the adult stage. We expressed both the Green Fluorescent Protein (GFP) and *dodo RNAi* in the adult outer photoreceptors neurons (PRs) with the *rh1-Gal4* driver, and we monitored the survival of Dodo depleted PRs by fluorescence live imaging (Pichaud and Desplan 2001). An *RNAi* targeting the firefly *luciferase* gene, which does not downregulate any *Drosophila* endogenous protein, was expressed as control. As shown in **Figure 26C-E,** RNAi-mediated silencing of *dodo* led to premature and progressive degeneration of adult PRs, suggesting that Dodo was required to preserve neuronal

homeostasis during aging. To monitor the survival of adult central brain neurons we performed TUNEL assays upon RNAi-mediated silencing of *dodo* with the *elav-Gal4* driver. We observed increased TUNEL staining in Dodo-depleted brains (**Figure 26J**), suggesting that Dodo is required for the survival of adult central brain neurons.

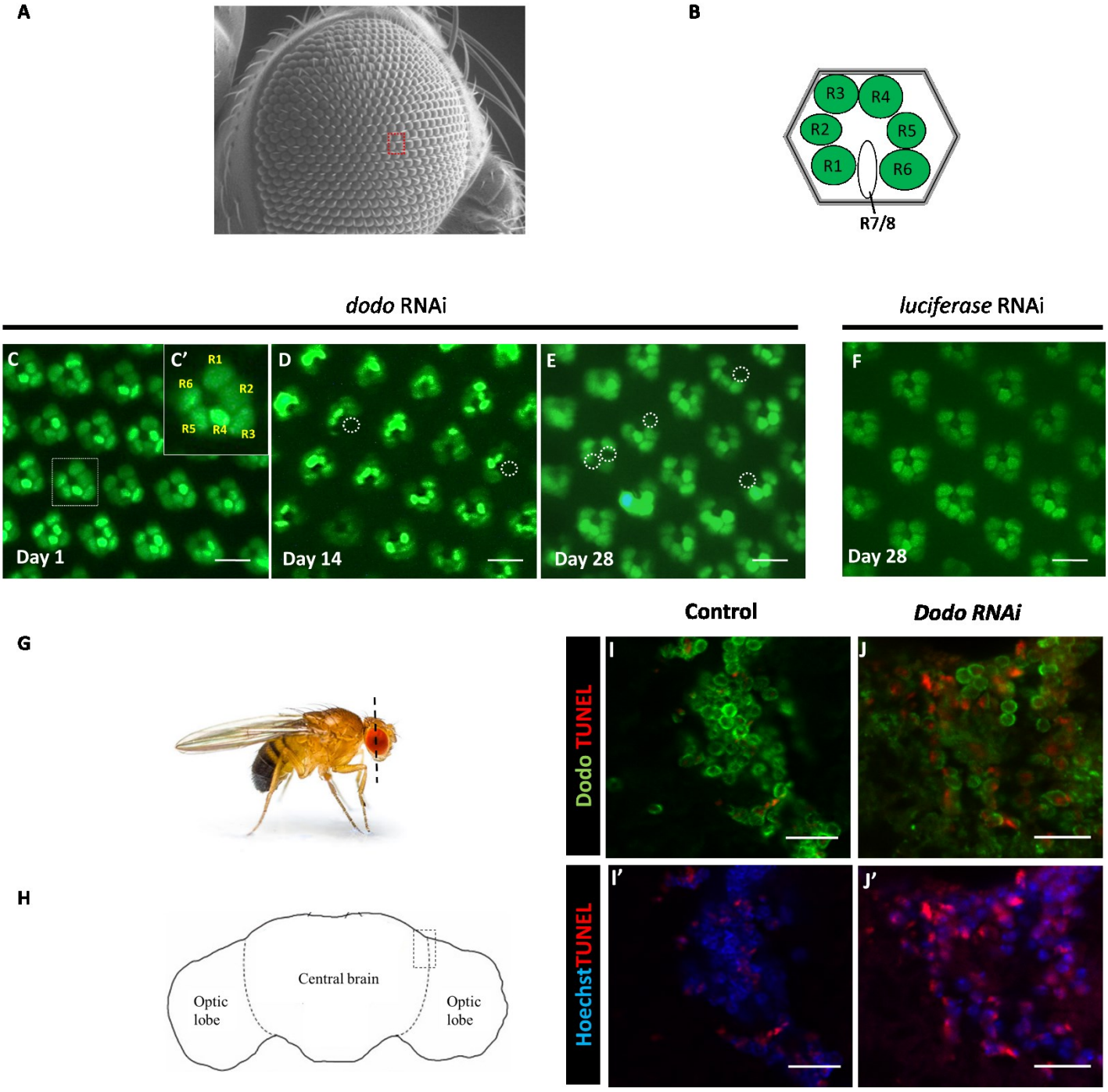



**Figure 26. Silencing of *dodo* led to neuronal death in the fly retina and brain. A)** Scanning electron micrograph of a *Drosophila* eye. **B**) Schematic representation of an ommatidium (dashed square in **A**). Outer (R1-R6, green) and inner (R7/8, white) photoreceptors are shown. **C-F**) Live imaging shows loss of GFP labelled outer photoreceptors (white circles) upon *dodo RNAi (rh1-Gal4;UAS-GFP/UAS-dodo$^{KK108535}$;)*

compared to control (*rh1-Gal4;UAS-GFP;UAS-luciferase*$^{RNAi}$), in 1 (Day 1), 14 (Day 14), 28 (Day 28), days old flies. Scale bar, 10 µm. **C'**) The inset shows a single ommatidium with the six outer photoreceptors expressing GFP (R1-6). The *rh1-Gal4* driver is expressed in adult outer photoreceptors. **G-H**) Transversal section of fly head **(G)** shows brain anatomy **(H). H)** Localisation of central brain neuronal nuclei shown in **I-J'** (black dashed square). **I-J')** Single confocal section immunofluorescence images of Dodo (green) and TUNEL staining (red) on transversal cryosection of central brain (of 35 days old flies). Nuclei are stained with Hoechst (in blue **I', J'**). Scale bar, 8 µm. Genotypes: control *FM7/+;UAS-dodo*$^{KK108535}$/+; *dodo* RNAi *elav-Gal4/+;UAS-dodo*$^{KK108535}$/+;. *FM7* is a wild-type *X* chromosome expressing selectable markers.

To assess whether *de novo* TE insertions contributed to the observed neurodegeneration, we monitored the death of Dodo-depleted PRs (by fluorescence live imaging) and central brain neurons (by TUNEL assays) in adult flies chronically treated with Lamivudine (3TC), a competitive inhibitor of the reverse transcriptase enzyme. This treatment prolonged the survival of Dodo-depleted PRs and central brain neurons (**Figure 27**), indicating that inhibition of TE insertions likely contributed to the neuroprotective function of Dodo.

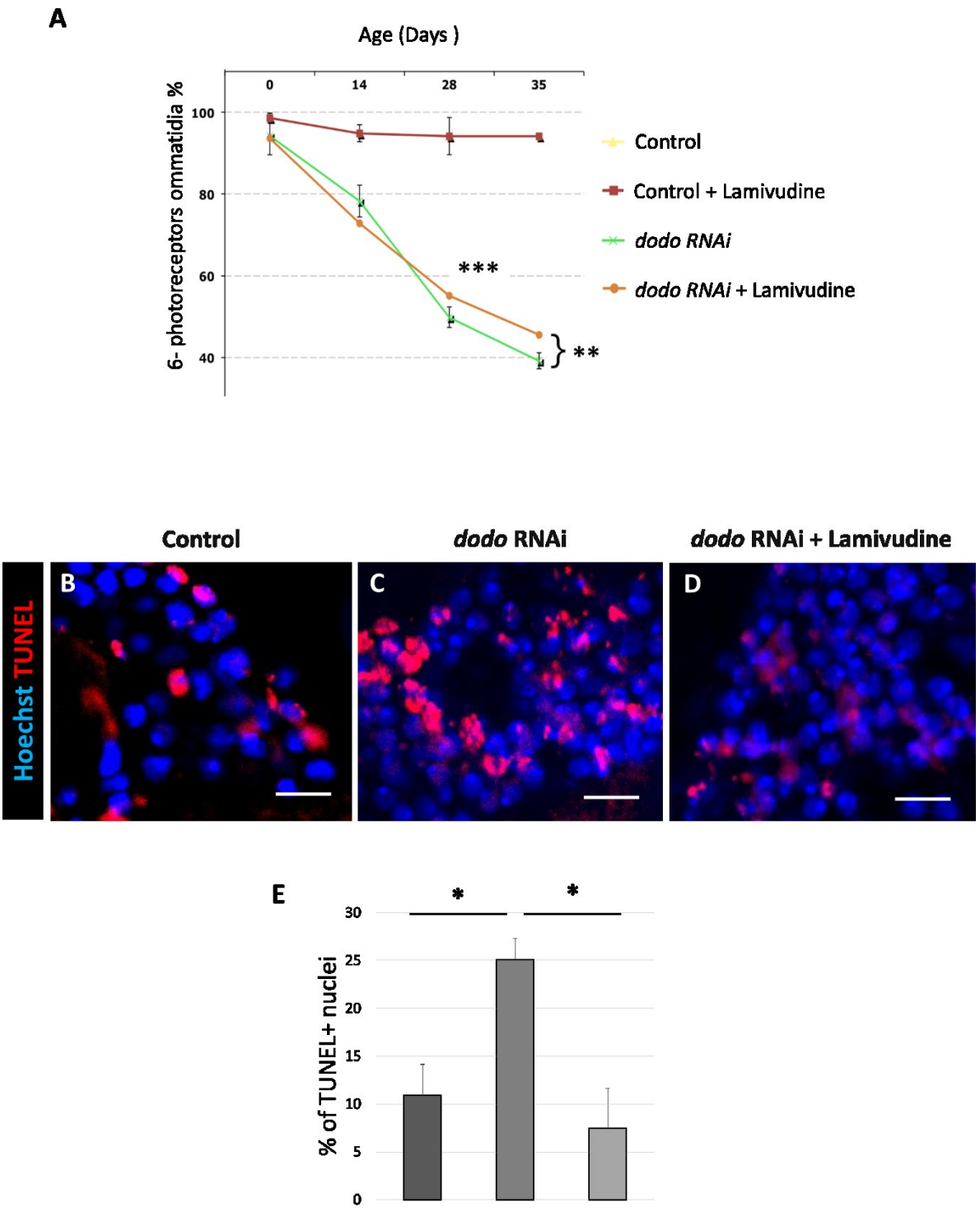



**Figure 27. *De novo* TE insertions contribute to the premature neurodegeneration caused by loss of Dodo.** **A)** Quantification of GFP-labeled photoreceptor neurons in the indicated conditions. Genotypes: *dodo RNAi*, *rh1-Gal4;UAS-GFP/UAS-dod*$^{KK108535}$; Control, *rh1-Gal4;UAS-GFP;UAS-luciferase*$^{RNAi}$. **B-D)** Single confocal section immunofluorescence images of TUNEL staining (red) on transversal cryosections of central brain of ageing flies (35 days old), in the indicated conditions. Nuclei are stained with Hoechst (in blue). Scale bar, 5 µm. **E**) Quantification of TUNEL positive signals in the brain of ageing flies in the same experimental conditions as **B-D**. Genotypes: control *FM7/+;UAS-dod*$^{KK108535}$*/+*; *dodo* RNAi *elav-Gal4/+;UAS-dod*$^{KK108535}$*/+;*. *FM7* is a wild-type *X* chromosome expressing selectable markers. Adult flies were chronically fed with 100 µM Lamivudine. *P<0.05, **P<0.01, ***<0.001 two-tailed unpaired t-test.

## HP1a neuroprotective function in *Drosophila* involves TE regulation

The above results prompted us to investigate if loss of HP1a may promote age-dependent neurodegeneration similar to Dodo. To this aim, we monitored the survival of photoreceptors in the retina of flies expressing both Green Fluorescent Protein (GFP) and *HP1a RNAi* in the adult PRs, by fluorescence live imaging. As observed for *dodo RNAi*-expressing PRs, photoreceptors expressing *HP1a RNAi* degenerated during ageing. To assess whether HP1a exerted its neuroprotective function through negative regulation of TE insertions, we monitored the survival of photoreceptors of *HP1a RNAi*-expressing flies upon treatment with Lamivudine. Similar to *dodo RNAi*-expressing fly PRs, the treatment with Lamivudine led to a partial but significant suppression of neurodegeneration (**Figure 28**). These results suggest that, similar to Dodo, HP1a may exert its neuroprotective function at least in part through inhibition of TE transposition.

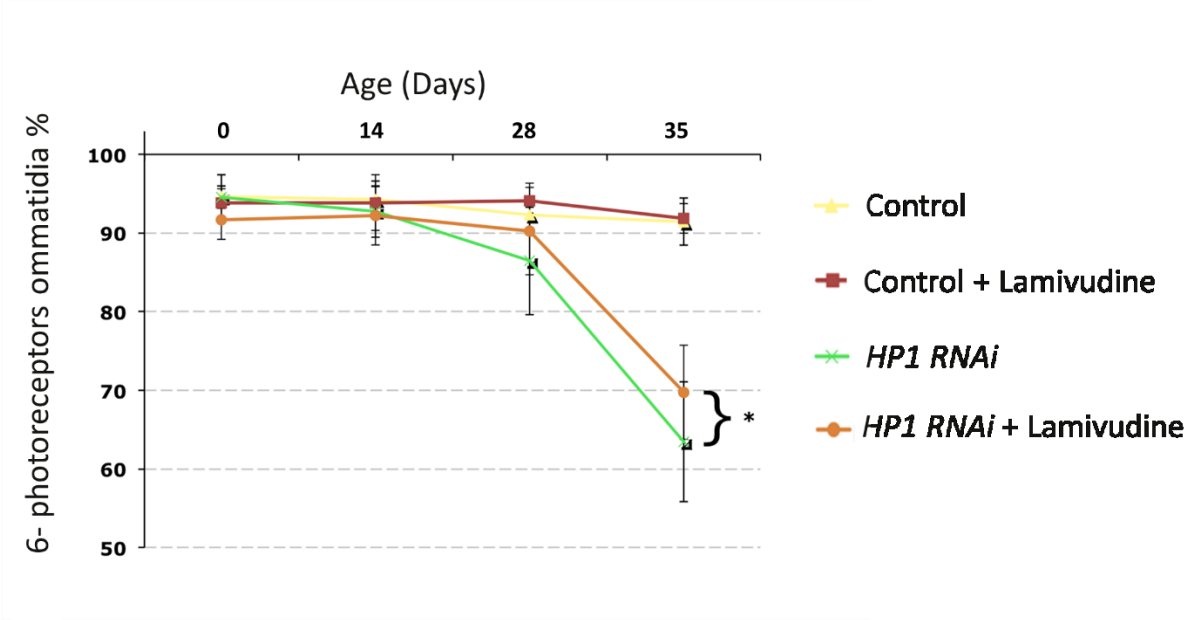



**Figure 28. *De novo* TE insertions contribute to the premature neurodegeneration caused by loss of HP1a.** Quantification of GFP labeled photoreceptor neurons in the indicated conditions. Genotypes: *HP1a RNAi*, *rh1-Gal4;UAS-GFP/ UAS-C1A9*$^{KK107477}$; control, *rh1-Gal4;UAS-GFP;UAS-luciferase*$^{RNAi}$. Adult flies were chronically fed with 100 µM Lamivudine. *P<0.01, two-tailed unpaired Student's t-test.

# Discussion

In this thesis, we have identified the Pin1 orthologue Dodo as a regulator of TE expression and mobility in *Drosophila melanogaster*.

We showed that depletion of Dodo licensed the expression of TEs belonging to different classes (LTR, non-LTR and DNA transposons) and that are normally repressed at the transcriptional level by the nuclear non-histone protein HP1a (Heterochromatin Protein 1a) in both germline and somatic tissues. Mechanistically, we observed that loss of Dodo leads to reduction of HP1a expression at the post-transcriptional level, with reduced formation of HP1a-containing heterochromatin foci and decreased recruitment of HP1a at TE regulatory sequences. Moreover, we observed that *dodo* mutant brains display increased *de novo* TE insertions especially in coding and regulatory sequences involved in neuronal function. Interestingly, we report that Dodo exerts a neuroprotective function in ageing flies and that TE mobilisation impairs maintenance of neuronal survival upon loss of Dodo.

## Dodo regulates HP1a and Lamin Dm0 at intraperinuclear heterochromatin domains.

Our chromatin immunoprecipitation (ChIP) experiments demonstrated that loss of Dodo was associated to a significant decrease of HP1a occupancy on the 5'UTR region of a representative retrotransposon. In further support of the idea that Dodo is required for HP1a-mediated chromatin silencing, we demonstrated that *dodo* mutant flies display strongly reduced appearance of HP1a-containing heterochromatin foci in both the ovary and brain. By performing co-immunoprecipitation (co-IP) and Proximity Ligation Assay (PLA) experiments in *Drosophila* brains, we demonstrated that Dodo interacts with both HP1a and the B-type Lamin Dm0, a component of the nuclear lamin complex involved in transcriptional repression of TEs (Chen et al. 2016).

Pin1 is a unique enzyme among prolyl-isomerases, in that its isomerase activity is highly specific for prolines following phosphorylated serine or threonine residues (pS/pT–P). Pin1 exhibited little isomerase activity also for substrates containing prolines following the acidic aminoacids aspartate or glutamate (D-P and E-P) (Yaffe et al. 1997). Lamin Dm0 bears several putative canonical Pin1/Dodo binding sites (S/T–P). Of note, interaction of Pin1 with phosphorylated human Lamin A/C has been reported to occur during infection of human fibroblasts with HCMV (Milbradt et al. 2010). In this context, Pin1 has been shown to promote phosphorylation-dependent Lamin A/C disassembly (Milbradt et al. 2016). However, the interaction of Dodo with *Drosophila* Lamin A/C proteins, as well as that of Pin1 with mammalian Lamin B proteins, remain unexplored and we are currently addressing these issues.

Inspection of HP1a primary sequence highlights no S/T–P sites, while it presents two E-P sites that may mediate binding to Dodo with low affinity. To identify the precise site(s) bound by Dodo, *HP1a* and *Lamin Dm0* point mutants at putative Dodo-binding sites should be tested for their ability to interact with Dodo by *in vitro* binding assays (GST-pulldown).
At present, it is unknown whether phosphorylation is indeed required for the binding of Dodo with either Lamin Dm0 or HP1a, and we are currently addressing this issue. In turn, identification of the kinases responsible for inducing the formation of these complexes may provide hints into the cellular pathways regulating TE repression. Alternatively, if phosphorylation of neither HP1a nor Lamin proteins will appear to be required for the interaction, it would be expected that the regulation of Dodo/HP1a/Lamin complexes may depend on the availability of active nuclear Dodo.

Our observations that Dodo interacts with both HP1a and Lamin Dm0, and that it is required for efficient complex formation between these proteins, support the idea that Dodo is recruited on lamina-associated heterochromatin domains and hereby contributes to chromatin silencing. To confirm that Dodo represses TE expression through heterochromatin-mediated transcriptional silencing, it will be however required to analyse its impact on the deposition of both active and repressive histone marks at genomic regions hosting mobile elements by ChIP experiments. Moreover, the observed decrease in HP1a occupancy at heterochromatic regions different from TEs suggests that the function of Dodo in maintaining heterochromatin may not be restricted to TE sequences. Genome-wide ChIP-sequencing surveys would reveal the impact of Dodo depletion on the global chromatin epigenetic landscape.
Interestingly, we observed that the formation of HP1a/Lamin Dm0 complex is stabilised by phosphorylation, as assayed by preliminary co-immunoprecipitation experiments upon treatment with lambda phosphatase (data not shown). In the future, it will therefore be interesting to assess whether Dodo catalytic activity is important for stabilisation of the HP1a/Lamin Dm0 complex.

Our results suggest that Dodo controls HP1a protein levels at the post-transcriptional level; moreover, experiments performed with the Pin1 inhibitor PiB suggest that Dodo may control HP1a protein levels through its isomerase activity. To confirm this result, generation of flies expressing *dodo* catalytically inactive mutant would be required. Interestingly, it has been reported that Pin1-dependent isomerisation regulates the stability of many key cellular proteins (Liou et al. 2011). We are currently investigating whether Dodo may regulate HP1a protein stability by inhibiting its degradation mediated by the ubiquitin-proteasome system or by alternative mechanisms.

### Consequences of heterochromatin relaxation and TE mobilisation upon Dodo loss

In addition to increasing TE expression, augmented chromatin relaxation may also impact TE transposition, which requires chromatin accessibility. We have indeed obtained evidence that loss of Dodo in the brain is associated with increased *de novo* TE insertions. New insertions mostly occurred at genomic regions characterized by HP1a occupancy. Hence, Dodo may suppress both TE expression and integration in the genome through heterochromatin maintenance. Whole-genome ChIP-sequencing analysis of specific chromatin marks in *dodo RNAi* brains compared to their parental genomes would be required to address this issue. Interestingly, our analysis of *de novo* somatic TE insertions in Dodo depleted brains highlighted that some of the targeted loci include coding/non-coding/regulatory sequences of genes involved in neuronal function. Thus Pin1-dependent, TE-driven alterations of gene expression may contribute to loss of neuronal homeostasis during ageing. We analysed the expression of some genes targeted by TE insertions in Dodo-depleted brains. Preliminary experiments highlighted that the mRNA levels of one of these genes, namely *dnc,* are reduced in Dodo depleted flies compared to the wild-type isogenic control. *dnc* encodes a cyclic nucleotide phosphodiesterase involved in several neuronal functions, such as memory and learning. Interestingly, the *dnc* gene has been found to be targeted by *de novo* TE insertions also in Ago2 mutant flies (Perrat et al. 2013). Clearly, to appreciate the overall contribution of Dodo-dependent TE mobility to transcriptional programs, global gene expression profiling will be required.

Augmented TE transposition rates have been associated to increased DNA damage (Belgnaoui et al. 2006). We have preliminarily observed that Dodo loss is associated to an increase of DNA damage in the germline, as judged by γ-H2AV staining (**Figure S. 5**) and we are currently investigating this issue also in the brain. Interestingly, DNA damage and specifically DNA Double Strand Breaks (DSBs) has been associated also with depletion of heterochromatin components known to regulate TE mobility, such as HP1a, Lamin Dm0 and histone H1 (Chen et al. 2016; Yuka W Iwasaki et al. 2016; White et al. 2012). Whether the protection from DNA damage is a primary function of Dodo and requires its interaction with HP1a and/or Lamins, will require further investigation.

In this view, it is also worth considering that DNA DSBs have been reported to promote LINE insertions. The presence of DSBs may allow TEs to integrate in an endonuclease-independent fashion; alternatively, enzymes involved in repair of damaged DNA may aid TE retrotransposition mechanisms (Farkash and Prak 2005). It has also been shown that Pin1 promotes the NHEJ DNA repair pathway (Steger et al. 2013). In light of the reported involvement of NHEJ in LINE retrotransposition (Suzuki et al. 2009), it is conceivable that Pin1/Dodo may facilitate TE integration also through this mechanism.

Coherently with our observation of increased TE activity and DNA damage in the germline of *dodo* mutant flies, our collaborator dr. V. Specchia observed decreased fertility in both male and female *dodo* mutant flies as compared to the wild-type counterpart. We have obtained preliminary evidence confirming this result. It will be interesting to address the role of *de novo* TE insertions in the infertility displayed by *dodo* mutant, and we plan to explore this issue by testing the effect of inhibiting TE transposition through administration of the reverse transcriptase inhibitor Lamivudine (3TC). It is tempting to speculate that inhibition of TE mobilisation in the germline and of the consequent DNA lesions, mutations and transcriptional effects may represent an unpredicted means of Pin1/Dodo-dependent protection of germ cells. In fact, reduced fertility of Pin1 knock-out mice has been associated with loss of progenitor germ cells (Atchison, Capel, and Means 2003). Proliferation and cell death assays in the germline of *dodo* mutant strains would be required to address this issue. It has been reported that mutations or RNAi-mediated knock-down of *HP1a* and *Su(var)3-9* cause loss of male germline stem cells (GSCs), accompanied by defects in cell division or survival (Xing and Li 2015; Zeng et al. 2013). These evidences suggest that Pin1 and HP1a may cooperate to promote germline stem cell survival.

Interestingly, infertility has been reported also in mutants of component of the piRNA pathway, such as Zucchini, Armitage, dFMR1, Aubergine and Ago3 (Bozzetti et al. 2011, 2015). The piRNA pathway is a genome surveillance mechanism regulating TEs, first discovered in the gonads of *Drosophila melanogaster*. It has been proved that the piRNA pathway restricts TE activity also in the brain, through Aubergine, Ago3 and dFMR1 (Bozzetti et al. 2015; Perrat et al. 2013). Intriguingly, several evidences suggests that Dodo may be also a component of the piRNA pathway. In fact, it has been reported that flies bearing homozygous deletion of the *dodo* locus display embryonic defects with fused or absent dorsal appendages (Hsu et al. 2001), as observed in *aub* mutant flies (Aravin et al. 2001). Moreover, in the germ cells of fly ovaries, we observed that Dodo protein localisation resembles that of both Aubergine and Ago3 protein at the nuage (Brennecke et al. 2007), a perinuclear, electron dense structure. Western Blot and immunofluorescence analysis of Aubergine and Ago3 in *dodo* mutant fly tissues should be performed to further investigate the interplay between Pin1/Dodo and components of the piRNA pathway.

In somatic tissues, the raise of genomic instability over time is considered as an important driver of ageing and age-related pathologies, and studies in *Drosophila* and mammalian organisms suggest that unscheduled activation of TEs contributes to DNA damage and genomic instability. Intriguingly, a recent study in *Drosophila* reported that loss of repressive heterochromatin integrity leads to the overexpression and mobilisation of TEs during ageing (Wood et al. 2016). Moreover, TE

overexpression has been found in the Central Nervous System of ageing organisms, such as in mice and flies, and has been associated to lifespan shortening at least in flies (Van Meter et al. 2014; Wood et al. 2016). Another study showed that age-related derepression of retrotransposons in *Drosophila* fat body correlates with decreased levels of Lamin Dm0 and increased DNA damage (Chen et al. 2016). Lamin mutations and/or defects in their expression or post-translational processing cause a heterogeneous group of diseases known as laminopathies (Camozzi et al. 2014). Interestingly, at least one of these diseases, namely Hutchinson-Gilford Progeria Syndrome (HGPS) is associated to alterations in chromatin status: HGPS cell culture models show decreased levels of heterochromatin marks, especially H3K9me3 and HP1 (Camozzi et al. 2014). It would be interesting to inspect TE expression levels and copy number in tissues of HGPS patients.

At present, very few scientific works have dissected the functions of the Pin1 fly orthologue Dodo, and the results presented in this thesis demonstrated for the first time that Dodo exerts a neuroprotective role during ageing that relies, at least partly, on inhibition of TE mobilisation. In addition, we provide evidence that also HP1a has a TE-dependent neuroprotective function. Heterochromatin relaxation, with alteration in HP1a and heterochromatin marks has been observed in the brain of patients affected by Alzheimer's Disease (AD) (Frost et al. 2014), where it has been proposed to be a consequence of oxidative stress and DNA damage (Frost et al. 2014) that represent early events in AD pathogenesis (Wang et al. 2006). Interestingly, it has been reported that oxidative stress can induce TE activation (Giorgi et al. 2011; Terasaki et al. 2013). In mammalian models, loss of Pin1 anticipates ageing phenotypes including AD type neurodegeneration (Lee et al. 2011). Moreover, the catalytic activity of Pin1 is either impaired by oxidation, or its expression reduced in AD patients' brains (Chen et al. 2015). It is tempting to speculate that, besides its well-described activities in regulating tau phosphorylation status and amyloid precursor protein processing, Pin1 might also protect against AD-related neurodegeneration by maintaining TE repression and genomic integrity in the brain. At present it is unknown whether TE activity is increased in AD neurons, similar to other neurodegenerative conditions. It would be certainly interesting to verify this possibility, and to investigate whether the function of Pin1 in TE repression is conserved also in mammalian organisms. Here we have shown that at least in *Drosophila*, the ability of Pin1 to restrain TE mobility contributes significantly to its neuroprotective activity. Future investigations on Pin1 function as repressor of TE expression and insertion in the nervous system of mammals may provide benefit for people affected by neurodegenerative disorders, such as Alzheimer's Disease and Progeria syndromes.

# Materials and methods

## 1. *DROSOPHILA* STABULATION

### 1.1 *Drosophila* maintenance

All *Drosophila* lines were maintained in plastic tubes on standard food prepared in deionized water, according to the following recipe: 9,2g/L *Drosophila* Agar, Type II,(cat#66-103); 83,3g/L brewers yeast (ACROS Organics 368080050); 83,3g/L commercial mais fluor; 4,8ml/L of propionic acid (SIGMA code#P5561) as antibacterial agents; 12,5ml/L of Tegosept, used as antifungal agent. The mixture were subjected to high-pressure saturated steam at 121 °C for around 20 minutes and aliquoted into clean tubes. Flies are then maintained at 25°C and transferred onto fresh food every 3-4 days. At these temperature flies have a generation time of 10 days.

### 1.2 Fly strains

The experiments present in this thesis were performed using the following fly stocks:

- $w^{1118}$, a reference strain derived from Canton-S wild-type *D. melanogaster*, that present mutation in the *white* gene that confers a typical white eye (this stock was provided from the laboratory of Dott.ssa V. Specchia, University of Salento);

- $dod^{EY03779}$ mutant (Bloomington Drosophila Stock Center, BDSC code#15677), generated in the *Drosophila* Gene Disruption Project (Bellen et al. 2004) by the insertion of a *P-element* in the 5'UTR of the *dodo* gene sequence;

- $Su(var)205^{04}$ mutant (Kyoto Stock Center, #101823), which produces a nuclear localisation defective HP1 protein;

- *UAS-*$dod^{KK108535}$ (Vienna Drosophila Resource Center, VRDC code#v110593), a line expressing an RNAi that targets the *dodo* transcript in a Gal4-dependent manner;

- *UAS-*$C1A9^{KK107477}$ (Vienna Drosophila Resource Center, VRDC code#v107477), a line expressing an RNAi that targets the *HP1* transcript in a Gal4-dependent manner;

- *UAS-*$luciferase^{RNAi}$ (Bloomington Drosophila Stock Center, BDSC code#35788), a line expressing an RNAi against the firefly *luciferase* gene in a Gal4-dependent manner;

- *UAS- UAS-HP1a*, a line constructed by inserting a *Drosophila HP1a* cDNA into the *Drosophila* transformation vector pUAST (generous gift from Willis X. Li. Xing and Li 2015);

- *c135* (Bloomington Drosophila Stock Center, BDSC code# 6978), a line used to drive the expression of UAS constructs in germ and somatic cells in the testis;

- *elav-Gal4,UAS-Syt-GFP/FM7* (Bloomington Drosophila Stock Center, BDSC code#6923), a line used to drive the expression of UAS constructs in all postmitotic neurons;

- *rh1-Gal4;UAS-GFP*, a line used to drive the expression of UAS constructs in adult outer photoreceptors.

**1.3** Drug administration

For experiments using Lamivudine (3TC), 3TC was added to the food at 100 μM final concentration. For experiments using PiB, either PiB dissolved in DMSO at the indicated concentration or DMSO alone (for controls) was added to the food. Flies eggs were hatched on food containing 0,2 μM, 1 μM PiB or DMSO. Adult females were fed with the drug (either 3TC or PiB) until they were sacrificed for the experiments. No more than 30 individuals per tube were exposed to the drugs.

**1.4** *Drosophila* crosses

Simplified scheme of crosses performed in this work are presented in Figure 21. In Figure 21A is present the mating scheme used to drive the expression of *dod* RNAi ($dod^{KK108535}$) in germ and somatic cells in the testis, by the *c135*-driver.

The RNAi contruct was expressed in neuronal cells using the promoter activity of the elav gene; the parental driver line expresses a synapotagmin (a marker of synaptic vesicles) tagged with GFP; in addition it segregates a FM7 X balancer chromosome, which does not recombine and expresses a selectable marker. The progeny of this cross is composed by flies that express the *dod-RNAi* construct (*elav-Gal4>dod RNAi*), which have a normal eye conformation, and siblings that do not express *dod-RNAi (FM7;UAS-dod-RNAi).*

In **Figure 29** is reported the mating scheme used to drive the expression of RNAi construct only in photoreceptors expressing *rhodopsin-1* gene (*rh1*) promoter. We used a *dod-RNAi* or a *luciferase-RNAi (lucIR)*. *luciferase-RNAi* has no specific target in *Drosophila*.

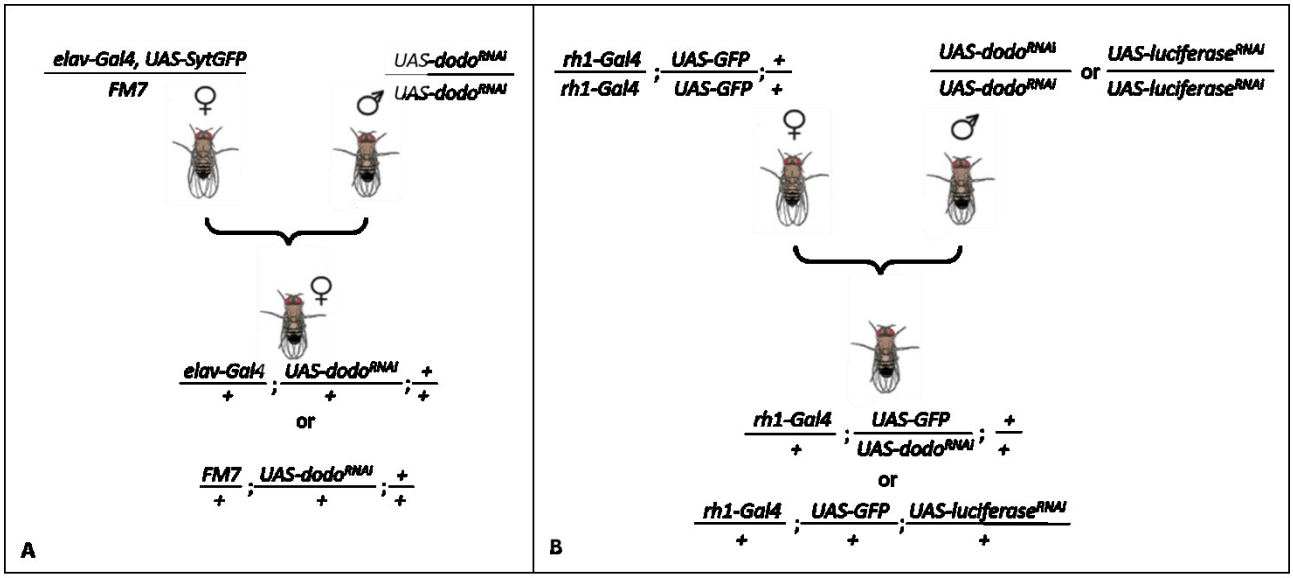



**Figure 29. Mating schemes and experimental design.**

## 2. MANIPULATION OF DROSOPHILA TISSUE

### **2.1** *Drosophila* ovary, testis and brain dissection

Female flies have been anesthetized with $CO_2$ and maintained asleep on ice. The dissection of indicated tissues from five female individuals, for protein recovering was made in Ringer modified solution supplemented with protease inhibitors (added freshly):

Ringer modified recipe: - KCl 183mM

- NaCl 77mM

- Tris-HCl pH=6,8 10mM

Protease inhibitors: - NaF 5mM

- PMSF 1mM

- CLAP 0.1Mm

- $Na_3VO_4$ 1mM

The collected tissue (n= 5 individuals per group) was then lysed in Laemmli Sample Buffer 2X, homogenized by sonication and boiled for 5 minutes at 95° for electrophoresis.

The dissection of adult brains (n=40) for RNA extraction was made in PBS 1x solution and the tissue is rapidly transferred in QIAZOL solution.

Both for protein and RNA extraction we used young flies (4 days old).

**2.2** Preparation for immunofluorescence staining on cryosection

Flies heads were cutted and then fixed in 4% paraformaldehyde in PBS 14-16h at 4°C, in agitation. After 3 washes of 15 minutes in PBS 1x, heads were equilibrated in 30% sucrose solution (in PBS) for 48-72 hours in agitation. Then, sucrose solution was removed and 3 washes of 15 minutes in PBS 1x were performed. At this point heads were ready for the inclusion in OCT embeddig medium and then snap freezing on isopentane (previusly solidificated upon liqid nitrogen). The embedded tissue was stored at -80°C. 10 μm slices of head tissue were placed on posivive-charged glass slides (Thermo Scientific). The slides were conserved at -80°C until immunostained.

## 3. IMMUNOFLUORESCENCE

**3.1** Immunofluorescence on frozen tissue

Slices of heads tissue air-dried at room temperature. Permeabilisation was performed with 3 washes of 10 minutes in PBS-Triton X-100 0,1% (PBST). Next, a blocking step of two hours at room temperature was made with 3% Foetal Bovin Serume (FBS) in PBST. Incubation with primary antibodies, at proper concentration, was made 14-16h at 4°C in a humid chamber. After 3 washes of 10 minutes in PBST we added secondary antibodies and performed a 1 hour incubation at room temperature in a humid chamber. Then 3 washes in PBST were performed and we stained the nuclei with Hochest (2μg/ml in PBS). After 3 washes in PBST and one in water, we mounted the slices with Prolong and added the coverslip. At least 4 individulas per group were analysed.

**3.2** TUNEL (terminal deoxynucleotide transferase dUTP nick end labeling) assay

TUNEL assay (In Situ Cell Death Detection Kit, TMR red, Roche) relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase (TdT), an enzyme that will catalyse the addition of dUTPs that are labelled with a marker. For TUNEL assay, after permeabilisation, it was required a permeabilisation step in 100mM sodium citrate in PBS-Triton X-100 0,3% at 65°C for 45 minutes. After 3 washes of 15 minutes in PBST at room temperature, accordingly to manufacture's instruction we performed the TUNEL reaction with a 1:10 enzyme:buffer ratio. The assay was performed at 37°C overnight in a dark humid chamber. At least 4 individuals per group were analysed.

**3.3** Proximity Ligation Assay (PLA) with Duo-link in situ kit

PLA assay was performed on slices of heads tissue. After a first permeabilisation with 0.3 % Triton X-100 in PBS, an additional permeabilisation in 100mM sodium citrate in PBS-Triton X-100 0,3% at 65°C for 45 minutes was required to better unmask the antigens. Then, the samples were rinsed in

0.1 % Triton X-100 in PBS (three changes, 10 min each) and incubated with the Blocking solution (0.1 % Triton X-100, 5% FBS in PBS) for 2 hours. The reaction with each primary antibody was carried out with coverslips, overnight at 4 °C in humid chamber. Then, the slices were rinsed with 0.1 % Triton X-100 in PBS. Next, PLUS and MINUS secondary PLA probes (Olink Bioscience), both rabbit and mouse immunoglobulins diluted in Blocking solution, were added for 1 h at 37 °C. The sliced were then washed twice with Wash A for five minutes each, and incubated in Ligation solution (DUO92008 – SigmaAldrich) for 30 min at 37˚C. Following two washes with Wash A for two minutes each, the slices were incubated in Amplification solution (DUO92008 – Sigma-Aldrich) for 100 min at 37˚C. The slices were then washed twice with Wash B for ten minutes each, followed by a single wash with 0.01x Wash B for one minute. The stained slices were stored in Duolink In Situ Mounting Medium (DUO82040 – Sigma-Aldrich) at -4˚C until ready for imaging. At least 4 individuals per group were analysed.

**3.4** Whole-mount immunofluorescence

Dissected tissues were collected in Schneider's Drosophila Medium and then fixed in 4% paraformaldehyde in PBS for 15 minutes in agitation. Permeabilisation was performed with 3 washes of 15 minutes in PBS-Triton X-100 0,1% (PBST). Next, a blocking step of one hour at room temperature was performed with 3% Foetal Bovin Serume (FBS) in PBST. Incubation with primary antibodies, at proper concentration, was performed overnight at 4°C in a humid chamber. After 3 washes of 15 minutes in PBST at room temperature, we added secondary antibodies and performed a 1 hour incubation at room temperature in a humid chamber. Then 4 washes in PBST were performed and we stained the nuclei with Hochest (2µg/ml in PBS). After 3 washes in PBST and one in water, we mounted with Prolong and added the coverslip. At least 3 individuals per group were analysed.

## 4. GFP REPORTER ASSAY IN FLY RETINA

Flies expressing specific *RNAi* constructs under the *rh1* promoter (crosses are shown in paragraph 1.3 of Material and Methods) were ice-anesthetized and placed in a 35mm cell culture dish half filled with 1,5% agarose at about 50°C, that was rapidly gelled on ice. Flies were then covered with cold water and eyes were properly oriented at stereomicroscope. The presence of a GFP reporter allowed the visualisation of outer phoreceptors using an upright fluorescence microscope Leica DM4000B and a 40X water immersion lens (Leica). For the time course study of neurodegeneration we monitored at least 6 individuals 1, 14, 28 and 35 days old. Where indicated, adult flies were fed with 100 µM Lamivudine (3TC).

## 5. DNAseq: INSERTIONS DETECTION AND ANNOTATION

DNA-Seq library were prepared following the Nextera DNA Library Preparation Kit protocol on 30 brains of adult individuals per condition. A total of 3000 genomes were analysed per condition. Raw Illumina reads were generated on HiSeq2000 platform following manufacturer's instruction. Base calling was performed with the CASAVA software, version 1.8.2. Raw reads were processed to remove contamination of adapter sequences and low quality bases using cutadapt (https://pypi.python.org/pypi/cutadapt/) and erne-filter (Del Fabbro et al., 2014), respectively. Illumina reads where first aligned on the curated canonical set of transposable elements of Drosophila melanogaster (available at http://www.fruitfly.org/p_disrupt/TE.html) using BWA-mem algorithm, version 0.7.10 (Li et al., 2010). A custom python script was developed to parse BAM alignments and retrieve the reads mapping on the borders of such elements and having some unaligned overhang. These reads were clipped by their TE part, while kept paired with the mapped or unmapped mates. Manipulated paired reads, collected as described above, were mapped on the reference genome of Drosophila melanogaster (release 5) using BWA-mem algorithm and candidate insertion breakpoint were catalogued on the basis of alignments layout. As an example, a split read mapping as forward on a 5'-TE border leaving 5'-overhang would generate a split alignment on reference genome either by aligning in forward orientation with some 3'-overhang or as reverse with some 5'-overhang. Non-candidate pairs were used in this second stage of alignment to reduce ambiguous mapping along the genome due to short reads after TE-clipping routine. Candidate breakpoints position were clustered by a maximum distance of 10bp. The detection procedure was applied on the two parental lines and the F1 progeny. F1 coordinates were filtered by any spanning coordinate detected in one of two parts or any repeat element already annotated on the reference genome (NCBI annotation; genome-build FlyBase r5.41) as "repeatmasker_dummy" or "transposable_element"; exclusion coordinates were extended by 10bp. Annotation of the insertion point was obtained with SnpEff software (http://snpeff.sourceforge.net/).

## 6. RNA MANIPULATION TECHNIQUES

### **6.1** Total RNA extraction

For total RNA extraction, the Qiagen lysis reagent was used: it is composed by phenol and guanidine isothiocyanate and allows the extraction of RNA, DNA and proteins. After chloroform addition there is the separation in three phases: taken the supernatant (upper phase), isopropanol addition allows RNA precipitation. Removed the supernatant, the RNA pellets were washed with ethanol 75%, air-dried and suspended in water. RNA was quantified at Nanodrop.

**6.2** RNA retrotranscription

The retrotranscription was performed using the QuantiTect Reverse Transcription kit (Qiagen). This kit allows the retrotranscription of extracted RNA through two sequential steps, a first step of genomic DNA removal and a second one of retrotranscription with a mix containing oligodT and random primers. cDNA obtained were then diluted 1:50 in water in order to proceed with RT-qPCR.

## 7. QUANTITATIVE PCR

RT-qPCR was performed using the *SsoAdvanced SYBR Green Supermix* (BIORAD) reagent. The RT-qPCR program is composed of a first step of denaturation (30 seconds at 95°C) and then 40 cycles of denaturation (95°C for 5 seconds), annealing and extension (60°C for 30 seconds) and dissociation. The instrument used is the BIORAD CFX96 Touch™ Real-Time PCR Detection System termocycler. The quantification is based on the $2^{-\Delta\Delta Ct}$ method using the housekeeping gene *elav* as normaliser. Primers sequences (FW= forward, RV= reverse) are listed in Table 2.

| **GENE TARGET** | **PRIMER SEQUENCE (5'→3')** |
|---|---|
| *elav* | Fw: ATGTTCTAAACGGCCTGCGA |
| | Rev: CAGCCCCGACACATAAAGGT |
| *dodo* | Fw: GAAAGTTCGGCAGAGGTCAG |
| | Rev: CTGGGCATTCCGTTTTATTC |
| *HP1* | Fw: CGCAAGGATGAGGAGAAGTCA |
| | Rev: TCCTGAAACGGGAATGGTGTC |
| *ZAM* | Fw: TCGTCGCCGCAGGAAACTCTC |
| | Rev: GTGGAGCGACGATTGGAAGAA |
| *QUASIMODO* | Fw: TCTACAGTGCCATCGAGAGG |
| | Rev: TAGTTCAGCCCAAGTGTTGC |
| *Mdg-1* | Fw: CAAAACTCCAACTCCCAATC |
| | Rev: AGTGGTCCTCGCAGTCGTT |
| *THARE* | Fw: ATCCAGGCCAAGGATATGAC |
| | Rw: TCTGATGATGACTCGGAAGC |
| *IVK* | Fw: ACTCTGGGTTCCCAGTCATC |
| | Rev: GGTCCTTGGAGTTAAACGGA |
| *R1* | Fw: TGGCGAAACTTGATGTAGGA |
| | RevGCGGCAAACACTCTCCTTCT |
| *LaminDm0* | Fw: ACTGGAGAGAGCATGTTGCC |
| | Rev: CGCCGCGAATACAAGAAGTG |
| *actin* | Fw: GCGTCGGTCAATTCAATCTT |
| | Rev: AAGCTGCAACCTCTTCGTCA |

## 8. IMMUNOPRECIPITATION

Co-immunoprecipitation of HP1a, Dodo and Lamin Dm0 was performed in 20 mM Tris-HCl pH 8, 150 mM NaCl, 0,2% NP-40, 0,2% Triton X-100, 5mM EDTA, with protease inhibitor cocktail

(Sigma), 1 mM PMSF, 5 mM NaF, 1 mM Na3VO4, using lysate from 80 *Drosophila* heads and 3 µg of anti-HP1a monoclonal antibody or anti-HA antibody as control.

## 9. CHROMATIN IMMUNOPRECIPITATION ASSAY

Ovaries (n=100 individuals) were dissected from 4-days-old females in 1X PBS and stored in 1.5 ml tube on ice during isolation (up to 2 h). PBS solution was removed after centrifugation (1000 rpm, 1 min). 50 ovaries were used for one IP reaction. Intact ovaries were crosslinked with 2% formaldehyde for 20 min. and washed in PBS. The tissue were lysed weakly in lysis buffer (SDS 0,1%, EDTA 2mM, Tris pH 8.1 20mM), with protease inhibitor cocktail (Sigma), 1mM PMSF and phosphatase inhibitors (NaF 5mM and Na3VO4 1mM), for 20 min on ice. Chromatin was sonicated with Branson Ultrasonics Sonifier™ S-450 to 500-1000 bp average fragment size and cleared by centrifugation. IP was performed overnight at 4°C with the indicated antibodies in IP buffer (SDS 0,08%, EDTA 1,6mM, Tris pH 8.1 16mM, NaCl 150mM, Triton X-100 1%). A negative control was performed in the presence of isotype-specific unrelated Ab. DNA– protein complexes were recovered by protein A/G PLUS-Agarose (Santa Cruz Biotech.) and washed sequentially with IP buffer, IP-250 buffer (SDS 0,08%, EDTA 1,6mM, Tris pH 8.1 16mM, NaCl 250mM, Triton X-100 1%) and LiCl solution (10 mM Tris-HCl pH 8, 1mM EDTA, 250 mM LiCl, 0.5% Na-Deoxycholate, 0.5% NP40), then resuspended in TE, digested with 2U Dnase-free Rnase (Calbiochem) for 30 min. at 37°C, and incubated 6h at 68°C with 300 mg/ml Proteinase K (Invitrogen) in 0.5% SDS, 100 mM NaCl to digest proteins and reverse crosslinks. After purification by phenol-chloroform extraction and ethanol precipitation, DNA was resuspended in water and 1/20 volume was used for quantification. DNA–protein complexes were recovered with protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Real-time PCR was performed on an StepOne Plus cycler (Applied Biosystems), using SYBR Green Universal PCR Master Mix (Applied Biosystems). Promoter occupancy was calculated as the percentage of input chromatin immunoprecipitated using the $2^{-\Delta\Delta Ct}$ method. Primers used are listed below.

| **TARGET** | **PRIMER SEQUENCE (5'→3')** |
|---|---|
| *H23heterochromatin* | Fw: CCAAGTTGGCCAGTTTTGAT |
| | Rev: AGTTCAAGCCCGGGTATTCT |
| *Nanos* | Fw: CTTTCGACCCGGATTTTCGC |
| | Rev: TTCCAGACTGAGCCAACGAG |
| *ZAM* | Fw: ATGTAGTGTACCTGCGTGGCAT |
| | Rev: TGTGATGTAGTACCGGGCCTTTA |

## 10. WESTERN BLOT

### 10.1 SDS-PAGE (SDS-polyacrylamide gel electrophoresis)

For the electrophoretic separation of protein samples in SDS-PAGE the running gel was composed by 15% acrylamide, 0,1% bisacrylamide, 0,374M Tris-HCl pH 8,7, 0,1% SDS. The stacking gel composition was: 5% acrylamide, 0,14% bisacrylamide, 0,125M Tris-HCl pH 6,9, 0,1% SDS. The electrophoretic run was performed applying a constant potential difference of 80V while the samples were in the stacking gel, increasing it to 180V for the running gel run.

### 10.2 Electrotransferring of proteins from gel to nitrocellulose membrane

For the transfer of proteins on nitrocellulose membrane the Wet transfer cell (BioRad) was used: the blotting buffer is composed by 0,2 M Tris, 0,2M Glycine. The protein transfer is achieved applying a constant potential difference of 100V for 1 hour and 30 minutes.

### 10.3 Western Blot

After incubation of nitrocellulose membrane for 30 minutes in blotto tween (5% milk, 0,2% Tween20 in PBS), the membranes were incubated with the primary antibodies diluted in blotto tween for 1 hour or overnight. After three washes, the membranes were incubated with the secondary antibodies (conjugated with HRPO) that target the constant region of the species of the corresponding primary antibody. After 30 minutes, the membranes were washed three times with blotto tween and twice with PBS. For the western blot development, *ECL plus* (Pierce) or *ECL* (Amersham) were used.

## 11. ANTIBODIES

The primary antibodies antibodies used for Western blot and immunofluorescence are:

- anti-Pin1 (homemade by Del Sal laboratory, 1:500 for WB, 1:100 for IF);
- anti-Vasa (DSHB, 1:100 for IF)
- anti-HP1 C1A9 (DSHB, 1:1000 for WB and IF) or W11 (gift from Sarah Elgin 1:100 for IF);
- anti-Elav 9F8A9 or 7E8A10 (DSHB, 1:100 for IF);
- anti-LamDm0 ADL6710 (DSHB, 1:1000 for WB, 1:500 for IF);
- anti-actin (Sigma, A2066, 1:1000 for WB);
- anti γH2Av UNC93-5.2.1 (DSHB 1:100 for IF)

The secondary antibodies used for Western blot and immunofluorescence are:

- anti-rabbit HRP conjugated (1:2000);

- anti-mouse HRP conjugated (1:2000);

The secondary antibodies used for immunofluorescence are:

- anti-rabbit Alexa Fluor conjugated (1:500);
- anti-rat Alexa Fluor conjugated (1:500);
- anti-mouse Alexa Fluor conjugated (1:500).

# Supplementary Information

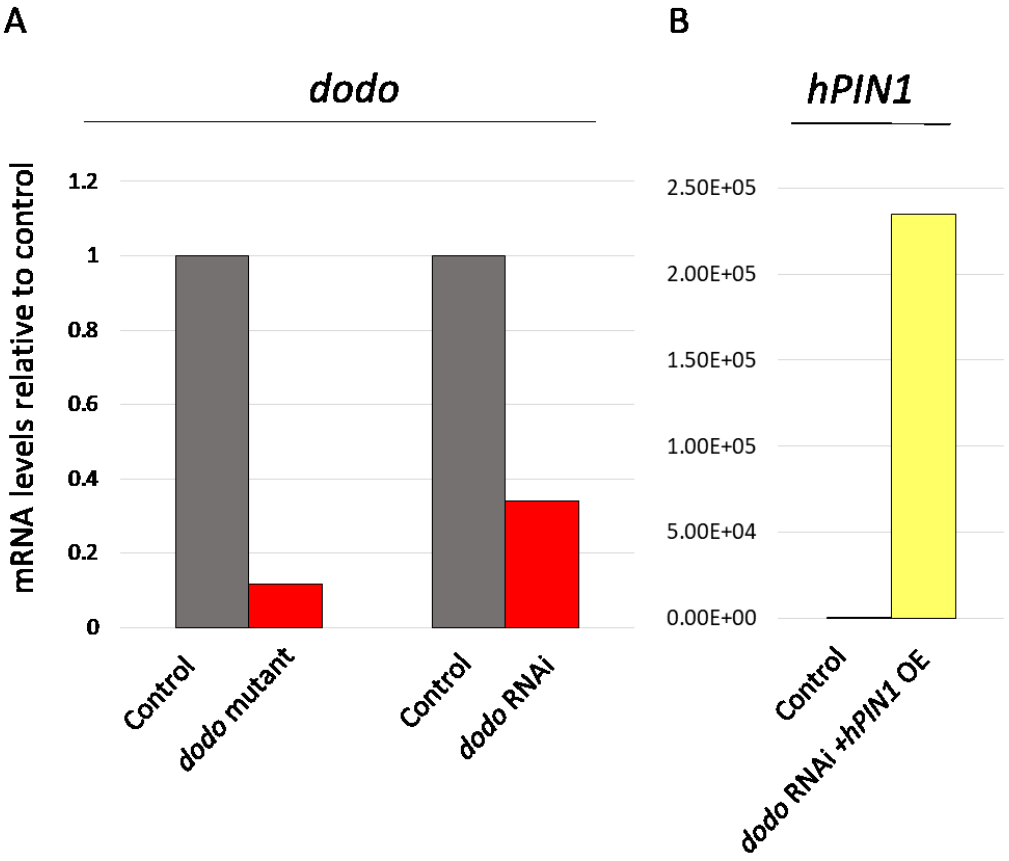



**Figure S. 1 mRNA levels of endogenous *dodo* or overexpressed *hPin1* in the testis of indicated flies.** RT-qPCR analysis of *dodo* **(A)** and *hPIN1* **(B)** expression in testes of the indicated flies. Genotypes: control, $w^{1118}$;*UAS-dod*$^{KK108535}$/+;;, *dodo* mutant *dod*$^{EY03779}$, *dodo* RNAi $w^{1118}$;*UAS-dod*$^{KK108535}$/+;*c135-Gal4*/+, *dodo* RNAi + *hPIN1* overexpression (OE) $w^{1118}$;*UAS-dod*$^{KK108535}$/+;*c135-Gal4/UAS-Pin1*. *c135-Gal4* drives expression in germ and somatic cells in the testis. *rp49* was used as reference for quantification.

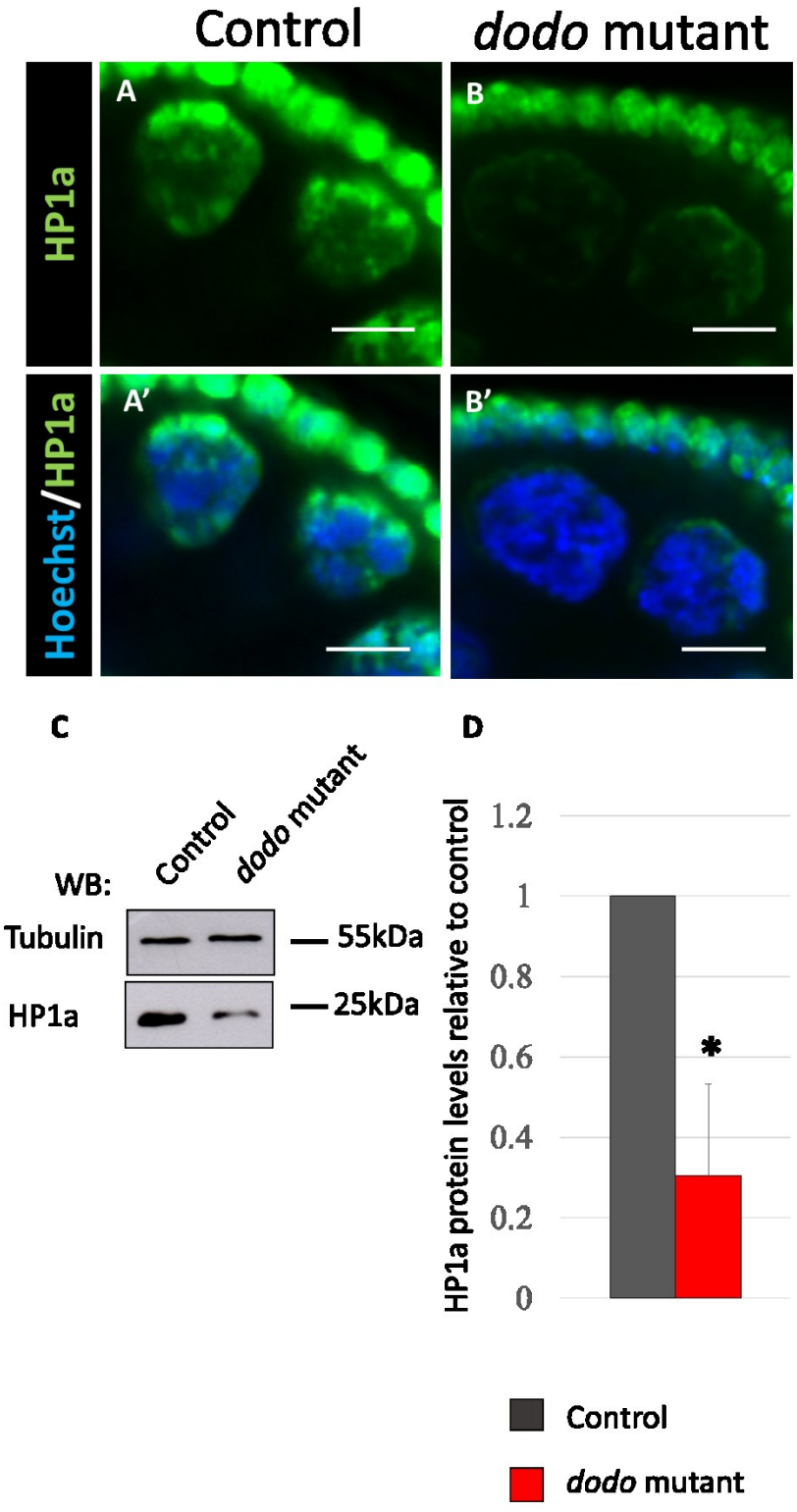



**Figure S. 2 *dodo* mutant flies exhibit decreased heterochromatic foci and HP1a total protein levels in the ovary. A-B'**) Single confocal section immunofluorescence images of HP1a in the brain of $w^{1118}$ (control, **A**) and $dod^{EY03779}$ (*dodo* mutant, **B**). Nuclei are stained with Hoechst (in blue **A', B'**). Scale bar, 5µm. **C**) Western blot analysis of HP1a protein from ovaries of $w^{1118}$ (control) and $dod^{EY03779}$ (*dodo* mutant) adult flies (4 days old). Actin was used as loading control. The image is representative of n=3 biological replicates. **D**) Quantification of Western blot analysis (**C**) of HP1a protein in $dod^{EY03779}$ (*dodo* mutant) relative to $w^{1118}$ (control) *Drosophila* ovaries. Actin was used as reference for quantification. Values represent mean ± s.e. of n=3 biological replicates. *P value < 0.05 by two-tailed unpaired Student's t-test.

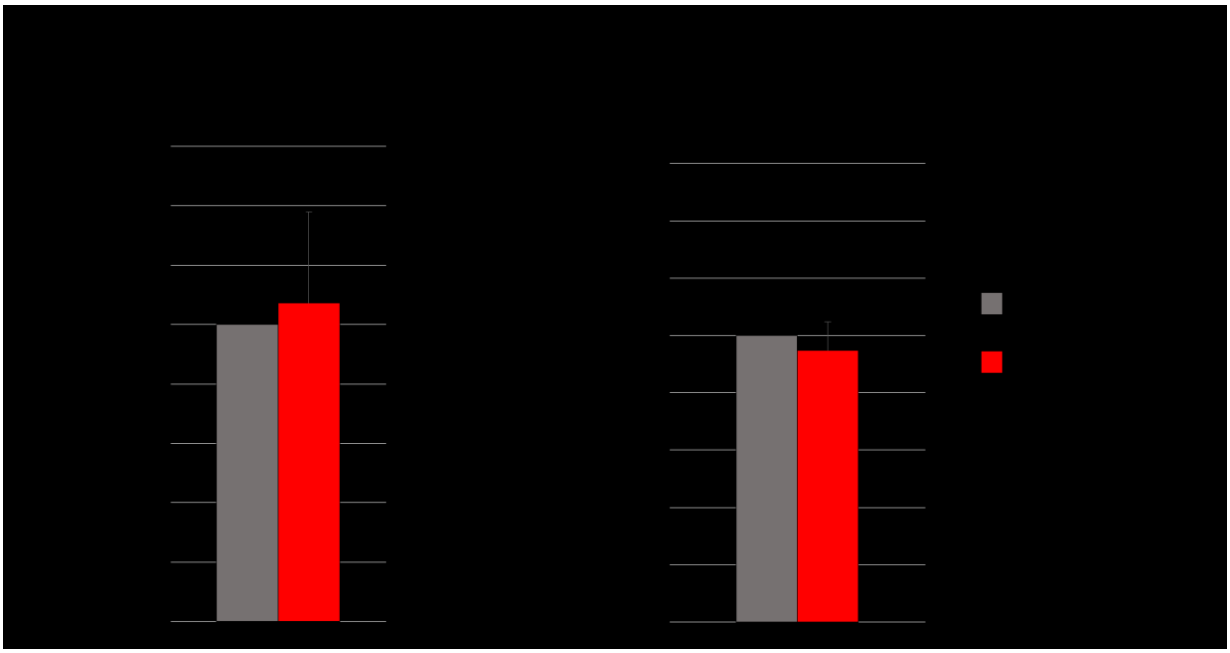


**Figure S. 3. HP1a transcript levels do not change in *dodo* mutant flies.** RT-qPCR analysis of the expression of HP1a in the ovary (**A**) and in the brain of *dodo* mutant ($dod^{EY03779}$) flies relative to control flies ($w^{1118}$); *actin* was used as reference for quantification. Values represent mean ± s.e. of n=3 biological replicates, calculated with the ΔΔCt method.


**Figure S. 4. H3K9me3 protein levels are reduceed in *dodo* mutant flies. A-B)** Western blot analysis of H3K9me3 protein from ovaries (**A**) and brains (**B**) of $w^{1118}$ (control) and $dod^{EY03779}$ (*dodo* mutant) young adult flies (4 days old). Actin or Histone H3 were used as loading controls.

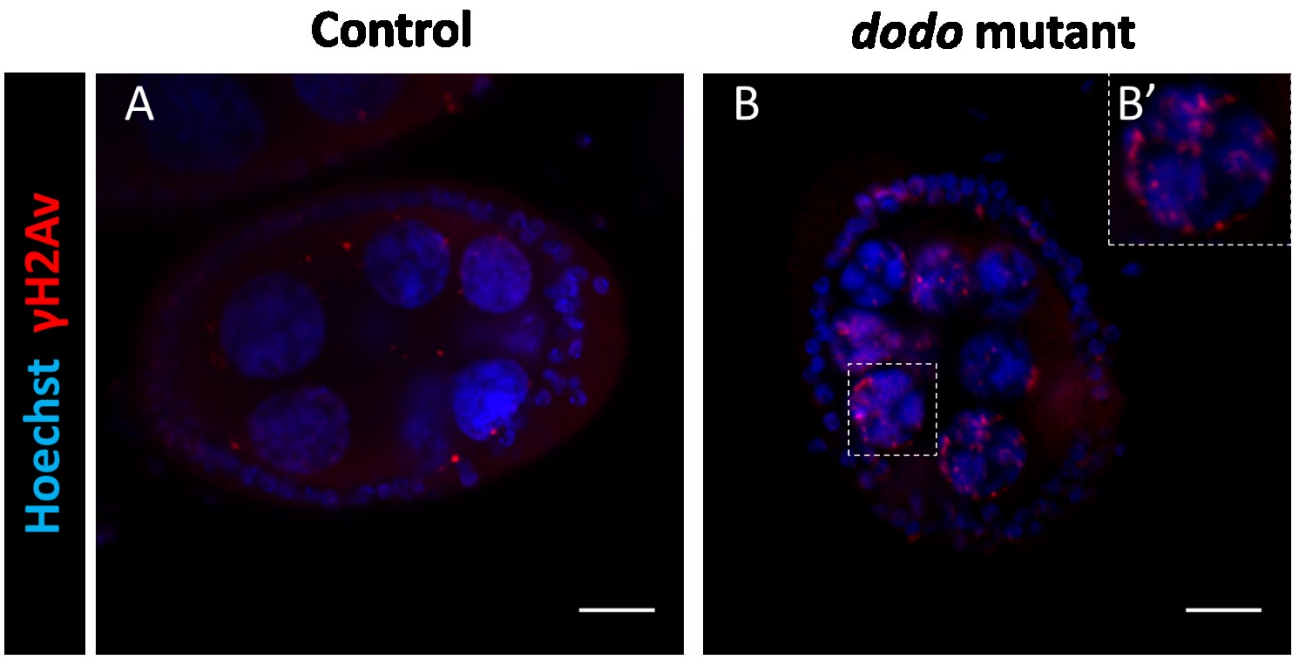



**Figure S. 5. *dodo* mutant flies exhibit increased levels of γ-H2Av foci in the germline. A-B**) Single confocal section immunofluorescence images of γ-H2Av (red) in the ovary of $w^{1118}$ (control, **A**) and $dod^{EY03779}$ (dodo mutant, **B**) flies. Inset **(B')** shows a single nurse cell. Nuclei are stained with Hoechst (in blue). Scale bar, 10μm.

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