JOINT PHD PROGRAMME

IN MOLECULAR BIOLOGY

PhD Thesis

The prolyl-isomerase Pin1 maintains heterochromatin by preserving nuclear envelope structure in brain tissue.

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ACADEMIC YEAR 2018/2019

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ABSTRACT

The organization of chromatin is tightly regulated, being crucial for the control of gene expression programs, both during organism development and in tissue homeostasis. In particular, perinuclear heterochromatin has an evolutionarily conserved function in restraining the activity of mobile genetic elements, also known as transposable elements (TEs), which are potentially mutagenic. Aging and several aging-related diseases have been proposed to involve a deregulation of chromatin organisation. A widespread relaxation of heterochromatin, leading to unscheduled TE expression and de novo genomic insertion, associated with accumulation of DNA damage, have recently emerged as key contributors of aging and aging-dependent pathologies, including several neurological disorders, such as Alzheimer's disease (AD). However, it is unclear how heterochromatin is challenged and its organisation is altered during aging and the pathogenesis of those disorders, including AD. Understanding the underlying molecular mechanisms could lead to the identification of potential biomarkers and therapeutic targets for prevention and treatment of some currently incurable diseases, such as AD, which are expected to become epidemic in the coming decades.

In this Thesis, we demonstrate that PIN1, a unique enzyme that isomerises phosphorylated-S/T-P aminoacid motifs, and whose activity promotes healthy aging and protects against AD, has a fundamental function, conserved from Drosophila to humans, in maintaining the formation of heterochromatin, thus preventing toxic hyperactivity of TEs. We further provide evidence that, in *Drosophila*, the PIN1 ortholog Dodo preserves the mechanical properties and the compartmentalization of the nuclear envelope, thus protecting Heterochromatin protein 1a (HP1a), a major regulator of heterochromatin, from proteasome-dependent degradation. Mechanistically, in both *Drosophila* and human cells, PIN1 promotes the anchoring of HP1a to the nuclear lamina, in line with data previously obtained in our laboratory suggesting that Dodo interacted with both HP1a and B-type Lamin proteins.

Our findings identify a mechanism by which PIN1 safeguards brain neurons from mechanical stress, which has been proposed as an important contributor to AD pathogenesis.

1 Introduction

1.1 Alzheimer's Disease

1.1.1 Hallmarks of Alzheimer's Disease

Alzheimer disease (AD) is a progressive neurodegenerative pathology associated with cognitive decline and is the most common form of dementia in the elderly. Approximately 13% of people over the age of 65 and 45% over the age of 85 are estimated to have late-onset AD (LOAD) (Alzheimer's Association. 2012). Early-onset familial AD (EOAD), which typically develops before the age of 65 years accounts for only a small portion (<1%) of AD cases (Hardy & Selkoe, 2002; Blennow et al., 2006).

Late-onset sporadic AD, or the so-called sporadic form of AD, is not associated with any known mutation. However, the presence of one or two alleles e_4 of the apolipoprotein E (APOE), a cholesterol transporter (Corder, et al., 1993;Bertram et al., 2007; Avramopoulos 2009; Huang & Mucke, 2012), as opposed to APOE e_2 or APOE e_3 increases the disease risk to develop sporadic AD by several fold (Corder et al., 1993). Neuroinflammation, head trauma, and diabetes have been also implicated as risk factors for sporadic AD.

Early-onset familiar AD (FAD) can result from autosomal dominant mutations in the genes encoding for the Amyloid Precursor Protein (APP), for Presenilin 1 (PSEN1) or Presenilin 2 (PSEN2) (O'Brien and Wong, 2011; Thies and Bleiler, 2013). Most mutations in the APP and presenilin genes increase amyloid β (Aβ) protein production, which is the main component of senile plaques, leading gradually to profound neuronal and glial alteration, synaptic loss and dementia (Selkoe 1999). Furthermore, FAD can result from APP locus duplication (Delabar et al., 1987; Rovelet-Lecrux et al., 2006). In addition, triplication of chromosome 21, which carries the APP gene, in Down syndrome (DS) leads to dementia due to Alzheimer's disease (Holland and Reinmuth, 1998). Approximately 40–80% of persons with DS develop AD-like dementia by age 40 (Holland and Reinmuth, 1998), a much younger age than is typically seen in sporadic AD (Zigman etal., 2004; Oliver and Holland,1986; Holland et al., 1998). The onset of dementia symptoms in DS parallels the development of classic brain neuropathological lesions *(i.e.,* amyloid plaques) similar to that evident in AD.

Both late- and early-onset AD are characterized neuropathologically by the accumulation of amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs), in addition to widespread synaptic loss, inflammation and oxidative damage, and neuronal death (Burns & Iliffe, 2009; Meng-Hui Dai et al., 2018).

1.1.1.1 Amyloid precursor protein in AD

Amyloid plaques are primarily composed of Amyloid-β peptides generated by differential proteolytic cleavage of the transmembrane Amyloid Precursor Protein (APP) (Selkoe 1999; Chow et al., 2010).

APP is a single-pass transmembrane protein expressed at high levels in the brain and cleaved by a series of sequential proteases. Non-amyloidogenic processing of APP involves α-secretase followed by intramembrane γ-secretase complex, consisting of Presenilin 1/2, Nicastrin, APH-1 and PEN-2 (Chow et al., 2010), while amyloidogenic processing of APP involves β- secretase (BACE) followed by γ-secretase complex (Fig. 1).

APP contains eight potential serine (Ser), threonine (Thr) and Tyrosine (Tyr) phosphorylation sites within its cytoplasmic domain (Lee et al., 2003). Seven of these were recently shown to be phosphorylated in AD brains, i.e., Tyr-653, Ser-655, Thr-668, Ser-675, Tyr-682, Thr-686, and Tyr-687 (APP695 isoform numbering) (Lee et al., 2003). A number of protein kinases and phosphatases have been shown to regulate APP phosphorylation, including cyclin-dependent protein kinase 5 (cdk5) (Iijima et al., 2000), p34cdc2 protein kinase (cdc2) (Suzuki et al., 1994), glycogen synthase kinase 3 (GSK-3) (Aplin et al., 1996), and c-jun N-terminal kinases (Standen et al., 2001 ; Taru et al., 2002 ; Taru and Suzuki, 2004; Kimberly et al., 2005) and protein phosphatase (PP) 2A (Sontag et al., 2007). In particular, Thr-668 phosphorylation has been reported to play a role in APP metabolism by facilitating the BACE cleavage of APP (Lee et al., 2003).

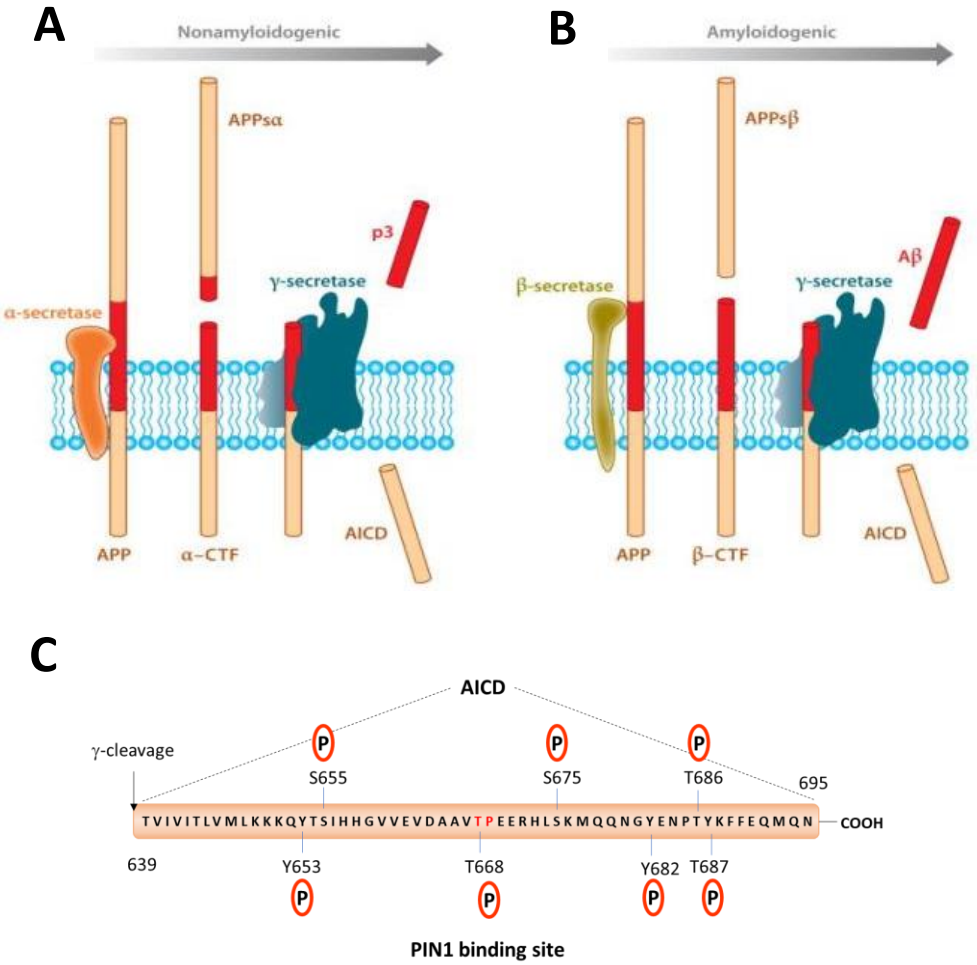


Figure 1 Cleavage of the amyloid precursor protein (APP). A) Nonamyloidogenic APP processing, B) amyloidogenic APP Processing. (O'Brien and Wong, 2011) C) Sequence of the APP intracellular domain (AICD) including important phosphorylation sites; the VTPEER site is involved in AD pathophysiology and includes the Thr668 residue, which is recognized by PIN1. The amino acid numbers are referring to the APP695 isoform (Ferrari Bravo G.).

1.1.1.2 Microtubule binding protein TAU in AD

NFTs are composed of hyperphosphorylated forms of the microtubule binding protein TAU (MAPT) and are found intracellularly in affected neurons.

TAU promotes the assembly of tubulin into microtubules (MTs) and MT structure stabilization (Melková et al., 2019). Structurally, TAU is subdivided into four regions: an N-terminal acidic projection region, a proline rich domain (PRD), a microtubule-binding domains (MTBD) characterized by repeat region and a basic C-terminal domain (CTD) (Mietelska-Porowska et al., 2014) (Fig. 2).

TAU contains 80 Ser and Thr residues and 5 Tyr residues that can be potentially phosphorylated (Chen et al., 2004), of these only 45 have been proved experimentally to be phosphorylated (Hanger et al., 2009). TAU phosphorylation sites are mostly concentrated in the flanking regions of the microtubule-binding repeats (PRD and CTD) (Morishima-Kawashima et al., 1995; Johnson and Stoothoff, 2004; Hanger et al., 2009), with only some sites inside the repeats. Importantly, phosphorylation of several Ser/Thr residues (notably Ser-214, Thr-231, Ser-262, Ser-324, Ser-320 and Ser-356) has been shown to reduce the binding of MAPT to microtubules (Hanger et al., 2009; Schneider et al.,1999).

A number of protein kinases and phosphatases have been shown to regulate TAU phosphorylation in vitro/ in vivo. These include PAR-1 kinase (Nishimura et al., 2004), GSK-3β, MAP kinase, Cdk2 and 5, PKA, CaMKII, microtubule-affinity regulating kinase (MARK), and PP1, PP2A, PP2B, and PP2C (Goedert et al. 1995, Lee et al. 2001, Mandelkow 1999). In AD patient brains, PP2A activity decreases (Gong et al., 1993, 1995), and this causes abnormal TAU hyperphosphorylation (Iqbal et al., 2016) and consequent TAU detachment from MTs (Iqbal et al., 2010). Decreased PP2A activity also induces APP phosphorylation at the Thr-668 site, leading to enhanced β-secretase processing of APP (Sontag et al., 2007). However, the endogenous pathophysiological mechanisms that inhibit PP2A activity in human AD have not been resolved.

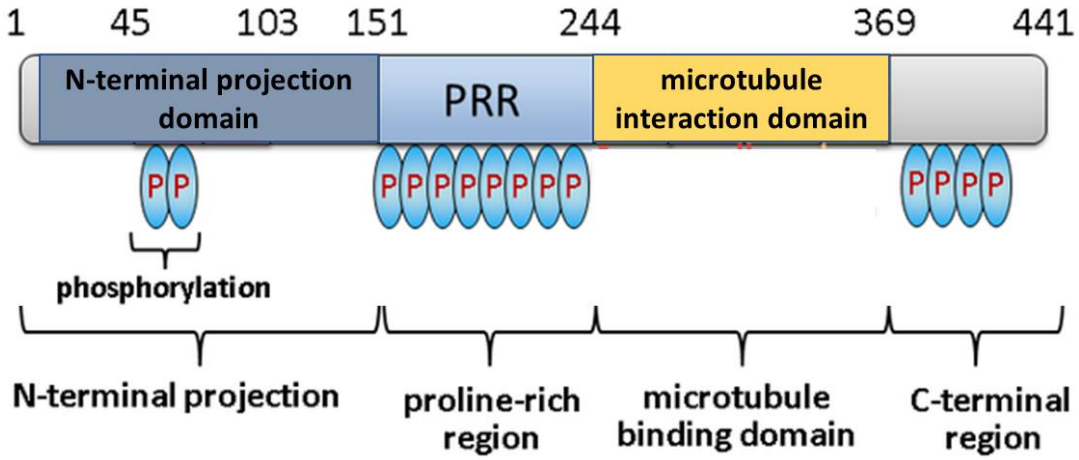


Figure 2 TAU structure. (A) TAU is characterized by a N-terminal domain, a proline rich region (PRR), a repeat domain region which binds MTs and a basic C-terminal domain (CTD). Some phosphorylation sites (P in blue ovals) are shown. (Modified from Heinisch and Brandt, 2016).

Compared to the 6 human isoforms, which harbor either 3 or 4 C-terminal microtubule binding domains (MTBD) and 0 to 2 N-terminal insertions, the dTAU protein contains 5 MTBD (Heidary & Fortini, 2001; Feuillette et al., 2010) with 46% identity and 66% similarity to the corresponding hTAU region but no N-terminal insertions (Heidary & Fortini, 2001).

1.1.2 Animal models for the study of AD

Transgenic models are promising tools to decipher the mechanistic importance of TAU phosphorylation and Aβ deposits, as well their relationship between each other and the other pathological changes. AD models range from yeast (Winderickx et al., 2008) to *Drosophila* and mammals (Gotz, et al., 2001; Hsiao, et al., 1996; Sturchler-Pierrat, et al., 1997).

1.1.2.1 Drosophila models

Drosophila represents a powerful animal model of human diseases, including neurodegenerative disorders such as AD (Driscoll and Gerstbrein, 2003; Napoletano et al., 2011; Tan and Azzam, 2017), thanks to conservation of genes and pathways, low genetic redundancy as well as practical reasons, including short life span and cheap maintenance.

Both APP and TAU have orthologues in *Drosophila*, known as Appl (APP-like) and dTAU (Tan and Azzam, 2017). However, the best characterized fly AD models were generated with human proteins. Ectopic expression of the human APP proteins in the fly nervous system induce significant neuron loss and impairment in associative learning and memory (Iijima and Iijima-Ando, 2008), while ectopic expression of the human TAU recapitulates primary features of tauopathy, including TAU hyperphosphorylation and TAU-induced neurodegeneration.

1.1.2.2 Mouse models

So far, the best mouse AD models combine amyloid and TAU pathology by overproducing APP, PS1, and TAU (Kathryn et al., 2009). Mouse models have provided insights into AD mechanisms even if they do not recapitulate the entire biology of the disease. For example, TAU transgenic mouse models display progressive NFT formation and neuronal loss, but not senile plaques (Kathryn et al., 2009).

1.2 The prolyl-isomerase PIN1

1.2.1 Regulation of APP and TAU by PIN1

Protein post-translational modifications (PTMs), in particular phosphorylation, play a pivotal role in modulating the activity of proteins involved in different cellular processes (Prabakaran et al., 2012; Walsh, 2006), including control of cell cycle, apoptosis, transcription, cell commitment, metabolism, DNA repair and neuronal homeostasis (Brenkman et al., 2008; Lu et al., 2007; Pinton et al., 2007; Rustighi et al., 2014; Yeh and Means, 2007). In particular PTMs are important to regulate the function of both APP and TAU, which harbor different Threonine (Thr)/ Serine (Ser) phosphosites where the Threonine or the Serine is followed by a Proline. These sites are important regulatory sites, as the Proline residue can adopt cis or trans configuration, thus affecting protein conformation and function.

Both APP and TAU can be phosphorylated and are functional in their trans configuration, while their cis isomers form plaques and NFTs, respectively.

Isomerization of Ser/Thr-Pro motifs is especially important because kinases like MAPK and Cdk2 and phosphatases PP2A specifically recognize the cis or trans conformation of the prolyl peptide bond of their substrates (Liou et al., 2011). In particular, PP2A dephosphorylates trans-Ser(P)-Pro motifs of TAU proteins (Zhou et al., 2000).

Cis-trans Proline isomerization is catalyzed by peptidyl-prolyl isomerases (PPIases). Among these, PIN1 is particularly important for its unique ability to catalyze the cis-trans conversion of Prolines following phosphorylated Serine or Threonine residues (pSer/Thr-Pro) (Lu et al., 1996, 2002; Yaffe et al., 1997).

In the APP protein, PIN1 can bind and isomerize the phosphorylated Threonine 668-Proline motif (pT668-P), promoting APP non-amyloidogenic processing and reduction of Aβ production (Pastorino et al., 2006), while in the TAU protein, PIN1 can bind the phosphorylated Thr-212-Pro motif (Smet, et al., 2004) and Thr-231-Pro motif (pT231-P), promoting TAU dephosphorylation and binding to microtubules (Lu et al., 1999). Moreover, PIN1 binds and stimulates dephosphorylation of TAU at Ser-202, Thr-205, Ser-235, and Ser-404 (Cdk5 sites) (Kimura et al., 2013). Furthermore, FTDP-17 mutant TAU (P301L or R406W) showed slightly weaker PIN1 binding than non-mutated TAU, suggesting that FTDP-17 mutations induce hyperphosphorylation by reducing the interaction between PIN1 and TAU (Kimura et al., 2013).

In AD, TAU and APP are hyperphosphorylated (Iqbal et al., 2016; Shentu et al., 2018), and loss of PIN1 levels or function (Pastorino et al., 2006) can lead to an accumulation of cis phosphorylated APP, which is processed by the amyloidogenic pathway and leads to an accumulation of amyloid β-42 (Aβ42), and the formation of amyloid plaques. Furthermore, loss of PIN1 can lead to an accumulation of cis phosphorylated TAU, which becomes resistant to PP2A dephosphorylation (Liou et al., 2011) and degradation (Poppek et al., 2006; Lim et al., 2008) and loses its ability to bind microtubules and to promote their assembly, thus forming NFTs (Fig.3).

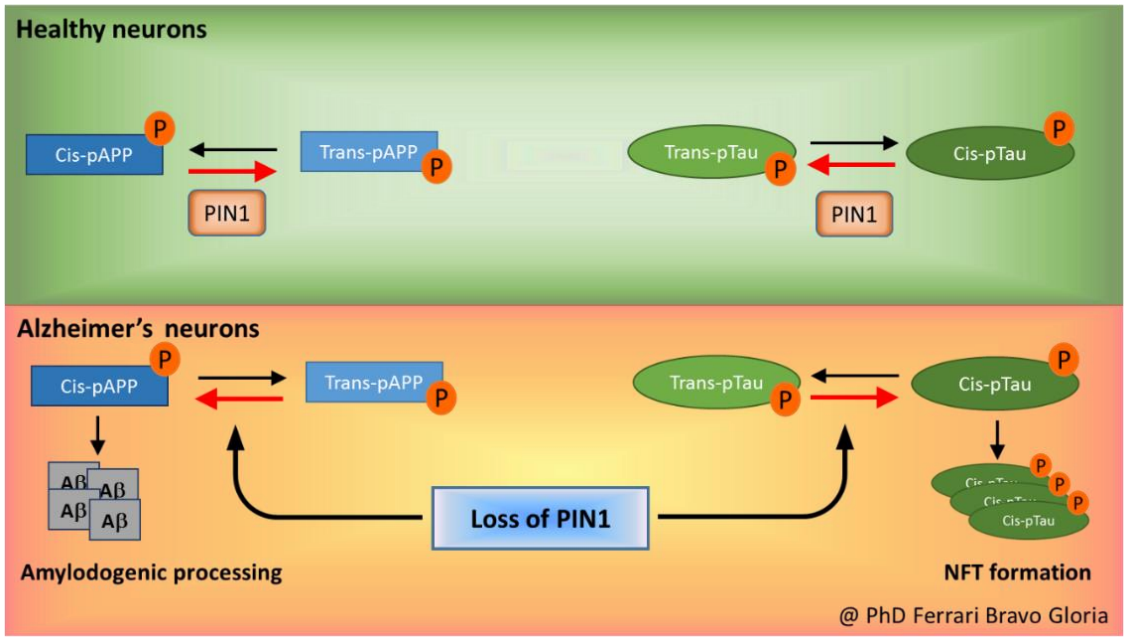


Figure 3. The regulation of TAU function and APP processing by PIN1 in healthy and Alzheimer's neurons. PIN1 promotes correct structure and function of APP and TAU. In healthy neurons, PIN1 promotes the cis-trans conversion of Prolines of both phosphorylated TAU and phosphorylated APP.

1.2.2 Structure, function and regulation of PIN1

PIN1 is a protein of approximately 18 kDa, evolutionarily conserved from *Saccharomyces cerevisiae* to *Drosophila melanogaster, Mus musculus and Homo sapiens* (Fujimori et al., 1999; Maleszka et al., 1996a). PIN1 proteins contain, connected by a short flexible linker region, an amino-terminal WW domain that binds to pSer/Thr-Pro motifs (Lu et al., 1999), and a carboxy-terminal catalytic PPIase domain that catalyses the cis/trans-isomerization between both conformations of the pSer/Thr-Pro bonds (Lu 2009).

The WW domain is characterized by two conserved tryptophan residues (Sudol and Hunter, 2000; Sudol et al., 2001), which mediate the interaction with the substrates and the pSer/Thr-Pro sites.

Subsequently the PPIase domain can interact with the pSer/Thr-Pro motifs and induce the cis/trans-isomerization.

The enzymatic activity of PIN1 is mediated by a specific basic triad, composed by Lys63-Arg68-Arg69, that recognizes the negative charge present in the phospho-residues (Zhou et al., 2000). PIN1 residues involved in substrate recognition and catalytic activity are conserved in *Drosophila m.* and *Homo sapiens* (Ranganathan et al., 1997) (Fig.4).

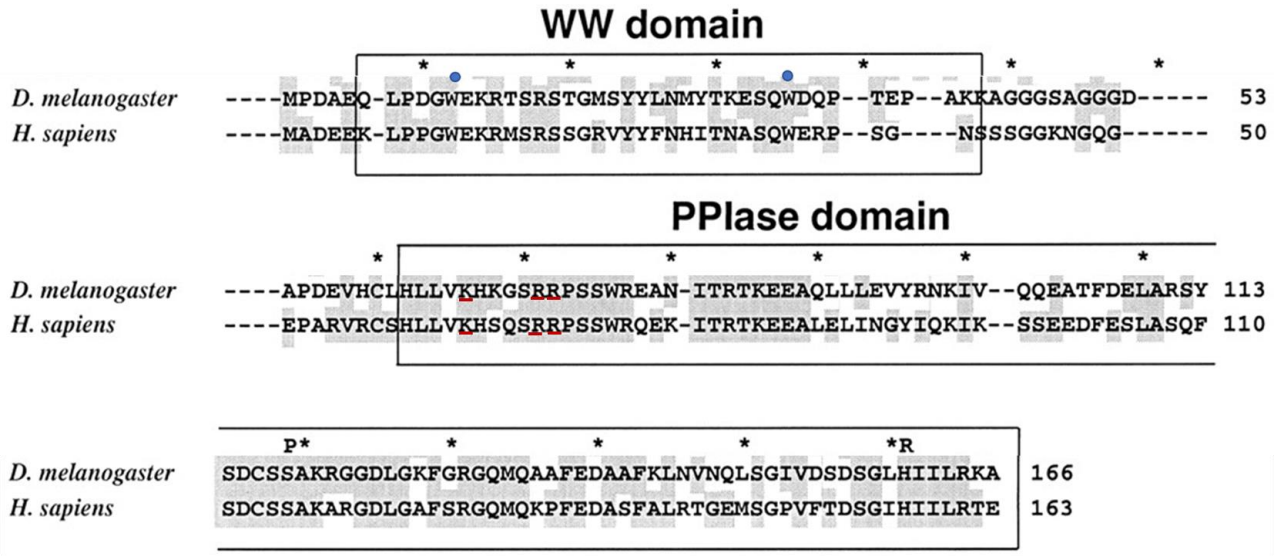


Figure 4. Alignment of D. melanogaster Dodo (P54353) with Homo sapiens PIN1 (XP_00 9024). The alignment was done using ClustalW multiple sequence alignment program, version 1.7 (Thompson et al. 1994). Dashes indicate gaps. Shaded areas indicate regions of identity between the proteins. The signature tryptophans of the WW domain are indicated by blue dots. The residues underlined in read in the PPIase domain are residues that mediate enzymatic activity (modified from Devasahayam, et al., 2002).

Although PIN1 exhibits a very high specificity for pSer/Thr-Pro motifs, it exhibited little isomerase activity also for substrates containing prolines following the acidic aminoacids aspartate or glutamate (Asp-Pro and Glu-Pro) (Yaffe et al. 1997). Furthermore, PIN1 can bind others non-canonical motifs — 186-Pro-Pro-Leu-Pro-189 in the PRR and 256-Asp- Asp-Tyr-Gly-Asp-260 in the RhoGAP domain of BPGAP1 (Pan et al., 2010), BNIP-H (or caytaxin) (Buschdorf et al., 2008), A3G cytidine deaminase (Watashi et al., 2008), the Pro-X-Thr-Pro recognition motif of phosphatase inhibitor-2, but independent of phosphorylation (Li et al., 2008), and the pThr-Gly motif of cyclin E (Yeh et al., 2005).

The *Drosophila melanogaster PIN1* orthologue *Dodo* is a 166 amminoacids long protein encoded by the *dodo* 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).

1.2.2.1 Mechanisms through which PIN1 impinges on specific cellular processes

The conformational changes induced by PIN1 on its protein substrates can have profound effects mainly on their stability, but also modulation of the catalytic activity, other PTMs like dephosphorylation, protein–protein interactions and subcellular localization have been described (Lu et al., 2002).

Isomerization of Ser/Thr-Pro motifs is especially important because the MAPK and Cdk2 kinases and PP2A phosphatase specifically recognize the cis or trans conformation of the prolyl peptide bond of their substrates (Werner-Allen et al., 2011; Zhou et al., 2000).

Furthermore, PIN1 has been shown to control protein turnover of many substrates by either favoring or blocking their recognition by several E3 ubiquitin-ligases. For example, the SCF (Skp1-Cullin-F-box) complex directs phosphoproteins to be recruited for ubiquitylation-mediated degradation (Gutierrez and Ronai, 2006). Recent structural studies indicate that F-box proteins preferentially bind substrates via a trans conformation of the phospho-Ser/Thr-Pro motif (Orlicky et al., 2003), suggesting that the cis or trans conformation of Proline could be a critical determinant in regulating protein degradation. Consequently, PIN1 plays a major role in modulating the conformation of these proteins thereby affecting their interaction with the SCF complex and proteasomal degradation (Gutierrez and Ronai, 2006; Liou et al., 2011). Via the SCF complex PIN1 regulates a plethora of targets in many tissues such as Notch1, Notch4, c-myc, cyclin E, c-jun, MCL-1 and SREBP (Cheng and Li, 2012; Welcker et al., 2004). Besides the action on SCF complex in the control of protein degradation, PIN1 is able to control the stability of many other proteins, such as cyclin D1, NF-κB, β-catenin, p53 family members, Nanog, Oct4, ErbB2, Akt, Smad proteins and TAU by different mechanisms (Liou et al., 2011).

Not surprisingly, due to its vast repertoire of protein targets, PIN1 is important in many cellular processes involving Proline-directed phosphorylation, including the cell cycle, cell signaling, transcription and splicing, DNA damage response, germ cell development and neuronal survival (Fig.5) (Lu & Zhou, 2007; Girardini *et al.*, 2011; Lee et al., 2011b; Liou et al., 2011; Yuan *et al.*, 2011; Brenkman et al., 2008; Lu et al., 2007; Pinton et al., 2007; Rustighi et al., 2014; Yeh and Means, 2007).

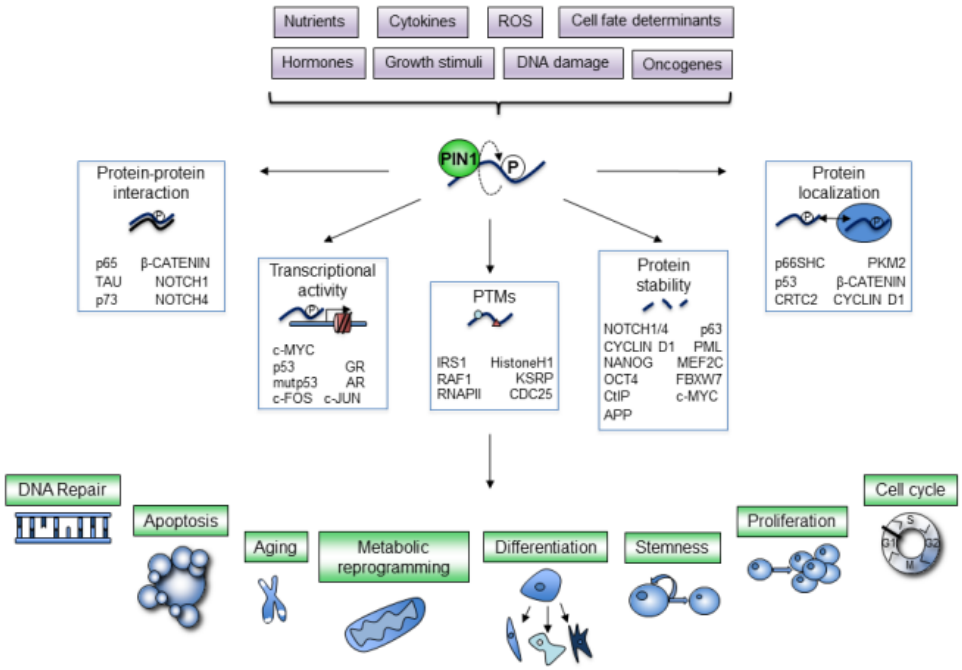


Figure 5. PIN1 modulates phosphorylation signaling transduction (Zannini et al., 2019).

1.2.2.2 PIN1 in cell cycle and chromatin regulation

Since its discovery, PIN1 has been shown to be a regulator of mitotic events by acting on numerous substrates, such as the mitotic inducer CDC25C phosphatase (Okamoto & Sagata 2007), the inhibitory kinase of cyclin B–CDC2 WEE1 (Zhou et al. 2000), and the 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 also plays a role in the G0/G1–S phase transition, where it increases the transcription and protein stability of cyclin D1, respectively boosting c-Jun N-terminal kinases (JNKs)(Wulf et al.,2001), β-catenin and NF-κB (Ryo et al. 2001; Ryo et al. 2003) and preventing cyclin D1 nuclear export and ubiquitin-mediated degradation (Liou et al., 2002), leading to the entrance in the cell cycle. Furthermore, PIN1 destabilizes the transcription factor c-Myc and cyclin E, which are recognized by the Cdc4 E3 ligase of the Skp1/Cul1/Rbx1 (SCF) complex, allowing the G1–S phase transition.

During mitosis, PIN1 has an important function in chromosome condensation. 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 & Manley 2007). PIN1 can control chromatin organization also by the interaction with the histone H1, which plays a crucial role in stabilizing higher

chromatin structure (Raghuram et al., 2013). In fact, PIN1 can recognize and bind phosphorylated residues on histone H1 (pS173 and pS187), modulating the conformation of its C-terminal domain and stabilizing its binding to chromatin (Raghuram et al., 2013). In absence of PIN1, transcriptionally active and inactive sites are decondensed as a result of increased H1 mobility (Raghuram et al., 2013). On the other hand, PIN1 can promote gene expression, through the induction of dephosphorylation of RNA Polymerase II C-terminal domain (CTD) (Zhang et al., 2012), and mRNA stability, by preventing AUF1-mediated RNA degradation (Esnault et al., 2006).

1.2.2.3 PIN1 in DNA repair

DNA damage severely affects replication and transcription and plays a major role in age-related diseases and cancer. The DNA damage response (DDR) is an elaborate and specialized cascade that cells activate in response to DNA damage to maintain genome integrity (Jackson and Bartek, 2009). The DDR coordinates cell-cycle checkpoints and DNA repair or, if the damage cannot be repaired, triggers specialized programs such as apoptosis and senescence (Ciccia and Elledge, 2010).

The regulation of DNA double-strand break (DSB) repair by phosphorylation-dependent signaling pathways is crucial for the maintenance of genome stability. DSBs activate ATM/ATR kinases, which trigger a signaling cascade that phosphorylates the repair factor CtIP, arrests cell cycle and triggers repair of DNA lesions (Matsuoka et al., 2007; Yu and Chen, 2004; Huertas et al., 2008; Peterson et al., 2013). DSBs are repaired by two major pathways: homologous recombination (HR) or canonical non-homologous end joining (c-NHEJ) (Jackson and Bartek, 2009; Lukas and Lukas, 2013). HR represents a highly accurate, error-free repair mechanism, whereas NHEJ ligates together the two DNA ends with little or no processing, thus being more error-prone. NHEJ is faster than HR and functions throughout the cell cycle, while HR is a multistep process restricted to the S and G2 phases of the cell cycle, in which homologous sequences on the sister chromatid serve as a template for repair (Moynahan and Jasin, 2010). The activation of HR in S phase requires 5’ to 3’ nucleolytic degradation of DSB ends to generate long stretches of single-stranded DNA (ssDNA), a mechanism known as DNA end resection which involve CtIP, a key protein that stimulates resection. By contrast HR is suppressed during G1 by CtIP proteasome-mediated degradation (Germani et al., 2003; Steger et al., 2013). PIN1 is fundamental in coordinating DNA repair pathway choice between HR, and NHEJ. This is due to the fact that PIN1 mediates the

isomerization of the repair factor CtIP after its phosphorylation at two conserved Serine/Threonine-Proline motifs (S276 and T315) by cyclin-dependent kinase CDK2 (Steger et al., 2013). This conformational change promotes PIN1 dependent CtIP poly-ubiquitination and subsequent proteasomal degradation. As a result of the activity on CtIP, PIN1 overexpression attenuates HR and promotes NHEJ, while PIN1 depletion reduces NHEJ as a result of increased DNA end resection (Steger et al., 2013).

Furthermore, PIN1 interacts with other key DSBs repair factors including 53BP1, BRCA1-BARD1(Steger et al., 2013), suggesting that PIN1 can influence DNA repair pathway choice between HR, and NHEJ in more than one way.

Moreover, PIN1 can isomerize the tumor suppressor p53 on multiple S/T-P sites that are phosphorylated following genotoxic insults, thereby increasing p53 protein stability, and transcriptional activity, and consequently its ability to induce cell-cycle arrest and apoptosis in response to stress (Zacchi et al., 2002; Zheng 2002, Mantovani et al., 2007). In particular, PIN1 is essential for inducing dissociation of p53 from the Mdm2 ubiquitin ligase, which regulates p53 degradation (Zacchi et al., 2002), as well as from the apoptosis inhibitor iASPP (Mantovani et al., 2007).

1.2.2.4 PIN1 in Model Organisms

Studies in model organisms have been instrumental to understand the function of PIN1.

Important evidence comes from genetic approaches in yeast, *Drosophila*, mouse and recently also in Zebrafish (Solange Ibarra et al., 2017).

PIN1 Knock-Out (KO) mice are viable and develop normally until midlife (Fujimori et al., 1999), suggesting that PIN1 is dispensable for organism development. However, these mice later show phenotypes reminiscent of those due to loss of Cyclin D1 function, such as retina hypoplasia and mammary gland impairment (Lee et al., 2011a; Liou et al., 2002, 2003). Loss of PIN1 in mice causes markedly increased levels of hyperphosphorylated TAU and increases amyloidogenic APP processing and elevates the toxic species of Aβ in an age-dependent manner (Pastorino, et al., 2006). Besides AD-like neurodegeneration, these animals also develop multiple signs of premature aging, including osteoporosis, skin retina and gonad atrophy, loss of body mass and accelerated telomere shortening (Lee et al., 2011a; Liou et al., 2002, 2003).

In *Drosophila melanogaster*, deficiency of the *PIN1* ortholog *Dodo* causes embryonic defects consisting in fused or absent dorsal appendages, which have been associated to alterations in the MAPK signaling that determines egg dorso-ventral polarity (Hsu et al., 2001). In addition, Dodo has been proposed to regulate the circadian clock machinery by stabilisation of phosphorylated period (PER) protein (Kang et al. 2015).

1.2.2.5 Regulation of PIN1 function

Different studies have shown that gene transcription, protein level and catalytic activity of PIN1 are tightly regulated by several mechanisms (Liou et al., 2011).

At the transcriptional level, PIN1 is positively modulated by the transcription factors E2F (Pulikkan et al., 2010; Ryo et al., 2002) AP4 (Ling Ma et al., 2012), the Notch signalling pathway (Rustighi et al., 2009), the Insulin-like growth factor (You et al., 2002), and in the hippocampus by the apolipoprotein E4 (Lattanzio et al., 2014).

PIN1 is also controlled by micro-RNAs, in particular it is negatively controlled by miR-200b in breast cancer cells (Zhang et al., 2013).

At the post-translational level, several modifications (PTMs), in particular phosphorylation, regulate PIN1 protein levels and activity. PIN1 phosphorylation on Ser16 in the WW domain by Aurora A abolishes its ability to interact with substrates (Lee et al., 2013), phosphorylation on Ser71 in the PPIase domain by the Death-associated protein kinase1 (DAPK1) blocks PIN1's catalytic activity and nuclear localization (Lee et al., 2011a), the phosphorylation on Ser65 by Polo-like kinase-1 (PLK1) increases the stability of PIN1 by reducing its ubiquitylation (Eckerdt et al., 2005), and the phosphorylation on Ser138 by mixed-lineage kinase 3 (MLK3) promotes PIN1 catalytic activity and nuclear localization (Lee et al., 2011a; Rangasamy et al., 2012). Furthermore in the context of AD, the protein phosphatase Calcineurin inactivates PIN1 by dephosphorylating S111 (Stallings et al., 2018). SUMOylation is another important PTM that affects PIN1's activity, in particular it inhibits PIN1 functions when it occurs on Lys6 or Lys63. This inhibitory effect is reverted via deSUMOylation by SENP1 which, in turn, increases PIN1 protein levels (Chen et al., 2013). Finally, PIN1 enzymatic activity is inactivated by oxidative modifications (Chen et al., 2015).

1.2.3 PIN1 in AD

Unlike the most differentiated cells in the body, where PIN1 expression exists at low levels, and its overexpression can contribute to cancer (Bao et al., 2004), PIN1 expression is induced during

neuronal differentiation and is mainly expressed in the nucleus of neurons at unusually high levels (Lu et al., 1996; Lu et al., 1999; Ryo et al. 2001; Wulf et al., 2001; Thorpe et al., 2004). However, in AD brains the levels of PIN1 are reduced. In particular, in AD PIN1 binds to pTAU and co-localizes and co-purifies with NFTs, resulting in depletion of soluble PIN1 (Lu et al., 1999; Thorpe et al., 2001, 2004; Ramakrishnan et al., 2003). A recent study also demonstrated that Aβ accumulation leads to reduced PIN1 activity in dendritic spines by increasing the activity of Calcineurin (Stallings et al., 2018). Subregions of the hippocampus with low expression of PIN1 are more prone to neurofibrillary degeneration in AD (Liou et al., 2003a) and brains at late stage of AD show reduced amounts of soluble PIN1 protein (Lu et al., 1999). PIN1 also appears to be inactivated by oxidation or delocalized in AD (Butterfield et al., 2006).

Another evidence of association between *PIN1* function and AD comes from the identification of a late-onset familiar AD locus in 19p13.2, which includes the *PIN1* gene (Wijsman et al., 2004). Moreover, a *PIN1* promoter polymorphism associated with reduced PIN1 protein levels has been found to be enriched in AD patients compared to healthy controls (Segat et al., 2007), while another *PIN1* promoter polymorphism, disrupting the binding site of the AP4 repressor, has been associated with a delayed AD onset (Ling Ma et al., 2012).

1.3 Emerging concepts in AD pathogenesis

AD pathogenesis is highly complex and includes interaction among multiple factors, the contribute of each in impairing neuronal functions and survival remains to be fully elucidated (Huang and Mucke, 2012). Besides amyloid plaques and NFTs, inflammation, oxidative stress and DNA damage have also proposed to play important roles. Moreover, different studies highlight the possible role of mechanical stress, osmotic pressure and extracellular matrix in AD.

1.3.1 Role of Inflammation in AD

Inflammation plays a role in Alzheimer's disease (Kinneya et al., 2018) although the extent of its contribution is unclear (Wyss-Coray and Rogers, 2012). In the brain of AD patients, astrocytes and microglia are chronically activated in areas surrounding Aβ deposits and release cytokines such the tumor necrosis factor (TNF)- α, interleukin (IL)-1, transforming growth factor (TGF)-β, fibroblast growth factor (FGF) and other molecules such as metalloproteases, complement

proteins, and oxygen radicals (Mattson et al., 1997; Griffin et al., 1998). Furthermore, during inflammation, there is extravasation of proteins from the vascular space to the extracellular space, resulting in increased osmotic pressure (Wiig 2011).

In AD, oxygen radicals can induce oxidative damage (Galasko and Montine, 2010) of membrane lipids, proteins and nucleic acids (Cheignon et al., 2018). Oxidation of lipids can disrupt membrane integrity (Castegna et al., 2004), and lipid peroxidation products can alter membrane proteins, provoking neurotoxicity (Subramaniam et al., 1997). Oxidation of nucleic acids can directly cause DNA damage, including DNA double strand breaks (DSBs), moreover it can also affect the efficiency of DNA repair systems (Coppedè and Migliore, 2010; Weissman et al., 2007). DNA DSBs are the most lethal form of DNA damage especially in neurons, which have a reduced DNA repair capability compared to proliferating cells (Merlo et al., 2016), and can only rely on NHEJ for repair DNA lesions.

Furthermore, oxidative stress (Satoh & Kuroda 2000), FGF2 (Lahiri & Nall, 1995), cytokines and growth factors (Mattson et al., 1997; Griffin et al., 1998) might result in facilitating the amyloidogenic pathway, contributing to Aβ overproduction/accumulation and TAU hyper-phosphorylation. Moreover, FGF2 through the binding to Heparan sulphate PGs (HSPG, a component of the ECM), plays a role in transforming normal soluble TAU protein into the insoluble form present in the neurofibrillary tangles (Perry G et al., 1991).

1.3.2 Role of the mechanical stress in AD

Accumulation of mechanical stress or osmotic pressure have been suggested to play a role in the development of diseases of old age, such as cardiomyopathy (Modesto and Sengupta, 2014), atherosclerosis (Heo et al., 2014), osteoarthritis (Visser et al., 2014) and AD (Robicsek et al., 2002). Similarly, the hypothesis that AD pathology could be driven by mechanical forces and intracranial pressure has been proposed (Wostyn 1994; Wostyn 2004; Silverberg et al., 2006; Wostyn et al., 2008; Wostyn et al., 2010).

Epidemiological and neuropathological data suggest a tight association between AD and the exposure to mechanical stress factors. Extracranial mechanical stressors predispose an individual to AD later in life, as observed in traumatic brain injury (TBI) (Chauhan 2014) and occupational exposure in athletes (boxers, football, and soccer players) and military personnel (Stein et al., 2014). Neuropathological analysis of TBI tissues in humans reveal abnormal accumulation of Aβ

deposits (Gatson et al., 2013) and TAU proteins, after isolated or/ and repetitive cranial impacts (Kanayama et al., 1996; Kane et al., 2012).

As observed in humans, AD-like pathology is also present in numerous experimental models of TBI (mice, rats, rabbits, pigs, and monkeys; Breunig et al., 2013).

Neuropathological evidence of AD has also been observed in normal pressure hydrocephalus (NPH) patients (Savolainen et al., 1999; Bech et al., 1999; Golomb et al., 2000; Cabral et al., 2011), a disease characterized by an accumulation of cerebrospinal fluid. As observed in humans, animal models of NPH have revealed accumulation of TAU and Aβ deposits in brain tissues subjected to hydrodynamic stress (Klinge et al., 2006; Silverberg et al., 2010). Moreover, in rodent models, subject to high levels of blood pressure, or hemodynamic stress, has been observed an accumulation of Aβ and TAU aggregates (Carnevale and Lembo, 2011; Schreiber et al., 2014).

These evidences suggested that continuous and repetitive exposure to environmental mechanical stress and/or intracranial pressure becomes a potential driving force for Aβ and TAU aggregation (Hachiya et al., 2008).

1.3.3 Role of the extracellular matrix in AD

ECM contains several glycoproteins such as laminin, fibronectin (Narindrasorasak et al., 1992), entactin, asymmetric acetylcholinesterase and also other specialized molecules including collagens and proteoglycans (PGs) (Brandan & Inestrosa, 1993). As a result of the multiple interactions among these components the resulting ECM may present different molecular architectures, that can be modulated through variations in compositions, isoform substitutions, and the addition of several exogenous macromolecules.

Brain ECM contains different types of proteoglycans localized to intercellular spaces between neurons and glia, while fibronectin and collagen, ECM components of systemic organs, are virtually absent from the adult brain ECM (Yamaguchi and Lecticans 2000; Bandtlow and Zimmermann, 2000; Dityatev and Schachner, 2003).

In AD, the slow cumulative changes in the microarchitecture of the brain could affect its mechanical properties (Bonneh-Barkay and Wiley, 2009; Tyler 2012; Simon and Iliff, 2016). Several studies have shown that modulations of the neuronal matrix affect synapse morphology, function [eg, interfering with induction or maintenance of long-term potentiation (LTP)] (Brakebusch et al., 2002; Evers et al., 2002 ; Ethell et al., 2007) and neuronal survival (Achim et al.,

1996; Baig et al., 2005 ; Bruckner et al., 1999 ; Crocker et al., 2004 ; Jourquin et al., 2005 ; Kaminska et al., 1997 ; Lo EH et al., 2002 ; Lorenzl et al., 2003; Lorenzl et al., 2004 ; Nagase et al., 1999 ; Okamoto et al., 2003 ; Osman et al., 2002 ; Satoh et al., 2000 ; Schuppel et al., 2002 ; Weber et al., 1999 ; Yoshihara et al., 2000). However, the extent to which mechanical dynamics influence brain structure and homeostasis in AD still remains unclear. Recently, evidence of changes in cell surface proteoglycans and/or ECM components in AD patients has been observed. It has been reported accumulation of adhesive ECM filaments (Klier et al., 1990; Enam et al., 1990), such as Heparan sulphate PGs (HSPGs; Berzin et al., 2000; Van Horssen et al., 2002), laminin, fibronectin (Narindrasorasak et al., 1992), chondroitin sulfate proteoglycans (CSPGs), dermatan sulfate (DS) proteoglycans (Snow et al.,1992; DeWitt et al., 1993) and collagen (Van Horssen et al., 2002) in senile plaque of AD patient (Berzin et al., 2000). HSPGs , moreover, were found associated with NFTs (Verbeek et al., 1999). Importantly, early accumulation of heparan sulfate (HS) was observed in primitive plaques both in AD and in Down's syndrome, (Snow et al., 1990), suggesting that ECM alterations precede neuronal dysfunction.

1.3.4 Chromatin alterations in AD

1.3.4.1 Chromatin organization

Chromatin organization is crucial for cell homeostasis. Changes in chromatin organization are common during cellular senescence and organismal aging, and wide epigenetic changes have been reported in age-related diseases, including AD (Winick-Ng and Rylett, 2018).

In eukaryotic cells, 147 base pairs of DNA wraps around the 8 core histones (H3, H4, H2A, H2B) to form the basic chromatin unit, the nucleosome (Rothbart and Strahl, 2014). Nucleosomes compact to form a 30nm diameter fiber, which is maintained by the linker histone H1 (Beckers et al., 2007). The function of chromatin is to efficiently package DNA into a small volume to fit into the nucleus of a cell and protect the DNA structure and sequence, prevents chromosome breakage and controls gene expression and DNA replication (Beckers et al., 2007).

In non-dividing cells, the genome is organized in two transcriptional states: euchromatin, in which the DNA is loosely packed and is available for transcription, and heterochromatin, with highly compacted DNA and usually transcriptionally repressed (Croft et al., 1999; Harničarová et al.,

2006; Zink et al., 2004). Heterochromatin can be classified in facultative, in which the transcription is transiently repressed, and constitutive, a more permanent repressive state.

A crucial layer of regulation of chromatin state is represented by PTMs of histone N-terminal tails. These include phosphorylation, methylation, acetylation, ubiquitination, SUMOylation and GlcNAcylation (Fig. 6) (Gillette and Hill, 2015). Enzymes that catalyze addition and removal of histone PTMs are known as “writers” and “erasers”, respectively (Gillette and Hill, 2015), while factors that recognize PTMs and direct transcriptional outcome are known as “readers” (Gillette and Hill, 2015). Post-translational modifications of the histones regulate gene expression by organizing the genome into active regions of euchromatin, where the DNA is less condensed and can be transcribed, or inactive heterochromatin regions, where DNA is more compact and usually transcriptionally repressed (Croft et al., 1999; Harničarová et al., 2006; Zink et al., 2004). Histone H3 is the most modified histone. Modifications to histone H3 can predict the type of chromatin (heterochromatin vs euchromatin), distinguish between functional elements of the genome (promoters, enhancers, gene bodies), and determine whether these elements are in an active or repressed state. Furthermore, modifications of the histone H3 can distinguish between facultative heterochromatin (H3K27me3), in which the transcription is transiently repressed, and constitutive heterochromatin (H3K9me3), a more permanent repressive state. Transcriptionally active euchromatin largely resides in the center of the nucleus and near nuclear pores (Solovei et al., 2013).

The spatial organization of the genome provides a way to regulate gene expression by altering the localization of specific chromatin regions. Genomic regions that contain mostly inactive genes, and those which are enriched in repeat sequences (e.g. transposable elements, TEs), tend to be clustered as heterochromatin and localized at the intranuclear periphery, associated with the nuclear lamina, or in peri-nucleolar regions (Peric-Hupkes et al., 2010; Shah et al., 2013). In contrast, euchromatic regions mainly localize in the nuclear interior (Luperchio et al., 2014).

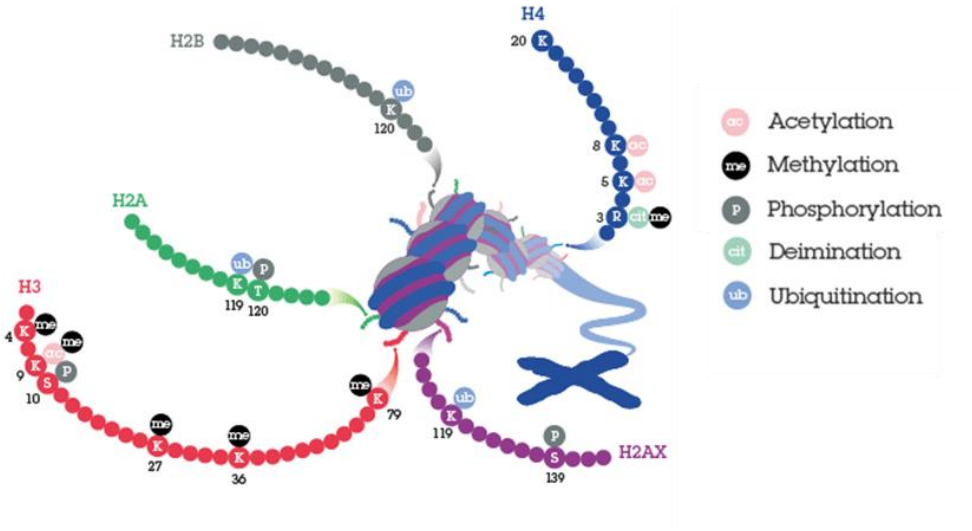


Figure 6. Schematic representation of the histone PTMs. Histone PTMs. https://www.abcam.com /epigenetics/histone-modifications-a-guide

1.3.4.2 The Heterochromatin Protein 1

Constitutive heterochromatin is characterized by high levels of histone 3 Lysine 9 di- and tri-methylation (H3K9me2 and H3K9me3); this modification is catalyzed by histone methyltrnasferases HMTs (SUV39, SETDB1, Schotta et al., 2002; Schultz et al., 2002) and is recognized by the reader proteins belonging to the heterochromatin protein 1 (HP1) family (Allis et al., 2007). These are conserved from flies to humans (Norwood et al., 2004), and include three members in mammals, alpha (α), beta (β), and gamma (γ), five in Drosophila (a, b, c, d, and e), and two in yeast (Swi6 and Chp2). HP1-mediated formation of heterochromatin involves the recruitment of HMT, which methylates neighboring H3K9 residues. These in turn serve as HP1-binding sites, and this self-enforcing loop allows the spreading of heterochromatin (Bannister and Kouzarides, 2011).

HP1 is a non-histone chromosomal protein that was initially discovered in Drosophila melanogaster (James and Elgin, 1986), and highly conserved from Schizosaccharomyces pombe to human (Norwood et al., 2004). Despite high sequence conservation, the HP1 paralogs achieve diverse functions. For example, human HP1α and β primarily associate with heterochromatic regions of the genome, such as centromeres and telomeres, and help mediate transcriptional gene silencing (Hayakawa et al., 2003; Smallwood et al., 2012). In contrast, hHP1γ largely localizes to euchromatic regions and plays roles in transcriptional elongation and RNA processing (Hayakawa et al., 2003; Smallwood et al., 2012). In Drosophila HP1a is associated with both

heterochromatin and euchromatin (Piacentini et al. 2009), while Drosophila HP1γ associates to euchromatic regions and is involved in RNA transcriptional elongation and processing (Hayakawa et al., 2003; Smallwood et al., 2012). All HP1 proteins possess two functionally distinct globular domains with a very similar structures, the N-terminal chromodomain (CD) (Liu et al., 2017), which binds H3K9me3, and the C-terminal chromoshadow domain (CSD) (Liu et al., 2017), which mediate interaction of HP1 with proteins containing ether the PxxVxL or the PxVxL motif (where P = Proline, V = Valine, L = Leucine and x is any amino acid) (Liu et al., 2017; Zeng et al., 2010). The CD and CSD are linked by an unstructured hinge region, which mediates the interaction of HP1 with Lamina-associated polypeptide 2 (LAP2β), Lamin B Receptor (LBR) and B-type lamins (Kourmouli et al., 2000). By this interaction HP1 is tethered to the nuclear lamina, forming large-scale repression chromatin domains (Ye et al., 1997). In Drosophila, it has been recently demonstrated that a large fraction of heterochromatin is bound by Lamin B of Drosophila called LamDm0 and dHP1a and is localized in proximity of the nuclear envelope (Pindyurin et al., 2018).

1.3.4.3 Nuclear Lamina

The nuclear lamina is a meshwork of intermediate protein filaments (lamins) (Goldman et al., 2002) that assemble just under the inner nuclear membrane (INM; Gruenbaum et al., 2005) and provide mechanical support to the nucleus and a platform for the binding of proteins and chromatin, in eukaryotic organism.

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. Independently of the eukaryotic organism of origin, all discovered lamins share the same domain structure: a short N-terminal head domain, a central rod domain, and a long C-terminal tail domain that includes a nuclear localization signal (NLS) (Gruenbaum and Foisner, 2015).

The nuclear lamina anchors the transcriptionally repressed heterochromatin at the nuclear periphery (Solovei et al., 2013). The absence of LaminA and LBR leads to loss of peripheral heterochromatin and localization of the heterochromatin in the center of the nucleus (Solovei et al., 2013). In Drosophila, loss of LamB/Dm0 is associated with a reduction of H3K9me3 and with heterochromatin relaxation (Chen et al., 2016).

1.3.4.4 Heterochromatin alterations in AD

Chromatin alterations have been proposed to contribute to aging and aging-related diseases. The use of *Drosophila* models has been instrumental to reveal that AD pathogenesis may involve global chromatin relaxation and aberrant transcriptional activation of many genes that are usually repressed in heterochromatin (Frost et al., 2014). Ectopic expression of the hTAU protein in the fly brain is associated with a reduction of heterochromatin foci and of dHP1a and H3K9me2 protein levels (Frost et al., 2014, 2016). These phenotypes correlated with aberrant gene expression and were observed also the brain of AD patients (Frost et al., 2014, 2016).

1.4 Transposable Elements in AD

1.4.1 Classification of Transposable elements

TEs are DNA sequences that are able to move in the genome using the host transcriptional and translational machinery (González & Petrov 2009). TEs constitute a significant fraction of the genome in all organisms, and comprise approximately 12% of Drosophila genome (Pimpinelli et al., 1995), 37% of the mouse genome (Waterston, et al., 2002), 50%-70% of the human genome (Lander, et al., 2001; de Koning et al., 2011) and almost 90% in some plants (Mascagni et al. 2015). According to their mechanism of transposition, TEs are historically subdivided into two major classes (Wicker et al. 2007). Class I TEs, also known as retro-transposable elementes (RTEs) or retrotransposons, comprise TEs that mobilize by a “copy-and-paste” mechanism involving an RNA intermediate (Garfinkel et al., 1985; Boeke JD, et al., 1985). Class II TEs, also known as DNA transposons, mobilize by a “cut-and-paste” mechanism.

1.4.1.1 Retrotransposons

RTEs are the only active Transposons in humans (Ewing & Kazazian, 2010), and are divided into two superfamilies, LTR and non-LTR, and further into subclasses (González & Petrov 2009).
Within this broader classification scheme, TEs can be described as those having the ability to self-mobilize (autonomous) and those relying on co-mobilization by the enzymatic machinery of other TEs (non-autonomous).

LTR retrotransposons are the predominant order in plants, as well as in a few animals, such as Drosophila melanogaster. Drosophila contains approximately 20 distinct families of LTR-retrotransposons that comprise ~1% of the genome (Bowen & McDonald 2001), while maize contains ~400 families of LTR-retrotransposons that comprise ~ 75% of the genome (Schnable et al. 2005). Numerous families of LTR retroelements are present in humans, but most of them are no longer active (Eickbush T. & Furano, A. 2002).

These elements undergo reverse transcription in virus-like particles by a complex multistep process. In particular, LTR retrotransposons contain two regions called Long Terminal Repeats (LTRs); Among the two LTRs they contain Open Reading Frames (ORFs) for GAG proteins involved in the formation of retroviral particles and for an aspartic protease, reverse transcriptase proteinase (AP), reverse transcriptase, RNase H and DDE integrase (IN) (Goodier and Kazazian, 2008) (Fig.7). The two major subclasses of LTR-retrotransposons are called Gypsy and Copia elements, and they differ in the position of integrase within the encoded polyprotein.

In humans, LTR elements are called human endogenous retroviruses (HERVs).

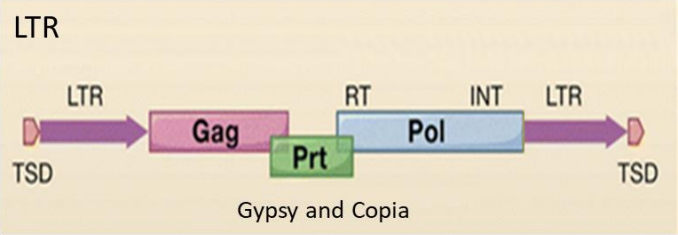


Figure 7. LTR transposon structure. Structure of LTR Transposable Elements in Mammals. Abbreviations: Gag, group-specific antigen (capsid proteins); Prt, protease; Pol, polymerase; TSD, target site duplication; RT, reverse transcriptase domain; INT, integrase domain; LTR, long terminal repeat. (Modified from Goodier and Kazazian, 2008).

LTR sequences regulate the activation and termination of transcription and the integration of the cDNA in the host genome. In particular, the 5′ LTR contains a promoter that is recognized by the host RNA polymerase II and produces the mRNA of the TEs. In the first step of the reaction, Gag proteins assemble into virus-like particles that contain TE mRNA, reverse transcriptase and integrase. The reverse transcriptase copies the TE mRNA into a full-length dsDNA. In the second step, integrase inserts the cDNA into the new target site (Fig.8).

Retrotransposon integrases create staggered cuts at the target sites, resulting in target-site duplication (TSDs).

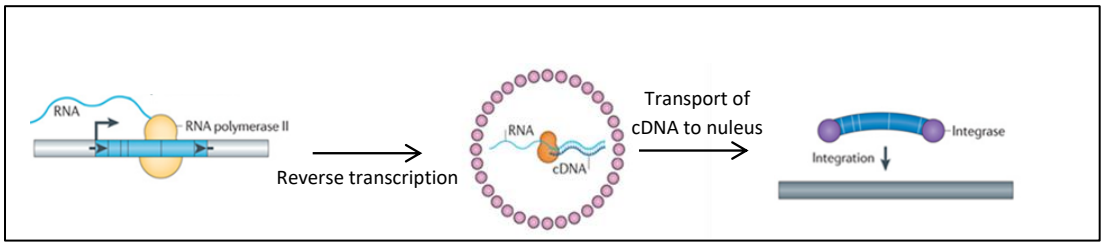


Figure 8. Mechanisms of LTR Transposable Elements mobilization (Levin & Moran 2011).

Some retrotransposons contain a third gene encoding the retroviral envelope (env) protein necessary for mobilization of retroelements outside of their host cells (Fig. 9) (Kim et al., 2004). Many of these retrotransposons are classified as endogenous retroviruses, or errantiviruses in Drosophila and other insects, as they either arose from retroviruses that lost infectivity or LTR retrotransposons that acquired env genes from exogenous sources (Stefanov et al., 2012).

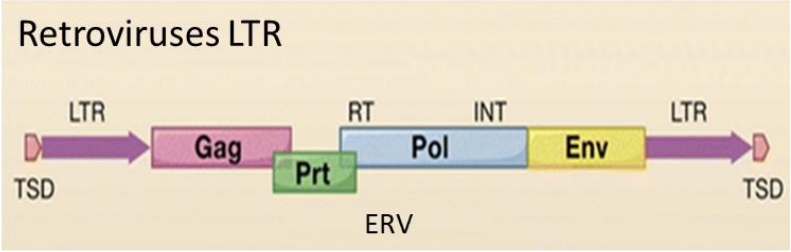


Figure 9. Retroviruses LTR structure. Structure of Retroviruses LTR Transposable Elements in Mammals. Abbreviations: Gag, group-specific antigen (capsid proteins); Prt, protease; Pol, polymerase; Env, envelope; RT, reverse transcriptase domain; INT, integrase domain; TSD, target site duplication; LTR, long terminal repeat. (Modified from Goodier and Kazazian, 2008).

The non-LTR superfamily is divided between autonomous and non-autonomous elements both lacks LTRs.

Autonomous non-LTR like LTR are transcribed by RNA polymerase II which bind a promoter in the 5′ LTR and encodes two Open Reading Frames (ORFs). ORF1 encodes a nucleic acid binding protein, and ORF2 encodes a protein with Endonuclease (EN) and Reverse Transcriptase (RT) activity (Fig. 10).

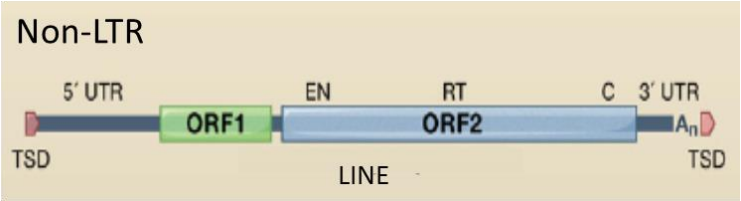


Figure 10. Autonomous Non-LTR structure. Structure of autonomous Non-LTR Transposable Elements in Mammals. Abbreviations: RT, reverse transcriptase domain; TSD, target site duplication; EN, endonuclease domain; C, zinc knuckle domain; An, poly(A). (Modified from Goodier and Kazazian, 2008).

Mammalian non-LTR retrotransposons are exemplified by long interspersed element class 1 (LINE-1 or L1) retrotransposons (Hancks and Kazazian, 2016; Muñoz-Lopez et al., 2016; Richardson et al., 2015).

As for LTR retrotransposons, the transcription of non-LTR retrotransposons generates a full-length mRNA. However, these elements mobilize via target-site-primed reverse transcription (TPRT) mechanism in which the transcript itself reenters the nucleus with the help of its protein products. Those proteins, usually including an endonuclease domain along with the reverse transcriptase. The endonuclease generates a single-stranded 'nick' the target site for integration in the genomic DNA, liberating a 3'-OH that is used to prime reverse transcription of the non-LTR RNA (Luan et al. 1993; Deininger and Batzer 2002) (Fig. 11).

Similarly, to LTR LINEs generally form Target Site Duplications (TSD) upon insertion (Eickbush and Malik, 2002).

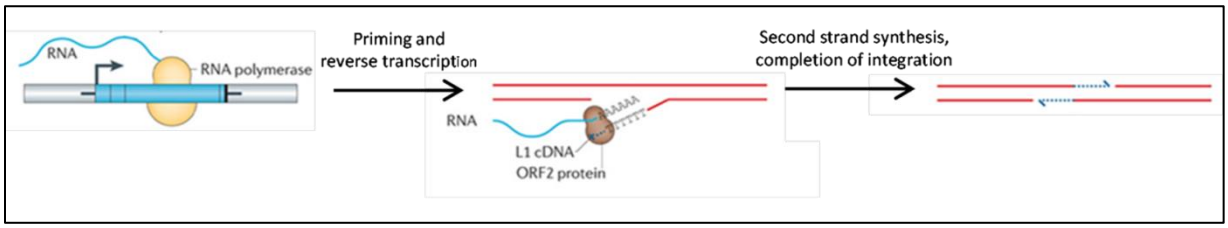


Figure 11. Mechanisms of autonomous Non-LTR Transposable Elements mobilization (Levin & Moran 2011).

Non autonomous non-LTR retrotransposons are segments provided with a transcriptional promoter at the 5' end and a sequence for recruitment of reverse transcriptase. The enzymes for reverse transcription are provided by a non-LTR autonomous retrotransposon which in practice are parasitized.

Two families of non-autonomous non-LTR retrotransposon are currently known:
Short INterspersed Elements (SINEs) and Sine Vntr Alu (SVA) (Rebollo et al. 2010) (Fig.12).
-SINEs are non-autonomous elements that are functionally related to LINEs. They are not deletion derivatives of any autonomous retrotransposon, but instead originate from the accidental retrotransposition of RNA polymerase III transcripts —tRNAs, 7SL RNAs and 5S rRNAs. Although they do not contain any ORF, they possess an internal promoter of RNApo1 III that allows them to be transcribed. Once expressed, they use LINEs' enzymatic machinery to transpose. In humans, the SINE Alu family alone has more than one million copies representing 11% of the genome (Lander et al. 2001).
-SVA elements are probably transcribed by RNA polymerase II (Ostertag EM, et al., 2003; Wang H,et al., 2005). However, SVA elements apparently contain no internal promoter and they might rely, at least partly, on promoter activity in flanking regions (Ostertag EM, et al., 2003; Wang H,et al., 2005).

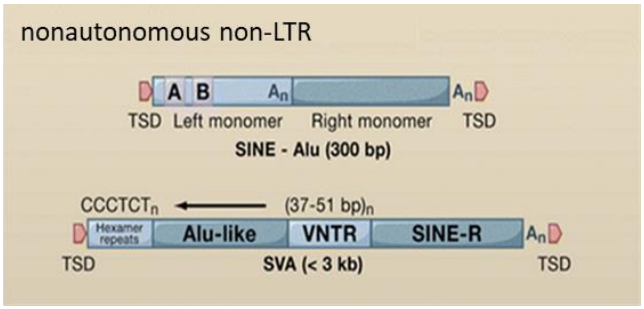


Figure 12. Non-autonomous non-LTR structure. Structure of non-autonomous non-LTR Transposable Elements in Mammals. Abbreviations: TSD, target site duplication; An, poly(A); SVA, (SINE-R, VNTR, Alu); VNTR, variable number tandem repeats. (Modified from Goodier and Kazazian, 2008).

In humans, LTR elements are thought to be inactive while the non-LTR retrotransposon LINE-1 is the only active autonomous TE (Ewing AD, et al., 2010). In addition to mobilizing its own RNA to new genomic locations via a “copy-and-paste” mechanism, LINE-1 is able to retrotranspose other RNAs including Alu, SVA, and occasionally cellular RNAs (Lander ES, et al.,2001; Ostertag EM, et al., 2003; Mills RE, et al., 2007; Buzdin A,et al., 2002; Garcia-Perez JL, et al., 2007).

1.4.1.2 DNA transposons

DNA transposons, or terminal inverted repeat (TIR) transposons, consist of a transposase gene flanked by TIRs, and move via a cut-and-paste mechanism. TIRs are repeating sequences found at both ends of these elements and are inverted with respect to each other. The transposase binds at or near the TIRs, excises the transposon from its existing genomic location and pastes it into a new genomic location (Fig.13). The cleavages of the two strands at the target site are staggered, resulting in a target-site duplication (TSD) typically of 4–8 bp as specified by the transposase.

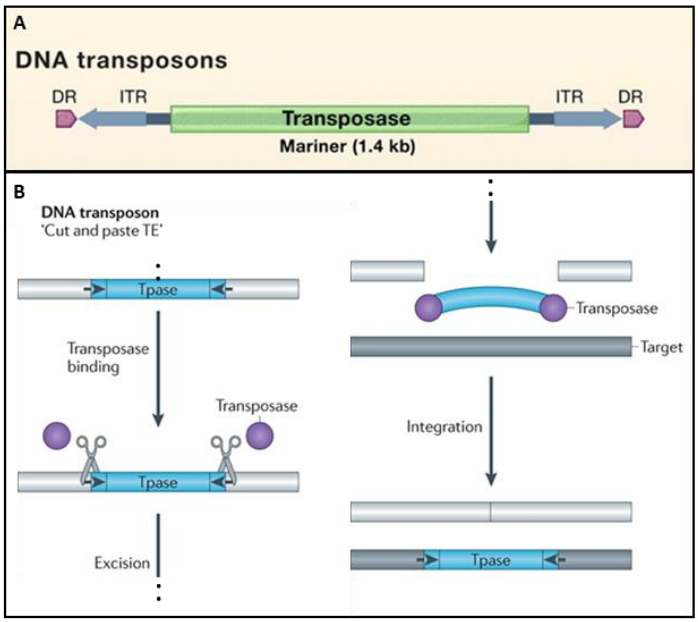


Figure 13. DNA trotransposons. Structure of DNA Transposable Elements in Mammals (Modified from John L. Goodier and Haig H. Kazazian Jr. 2008) and mechanism of DNA transposon mobilization (Levin & Moran 2011). Abbreviations: DR, direct repeat; ITR, inverted terminal repeat.

No active DNA transposons have been identified in mammalian due to lack of functional transposases, with the exception of at least one family of piggyBac elements in little brown bats (Mitra et al., 2013).

1.4.2 *Drosophila* Transposable Elements

There are 96 known families of transposable elements in *D. melanogaster*: 49 LTR families, 27 LINE-like families and 19 DNA transposons, or terminal inverted repeat (TIR) families (Fig. 14; Kaminker et al, 2002).

In *Drosophila*, at least 21% of non-LTR retrotransposons and 45% of LTR retrotransposons are full-length and potentially active, such as the LINE-like elements TART, jockey and Juan and the LTR retrotransposons roo, copia, blood, gypsy, and mdg1 (Sheen et al., 1994 ; Kaminker et al., 2002; Bowen et al., 2001).

Furthermore, at least 16% of the DNA transposons (1360, hobo, Bari1, pogo, and P elements) in *D. melanogaster* are full length and potentially active (Lander et al., 2001; Kaminker et al., 2002; Lerat & Capy, 1999; Muñoz-López et al., 2010; Palazzo et al., 2013).

These classes of element have different distribution in heterochromatin and euchromatin. LTR elements represent 61% of euchromatic transposable elements and approximately 78% of heterochromatic elements. LINE elements represent 24% of the euchromatic and 17% of the heterochromatic transposable element sequence. TIR elements represent 15% in euchromatin and 5% in heterochromatin.

Importantly, not all the families have equal distribution. Three of the 96 families have not been found in the euchromatic portion (Kaminker et al, 2002); these are the P element, R2 and ZAM. Furthermore, the telomere-associated HeT-A and TART have been found absent from the euchromatic portions of all chromosomes except chromosome 4 (Kaminker et al, 2002).

Class LTR		Class non-LTR	Class TIR	Class FB
17.6	invader 1	Baggins	1360	FB
1731	invader 2	Bs	Bari1	
297	invader 3	Crla	Bari2	
3S18	invader 4	Doc	HB	
412	invader 5	Doc2	H	
Accord	McClintock	Doc3	hopper	
aurora	mdg1	F	hopper 2	
blastopia	mdg3	G	looper 1	
blood	micropia	G2	mariner 2	
Burdock	opus	G3	NOF	
Circe	qbert	G4	P	
copia	Quasimodo	G5	pogo	
driver	roo	G6	S	
driver2	rover	Helena	S2	
Dm88	springer	Het-A	Tc1	
frogger	Stalker	I	transib 1	
GATE	Stalker 2	Ivk	transib 2	
gtwin	Stalker 4	jockey	transib 3	
gypsy	Tabor	jockey2	transib 4	
gypsy 2	Tirant	Juan		
gypsy 3	Transpac	R1		
gypsy 4	ZAM	R2		
gypsy 5		Rtla		
gypsy 6		Rtlb		
HMS-Beagle		TART		
Indefix		x		

Figure 14. List of different families of transposable element

1.4.2 Evolutionary potential vs. harmful effect of TEs

Evolutionarily, TE mobilization can provide a source of diversification in the population and thus exert a positive role in adaptation to changing environments. Genetic and epigenetic plasticity is indeed able to protect a population from extinction under increasing ecological stress (Lindsey et al., 2013). TEs could have been used as a source of non-coding sequence material, for example non-coding RNA, antisense mRNA or double stranded RNA, to fuel new regulatory strategies during vertebrate evolution (Chuong et al., 2017). Moreover, in the mouse, the expansion of TEs has been associated to the dispersal of enhancers with forebrain specific activity, possibly leading to the evolution of mammalian neocortex (Chuong et al., 2017).

TEs seem to have a relevant role in early embryonic development and in the Central Nervous System (CNS). In the brain of many species, LINE expression has been proposed to provide a source of neuronal diversity and sustain the complexity of neuronal networks (Muotri et al., 2005; Perrat et al., 2013).

However, the mobile property of TEs is the reason for their mutational potential. Both insertions and excisions of TEs can cause genomic instability, thus causing many human diseases, including genetic disorders, psychiatric problems, and cancer (Tighe et al., 2004; Miki et al., 1992 ; Miki et al., 1996 ; Johnston et al., 2001 ; Wallace et al., 1991 ; Guffanti et al., 2014). Furthermore, TE insertions may result in insertional mutations, non-allelic homologous recombination (NAHR), creation of novel regulatory sequences, alternative splicing and epigenetic changes (Belancio et al., 2009). In humans, there are at least 75 documented cases of diseases resulting from de novo TE insertions (Beck, et al., 2011; Goodier & Kazazian, 2008).

Furthermore, failure of TE regulatory mechanisms and TE iperactivity has been proposed to be at the basis of aging and of several diseases. Animal models, in particular *Drosophila*, have emerged as valuable systems to study the physiopathological roles of TEs.

A recent study that took advantage of a fly model of ALS, based on the ectopic expression of the TDP43 protein, provided the first evidence that TEs could contribute to neurodegeneration (Krug et al., 2017).

1.4.4 Regulation of Transposable element activity

For their genotoxic potential, mobile elements are usually kept repressed by epigenetic mechanisms. The heterochromatin structure represents one of the major players in the repression of TEs (Liang et al., 2002; Kato et al., 2007). This involves DNA methylation, in mammalian and plants, and deposition of histone repressive marks, including H3K9me3, H3K27me and H4 lysine 20 methylation (H4K20me3). In addition, deposition of HP1 protein, which binds H3K9me3, stabilizes the heterochromatin structure and further spreads this histone modification (Slotkin and Martienssen, 2007; Bulut-Karslioglu et al., 2014; Pezic et al., 2014). The heterochromatin structure is also stabilized by the anchoring of H3K9me3 and HP1 at the inner nuclear membrane (Towbin et al. 2012).

Expression of TEs is also opposed by different mechanisms involving small noncoding RNAs. These can act as a defense against TEs, both by preventing transcription via heterochromatin formation, and by degrading TE transcripts (Grewal & Elgin 2007; Matzke et al. 2007; Slotkin & Martienssen 2007; Zaratiegui et al. 2007).

These mechanisms involve interfering RNAs (RNAi) which act at the chromatin level and promote heterochromatinization of the mobile elements, and piwi-interacting RNAs which cause post-transcriptional gene silencing of the transposon's transcript (Levin and Moran, 2011; Rigal and Mathieu 2011).

1.4.4.1 Regulation of TEs in the germline

In *D. melanogaster*, movement of all TEs is tightly regulated in germline cells, where uncontrolled transposition events may impose significant genomic defects that would be inherited by successive generations. The primary germline regulatory pathway is mediated by PIWI (P-element induced wimpy testis), Aubergine (AUB), Argonaute 3 (AGO3) and small PIWI-interacting RNAs (piRNAs) that regulate TEs via RNAi and epigenetic mechanisms, including heterochromatin formation (Yin and Lin, 2007; Muerdter et al., 2013). The piRNAs derived from TEs are generated by processing of sense and antisense transcripts from TEs into small RNAs by PIWI and AUB (Vagin et al., 2006). piRNAs generated from transposon transcripts form PIWI-piRNA complexes that function to silence transposon transcripts via RNAi (Siomi et al., 2010). Furthermore, in fruit flies it is believed that the piRNA pathway induces transcriptional repression by initiating deposition of repressive histone marks (Klenov et al. 2007; Grewal & Elgin 2007).

Both the proteins and piRNAs required for this pathway are almost exclusively produced in germ cells (Brennecke et al., 2006).

1.4.4.2 Regulation of TEs in somatic cells

In *D. melanogaster*, the regulation of retrotransposons in somatic cells is mediated by esiRNAs, which are generated by Dicer2 (Dcr2) cleavage of long dsRNA precursors derived from convergent sense and antisense transcription of retrotransposons in the genome (Russo et al., 2016) (Russo et al., 2002). Data support a model in which esiRNAs regulate retrotransposons in the nucleus via heterochromatin formation in D. melanogaster and other eukaryotic organisms, such a S. pombe (Fagegaltier et al. 2009, ; Kloc et al., 2008). Mechanicistically, esiRNAs generated by Dcr2, interact with an argonaute-family protein (called RITS in Schizosaccharomyces pombe) and guides the cleavage of nascent transcripts, which are still attached to RNA polymerase II and the DNA strand. Cleavage of this nascent transcript targets this region of chromatin for modification by the recruitment of a H3K9 methyltransferase and other proteins (McCullers & Steiniger 2017).

A similar siRNA-mediated pathway has been reported in humans to regulate LINE-1 retrotransposons via RNAi, but the dsRNA precursors are generated by a different mechanism than in D. melanogaster (Yang et al., 2006).

1.4.5 Transposable elements in organismal aging and AD

The reasons why some TEs appear to be upregulated in certain disease conditions remain poorly understood, but environmental stimuli, such as infections (van der Kuyl, 2012) and cellular stress (Mourier et al., 2014), as well as natural cellular processes such as senescence (De Cecco et al., 2013), destabilize epigenetic marks that normally silence TEs in the genome, triggering sporadic transcriptional activation.

1.4.5.1 Deregulation of transposable elements in organismal aging

DNA damage (Shiloh and Ziv, 2013; Biton et al., 2008; Shull et al., 2009, Coufal et al. 2011), global chromatin relaxation (Levin and Moran, 2011), and reduction of *Drosophila* Lamin B (Chen et al., 2016) result in an increase in Transposable element (TE) expression.

Aberrant TE activation has been observed during organismal aging, in mice (De Cecco et al.,

2013; Van Meter et al., 2014), in human fibroblasts culture during replicative senescence (De Cecco et al. 2013b), in Drosophila fly heads (Li et al., 2013) and in the Drosophila fat body, an organ equivalent to the mammalian liver and adipose tissue (Chen et al., 2016).

Despite the evidence for genomic instability in AD (Frost et al., 2014); Gjoneska et al., 2015; De Jager et al., 2014), only a recent study implicates an altered Transposable elements (TEs) expression in AD (Guo et al., 2018).

1.4.5.2 Deregulation of transposable elements in AD pathogenesis

Two recent studies have provided evidence that TEs contribute to the pathogenesis of AD (Guo et al., 2018; Sun et al., 2018). The group of J. Shulman found increased TE expression in the brain of AD patients and showed that ectopic expression of the hTAU protein in the *Drosophila* brain was associated with increased TE expression and mobility (Guo et al., 2018). Using the same fly model, Sun and colleagues proposed that TEs contribute to neurodegeneration, showing that this was prevented by feeding the flies with RT inhibitors, which inhibited TE genomic insertion.

2. AIM OF THE THESIS

The organization of chromatin is tightly regulated, being crucial for the control of gene expression, both during organism development and in tissue homeostasis. In particular, perinuclear heterochromatin has an evolutionarily conserved function in restraining the activity of transposable elements (TEs), which are potentially mutagenic. Ageing and several ageing-related diseases have been proposed to involve a decondensation of chromatin. A widespread decondensation of heterochromatin, leading to unscheduled TE expression and de novo genomic insertions associated with accumulation of DNA damage, have recently emerged as key contributors of aging and aging-dependent pathologies, including several neurological disorders, such as AD. However, it is unclear how heterochromatin is perturbed and its organisation is altered during aging and the pathogenesis of those disorders. Understanding the underlying molecular mechanisms could lead to the identification of potential biomarkers and therapeutic targets for prevention and treatment of some currently incurable diseases, such as AD, which are expected to become epidemic in the coming decades.

PIN1 is the only enzyme that isomerases Serine/Threonine-Proline motifs in a phosphorylation-dependent manner, and its activity promotes healthy aging and protects against AD, regulates chromatin and transcription, and modulates DNA damage response. Results previously obtained in our laboratory showed that, in *Drosophila*, the PIN1 orthologue Dodo promoted the anchoring of heterochromatin to the nuclear lamina, and that loss of Dodo was associated with heterochromatin decondensation and TE neurotoxic hyperactivity.

We hypothesized that PIN1 preserves the organization of the nuclear lamina, thus maintaining heterochromatin condensation and preventing TE toxic hyperactivity. To test this hypothesis, in this work we aimed to:

1. Assess the role of PIN1 in the regulation of heterochromatin condensation and TE activity;
2. Elucidate the underlying molecular mechanisms and their relevance for AD pathogenesis.

3. RESULTS

3.1. Dodo has an evolutionarily conserved function in preserving brain neuron health by maintaining heterochromatin and restraining TE activity

3.1.1. Dodo has an evolutionarily conserved function in preventing TE activity

Recent work in our laboratory has suggested that Dodo, the Drosophila orthologue of PIN1, which is ubiquitously expressed in several adult fly tissues (Fig. 15 A), could have a role in the regulation of TEs. Analysis of a mutant fly strain harboring a P-element insertion in the 5'UTR region of the *dodo* gene (*dodo*^{EY3779}, hereafter referred to as *dodo* mutant), which expresses low levels of the Dodo protein, showed that loss of Dodo was associated, in the fly head and gonads, with increased mRNA levels of several DNA and RNA TEs, compared to control flies (Voto, PhD thesis 2016). Similar effects were observed, in the fly head, upon neuron-specific *dodo* RNAi, and correlated with neurodegeneration, which was prevented by administration of 3TC (also known as lamivudine), a competitive inhibitor of the reverse transcriptase enzyme (Voto PhD thesis 2016). This suggested that loss of Dodo was associated with a neurotoxic hyperactivity of TEs. In support of this, we found that, in the head of flies expressing *dodo* RNAi under control of the ubiquitous Tubulin promoter (Fig. 16 A), the number of copies of the LTR retrotransposon *ZAM* and LINE-like retrotransposon *IVK* (two hallmarks of TE deregulation associated with loss of Dodo) inserted in the genome was increased, compared to control flies, as demonstrated by Copy Number Variation (CNV) assay (Fig. 16 B). This increase in TE copy number correlated with DNA damage, as shown by western blot analysis of phosphorylated H2Av (commonly referred to as γH2Av), a marker of DNA double strand breaks formation, homologous to mammalian γH2AX (Fig. 16 D). This was in line with data previously obtained in the lab showing that, in *dodo* mutant fly ovary, loss of Dodo was associated with DNA damage (Voto PhD thesis 2016). We observed that, in *dodo* mutant fly heads, DNA damage was dependent on TE insertions in the genome, as demonstrated by the fact that it was suppressed by treatment of flies with 3TC (Fig. 16 D). Furthermore, in aged *dodo* mutant flies, loss of *dodo* was associated with a reduction of learning performance and memory, two hallmarks of neuronal function, in line with the previous data from our laboratory showing that, in the same conditions, loss of Dodo was associated with

neurodegeneration (Voto PhD thesis 2016). All these defects were suppressed by treatment of flies with 3TC (Fig. 16 E, F and Voto PhD thesis 2016), demonstrating that loss of Dodo is associated with TE neurotoxic hyperactivity.

These data suggested that Dodo could have a neuroprotective role by restraining TE activity.

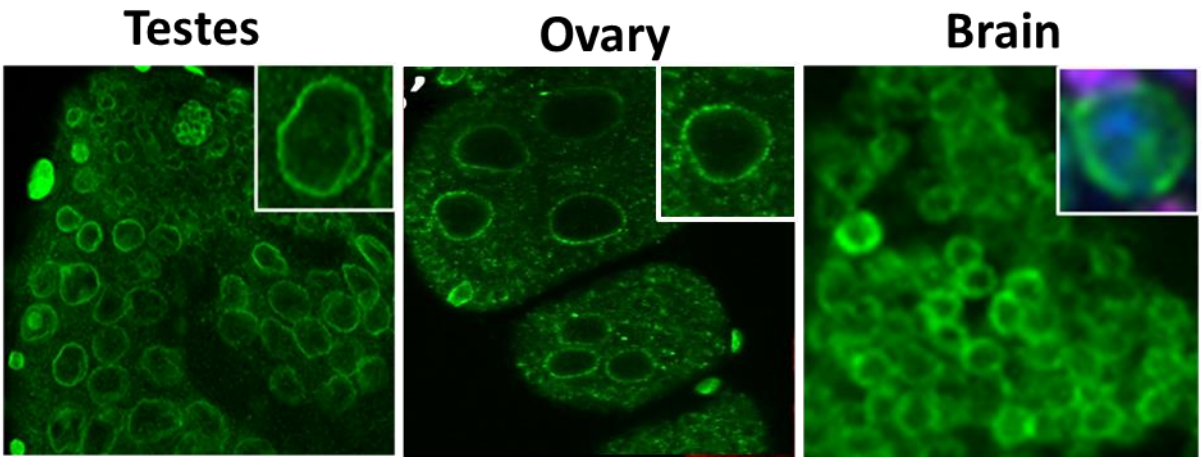


Figure 15. Dodo protein expression in adult fly tissues.
Single confocal section whole-mount immunofluorescence images of Dodo (green), using an anti-PIN1 antibody that recognizes the Dodo protein, in the indicated tissues of 4 days old wild type flies. Insets are representative of nuclei of testis spermatocytes, ovary nurse cells, and brain neurons.

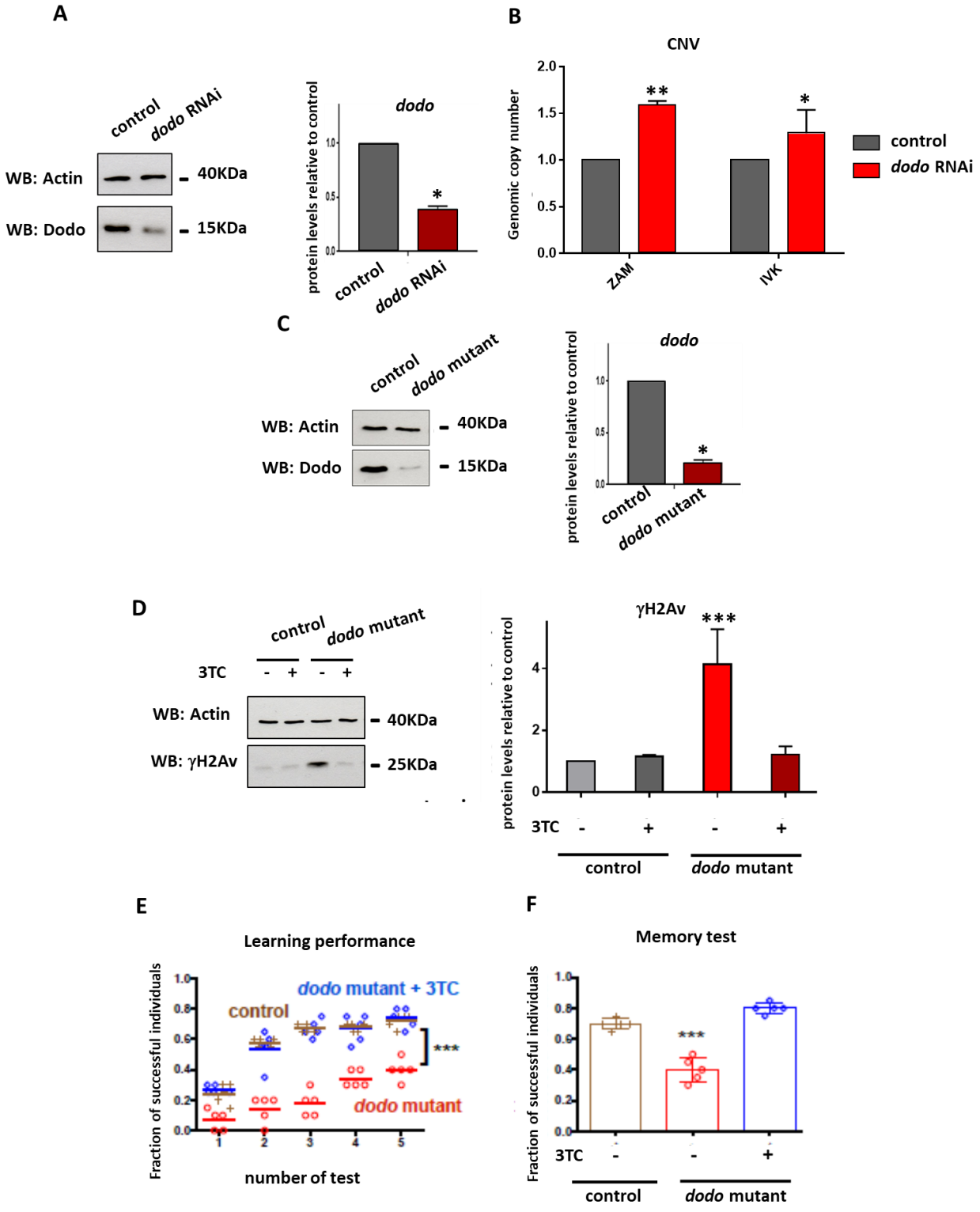

A
control
dodo RNAi
WB: Actin
40KDa
WB: Dodo
15KDa
dodo
protein levels relative to control
control
dodo RNAi
B
CNV
Genomic copy number
control
dodo RNAi
ZAM
IVK
C
control
dodo mutant
WB: Actin
40KDa
WB: Dodo
15KDa
dodo
protein levels relative to control
control
dodo mutant
D
control
dodo mutant
3TC
WB: Actin
40KDa
WB: γH2Av
25KDa
γH2Av
protein levels relative to control
3TC
control
dodo mutant
E
Learning performance
dodo mutant + 3TC
control
dodo mutant
Fraction of successful individuals
number of test
F
Memory test
Fraction of successful individuals
3TC
control
dodo mutant

Figure 16. Loss of Dodo is associated with TE mobilization leading to DNA damage and cognitive defects.

A) Western blot analysis with anti-PIN1 antibody in the heads of 4 days old control flies ((*w*^{1118}/*w*^{1118};*UAS-dodo*^{RNAi}/+;) and flies expressing *dodo* RNAi with the *Tubulin-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of Dodo protein level is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.

B) Copy number variation (CNV) assay in the heads of 4 days old control flies (*UAS-dodo*^{RNAi}) and flies expressing a *dodo* RNAi with the *Tubulin-GAL4* driver. Values represent mean ± s.d. of N=3 biological replicates. *P value <0.05 and **P value <0.01 by two tailed unpaired Student's t-test.

C) Western blot analysis with anti-PIN1 antibody in the heads of 4 days old control (*w*^{1118}) and *dodo* mutant (*dod*^{EY03779}) flies. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of Dodo protein level is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.

D) Western blot analysis in the heads of 4 days old control (*w*^{1118}) and *dodo* mutant (*dod*^{EY03779}) flies. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of yH2Av protein level is shown in the right panel. Values represent mean ± s.d. ***P value <0.001, by two tailed unpaired Student's t-test.

E) Learning assay in 24 days old control (*w*^{1118}) and *dodo* mutant (*dod*^{EY03779}) flies. Values represent mean ± s.d. ***P value <0.001 by two tailed unpaired t-test. Courtesy of Dr. Valeria Specchia, University of Salento, Italy.

F) Memory assay in 24 days old control (*w*^{1118}) and *dodo* mutant (*dod*^{EY03779}) flies. Values represent mean ± s.d. ***P value <0.001 by two tailed unpaired t-test. Courtesy of Dr. Valeeria Specchia, University of Salento, Italy.

Dodo protein sequence shows a high degree of similarity with that of human PIN1 (hPIN1) (Hsu et al., 2001), which suggests that Dodo functions are conserved from *Drosophila* to humans. To assess whether negative regulation of TE activity represents a fundamental function of Dodo, required for neuroprotection, we sought to generate transgenic flies expressing the hPIN1 protein and test if this could complement the function of Dodo (Supplementary Fig. S1 A, B, C) (Celora thesis 2019). Silencing of *dodo* by RNAi with the *Tubulin-GAL4* driver was associated with an increased number of dying cells in the brain of aged flies, compared to control flies, as assessed by TUNEL assay (Fig. 17 B), a common method for detecting DNA fragmentation that results from activation of nucleases during cell death. In these conditions, ectopic expression of hPIN1 reduced the number of $TUNEL^{+}$ cells to levels similar to control flies (Fig. 17 B). Furthermore, in aged flies expressing *dodo* RNAi with the *Tubulin-GAL4* driver, neurodegeneration was associated with a reduction of fly locomotor activity, a well-established readout of nervous system function (Frost et al., 2014), compared to control flies. In these conditions, ectopic expression of hPIN1 rescued fly locomotor activity (Fig. 17 C), demonstrating that Dodo neuroprotective function is conserved from *Drosophila* to humans. Then, we selected two hallmarks of TE deregulation associated with loss of Dodo, representative of each class, *i. e.* the LTR retrotransposon *ZAM* and LINE-like retrotransposon *IVK*. Silencing of *dodo* in fly brain neurons by RNAi, with the *Elav-GAL4* driver led to increased RNA levels of the *IVK* and *ZAM* retrotransposons (Fig. 17 A, previously reported in Voto PhD thesis 2016), compared to control flies. In these conditions, ectopic expression of hPIN1 led to repression of both *IVK* and *ZAM* (Fig. 17 A), demonstrating that regulation of TE RNA levels is a fundamental function of Dodo, conserved from *Drosophila* to humans.

Taken together, these data demonstrated that Dodo has a fundamental role, conserved from *Drosophila* to humans, in restraining TE activity and protecting the health of brain neurons.

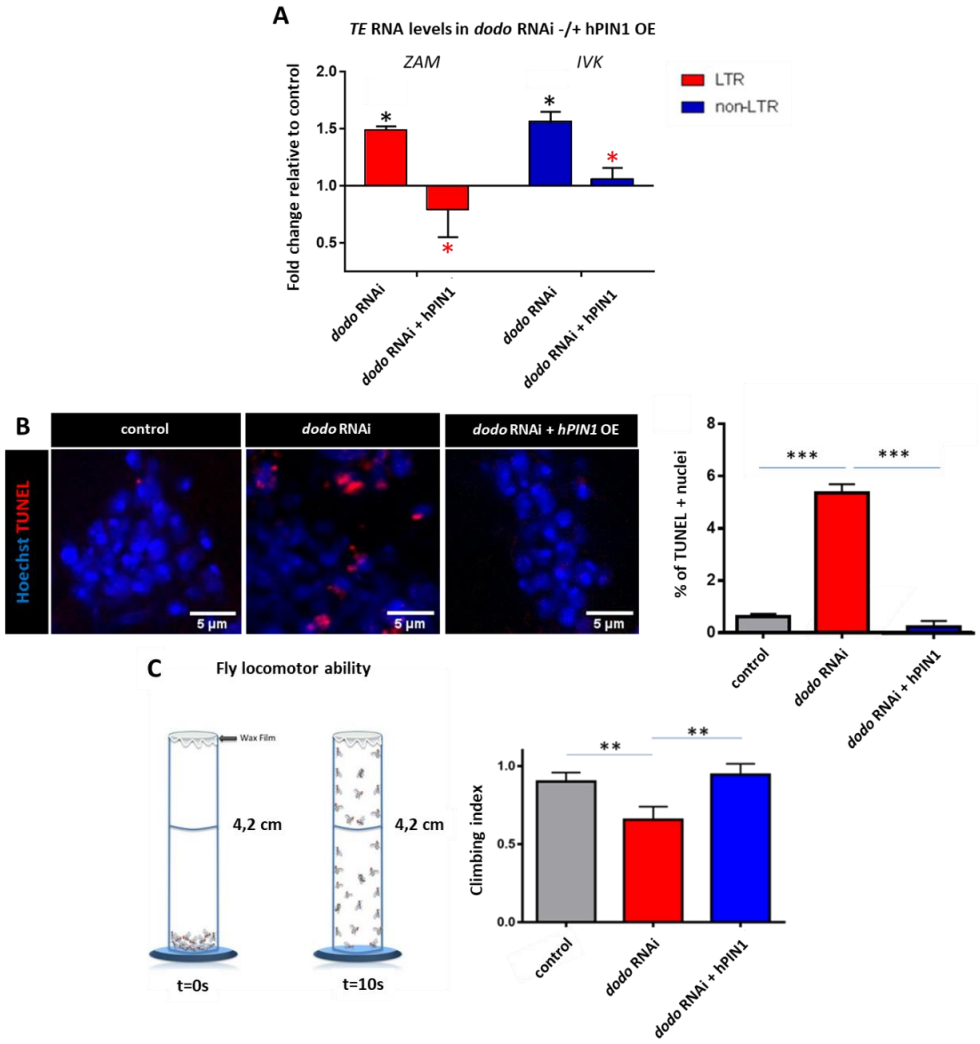


Figure 17. Dodo has an evolutionarily conserved function in restraining TE mRNA levels, and in preserving neuron survival and function during aging in the *Drosophila* brain.

A) RT-qPCR analysis in the heads of 4 days old flies expressing the indicated constructs with the *Elav-GAL4* driver, relative to control flies (w^{1118}/w^{1118};*UAS-dodo*^{RNAi}/+*UAS-hPIN1/+)*. *Actin* was used as reference for quantification. Values represent mean ± s.d. of N=3 biological replicates. Black and red asterisks indicate P value of *dodo* RNAi v_s control and *dodo* RNAi + *hPIN1* OE, respectively. *P value <0.05 by two tailed unpaired Student's t-test.

B) Whole-mount TUNEL staining in the brain of 20 days old control flies (w^{1118}/w^{1118};*UAS-dodo*^{RNAi}/+;) and flies expressing the indicated constructs with the *Tubulin-GAL4* driver. Nuclei are stained with HOECHST. Images are representative of N=6 individuals. Quantification of TUNEL^{+} nuclei is shown in the right panel. Values represent mean ± s.d. of…. ***P value <0.001 by two tailed unpaired Student's t-test.

C) Negative geotaxis assay in 14 days old control flies (w^{1118}/w^{1118};*UAS-dodo*^{RNAi}/+;) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Climbing index indicates the fraction of successful individuals. Values represent mean ± s.d. of N=5 biological replicates **P value <0.01 by two-tailed unpaired Sudent's t-test.

3.1.2. Dodo has an evolutionarily conserved function in maintaining heterochromatin

TEs are mostly clustered in repressive heterochromatin marked by tri-methylation of Histone 3 Lys9 (H3K9me3) (Peric-Hupkes et al., 2010; Shah et al., 2013). H3K9me3 is bound by Heterochromatin protein 1a (HP1a) (James & Elgin 1986), which recruits histone methyltranferases, thus sustaining H3K9 methylation (Bannister et al., 2001, Lachner et al., 2001). We observed that, in *dodo* mutant fly heads, loss of Dodo was associated with a reduction of dHP1a total protein levels, compared to control flies, as demonstrated by western blot analysis, suggesting that Dodo regulated dHP1a protein levels (Fig. 18 A, Ferrari Bravo data previously reported in Voto PhD thesis 2016). This was in line with data previously obtained in our laboratory showing that loss of Dodo was associated with reduction of dHP1a-containing heterochromatin foci, in the fly head and ovary, and of deposition of dHP1a in the regulatory sequences of the *ZAM* retrotransposon and in the TE-poor heterochromatin regions, in the fly ovary (Voto PhD thesis 2016). We further observed that, in *dodo* mutant fly head, loss of Dodo was associated with a reduction of H3Kme3 total protein levels, compared to control flies, as demonstrated by western blot and immunofluorescence analysis, suggesting that Dodo also regulated H3Kme3 protein levels (Fig. 18 B,C, Ferrari Bravo data previously reported in Voto PhD thesis 2016). This was in line with data previously obtained in our laboratory showing that loss of Dodo was associated with reduction of H3Kme3 total protein levels, in the fly head and ovary, and of deposition of H3Kme3 in the regulatory sequences of the *ZAM* and IVK retrotransposons and in the TE-poor heterochromatin regions, in the fly head (data not shown, Voto PhD thesis 2016).

Taken together, these results suggested that Dodo could have a fundamental function in preventing heterochromatin relaxation, thus restraining TE activity.

We analysed dHP1a and H3K9me3 total protein levels in the heads of flies expressing *dodo* RNAi under control of the neuron specific *elav* promoter, using the GAL4/UAS system (Supplementary Fig. S1 A, C). We observed a strong reduction of HP1a (Fig. 19 A) and H3K9me3 (Fig. 19 B) total protein levels, compared to control flies. In these conditions, ectopic expression of hPIN1 (Supplementary Fig. S1 A, B, C) rescued the protein levels of both HP1a (Fig. 19 A) and H3K9me3 (Fig. 19 B), demonstrating that maintenance of HP1a and H3K9me3 protein levels is a fundamental function of Dodo, conserved from *Drosophila* to humans.

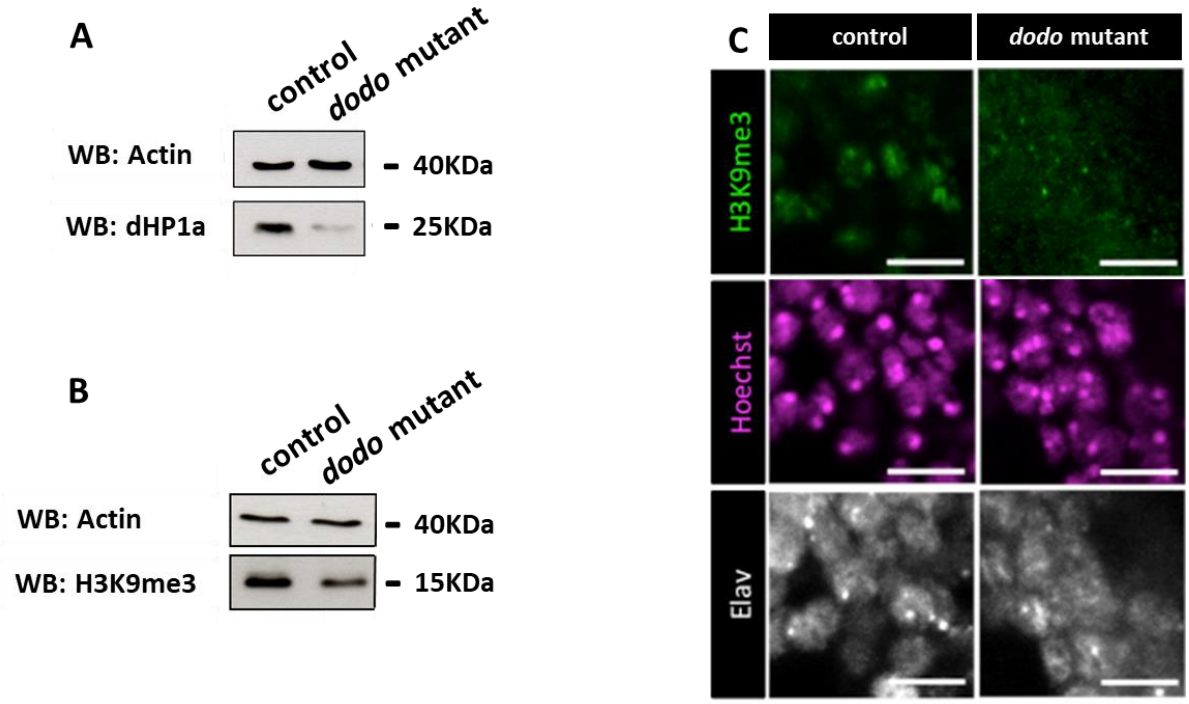


Figure 18. Loss of Dodo is associated with loss of heterochromatin marks in the *Drosophila* brain.

A-B) Western blot analyses in the heads of 4 days old control (w^{1118}) and *dodo* mutant ($dod^{EY03779}$) flies. Actin was used as loading control. Blots are representative of N=3 biological replicates.

C) Immunofluorescence analysis in head cryosections of 4 days old control (w^{1118}) and *dodo* mutant ($dod^{EY03779}$) flies. Nuclei are stained with HOECHST. Scale bar 4µm. Courtesy of Dr. F. Napoletano (University of Trieste).

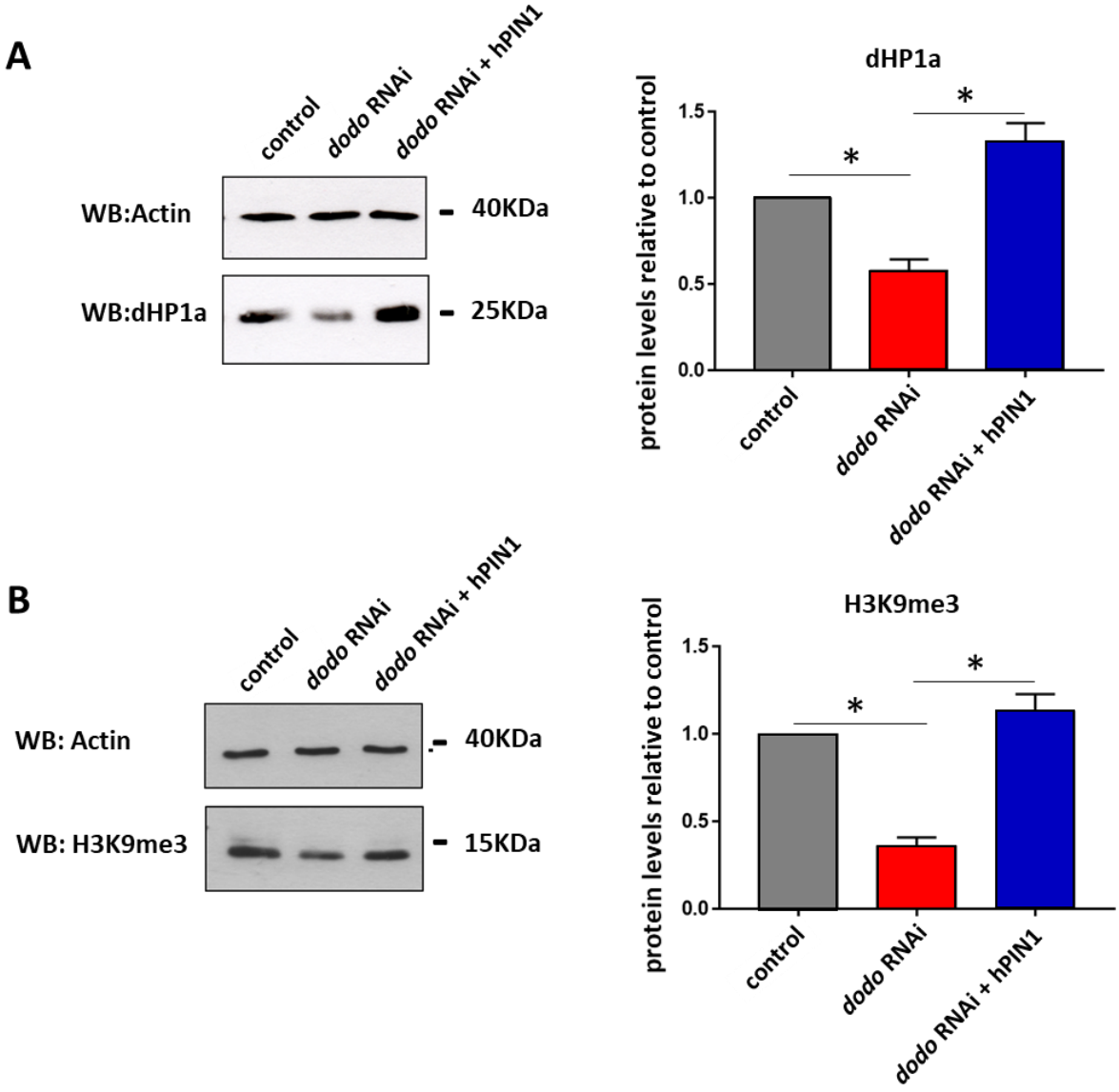


Figure 19. Dodo maintains dHP1a and H3K9me3 protein levels in the *Drosophila* brain.
A) Western blot analysis in the heads of 4 days old control flies (w^{1118}/w^{1118};UAS-$dodo^{RNAi}$/+;) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of dHP1a protein level is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.
B) Western blot analysis in the heads of 4 days old control flies (w^{1118}/w^{1118};UAS-$dodo^{RNAi}$/+;) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of H3K9me3 protein level is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.

3.1.3. Dodo restrains TE activity and preserves the health of brain neurons by heterochromatin-mediated transcriptional silencing

We hypothesized that Dodo could sustain H3K9 methylation and preserve heterochromatin formation, thus restraining TE neurotoxic hyperactivity, by maintaining dHP1a protein levels. To test this hypothesis, first we analysed *dhp1*$^{05/+}$ mutant flies expressing reduced levels of dHP1 protein (Fig. 20 A). In the brain of these flies, H3K9me3 total protein levels were reduced, compared to control flies, as demonstrated by immunofluorescence (Fig. 20 B) and western blot (Fig. 20 C) analyses similar to what we observed in Dodo-depleted neurons (Fig. 20 C). This correlated with increased mRNA levels of several TEs, including *ZAM* and *IVK* (Fig. 20 D). These results suggested that both formation of heterochromatin and negative regulation of TEs requires HP1 and led us to hypothesize that Dodo might prevent chromatin relaxation through maintenance of dHP1 protein levels. Hence, to assess if HP1a protein is required to maintain H3k9me3 levels upon loss of Dodo, we overexpressed HP1a in the brain of flies expressing *dodo* RNAi, using the control of the *Elav-GAL4* driver (Fig. 21 A). Silencing of *dodo* led to strong reduction of H3K9me3 (Fig. 21 B) protein levels, compared to control flies. In these conditions, ectopic expression of dHP1a rescued the protein levels of H3K9me3 (Fig. 21 B), demonstrating that maintenance of H3K9me3 protein levels is mediated by dHP1a. Furthermore**,** in the same conditions, overexpression of *dHP1a* suppressed all the other alterations associated with loss of Dodo: TE derepression, as shown by RT-qPCR analysis of the mRNA levels of *IVK* and *ZAM* retrotransposons (Fig. 21 C); DNA damage accumulation, as shown by western blot analysis (Fig. 21 D); neurodegeneration, as assessed by TUNEL assay (Fig. 22 A); cognitive and motor defects, as shown by learning (Fig. 22 B), memory (Fig. 22 C), and locomotor assays (Fig.22 D).

Taken together, these results demonstrated that Dodo, by maintaining dHP1a protein levels and heterochromatin formation, restrains TE activity and preserves neuronal health.

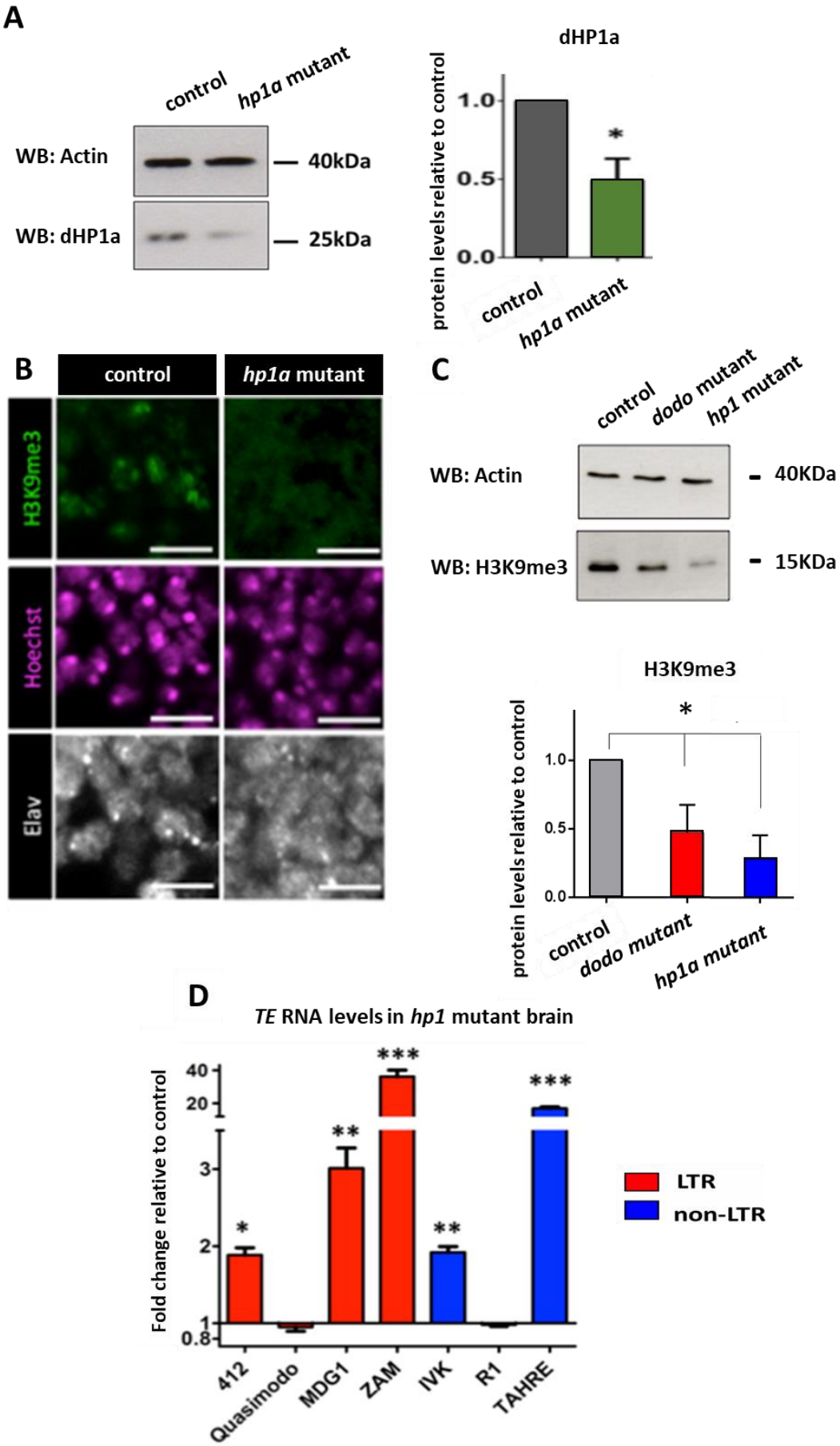

A
control
hp1a mutant
WB: Actin
40kDa
WB: dHP1a
25kDa
dHP1a
protein levels relative to control
1.0
0.5
0.0
*
control
hp1a mutant
B
control
hp1a mutant
H3K9me3
Hoechst
Elav
C
control
dodo mutant
hp1 mutant
WB: Actin
40KDa
WB: H3K9me3
15KDa
H3K9me3
protein levels relative to control
1.0
0.5
0.0
*
control
dodo mutant
hp1a mutant
D
TE RNA levels in hp1 mutant brain
Fold change relative to control
40
20
3
2
1
0.8
*
**
***
**
***
412
Quasimodo
MDG1
ZAM
IVK
R1
TAHRE
LTR
non-LTR

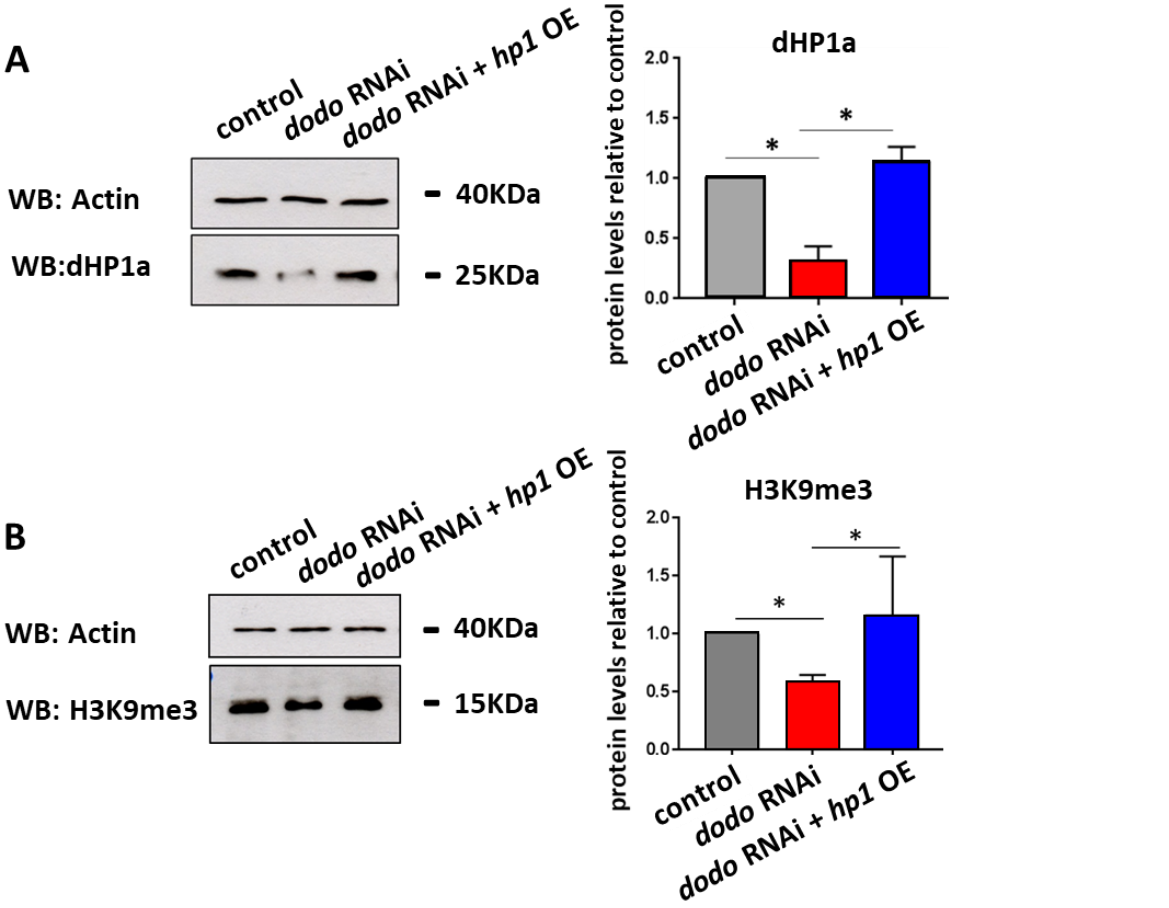
Figure 20. Loss of dHP1a is associated with loss of heterochromatin marks and increase of TE mRNA in the *Drosophila* brain.

A) Western blot analysis in the heads of 4 days old control (w^{1118}) and *dhp1a* mutant ($dhp1a^{05}/+$) flies. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of dHP1a protein level is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.

B) Immunofluorescence analysis in head cryosections of 4 days old control (w^{1118}) and *dhp1a* mutant ($dhp1a^{05}/+$) flies. Nuclei are stained with HOECHST. Scale bar 4µm. Courtesy of Dr. F. Napoletano.

C) Western blot analysis in the heads of 4 days old control (w^{1118}), *dodo* mutant ($dod^{EY03779}$) and *dhp1a* mutant ($dhp1a^{05}/+$) flies. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of H3K9me3 protein level is shown in the bottom panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.

D) RT-qPCR analysis in the heads of 4 days old *dhp1a* mutant ($dhp1a^{05}/+$) relative to control (w^{1118}) flies. *actin* was used as reference for quantification. Values represent mean ± s.d. of N=3 biological replicates. *P value <0.05, **P value <0.01; ***P value <0.001 by two tailed unpaired Student's t-test.


A
control
dodo RNAi
dodo RNAi + hp1 OE
WB: Actin
WB:dHP1a
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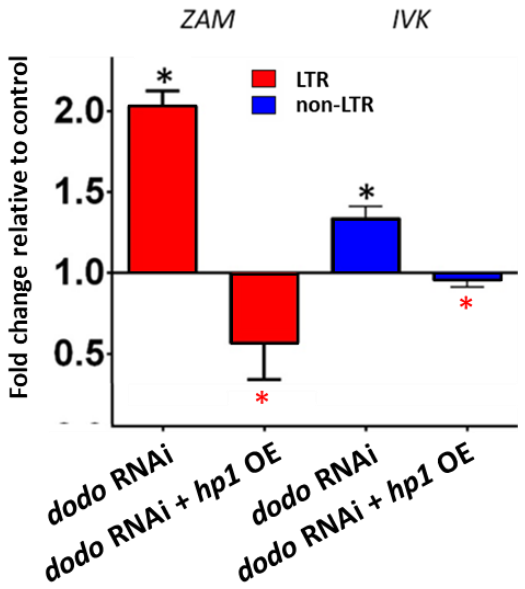
dHP1a
protein levels relative to control
B
WB: Actin
WB: H3K9me3
40KDa
15KDa
H3K9me3


C
TE RNA levels in dodo RNAi -/+ hp1 OE
ZAM
IVK
LTR
non-LTR
Fold change relative to control
dodo RNAi
dodo RNAi + hp1 OE

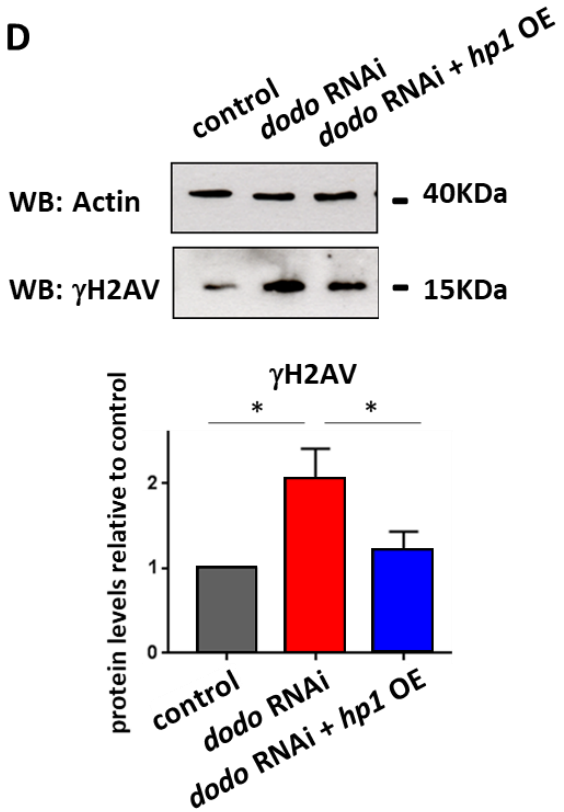

D
control
dodo RNAi
dodo RNAi + hp1 OE
WB: Actin
WB: γH2AV
40KDa
15KDa
γH2AV
protein levels relative to control

Figure 21. Loss of Dodo is associated with loss of dHP1a-dependent heterochromatin formation and inhibition of TE activity in the *Drosophila* brain.

A) Western blot analysis in in the heads of 4 days old control flies (*w^{1118}/w^{1118};UAS-dodo^{RNAi}/+;*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of dHP1a protein level is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.

B) Western blot analysis in in the heads of 4 days old control flies (*w^{1118}/w^{1118};UAS-dodo^{RNAi}/+;*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of H3K9me3 protein level is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.

C) RT-qPCR analysis in the heads of 4 days old flies expressing the indicated constructs with the *Elav-GAL4* driver, relative to control flies (*w^{1118}/w^{1118};UAS-dodo^{RNAi}/+UAS-dHP1a/+)*. *actin* was used as reference for quantification. Values represent mean ± s.d. of N=3 biological replicates. Black and red asterisks indicate P value of *dodo* RNAi v_s control and *dodo* RNAi + *dHP1a* OE, respectively. *P value <0.05 by two tailed unpaired Student's t-test.

D) Western blot analysis in the heads of 4 days old control flies (*w^{1118}/w^{1118};UAS-dodo^{RNAi}/+;*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of yH2Av protein level is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.

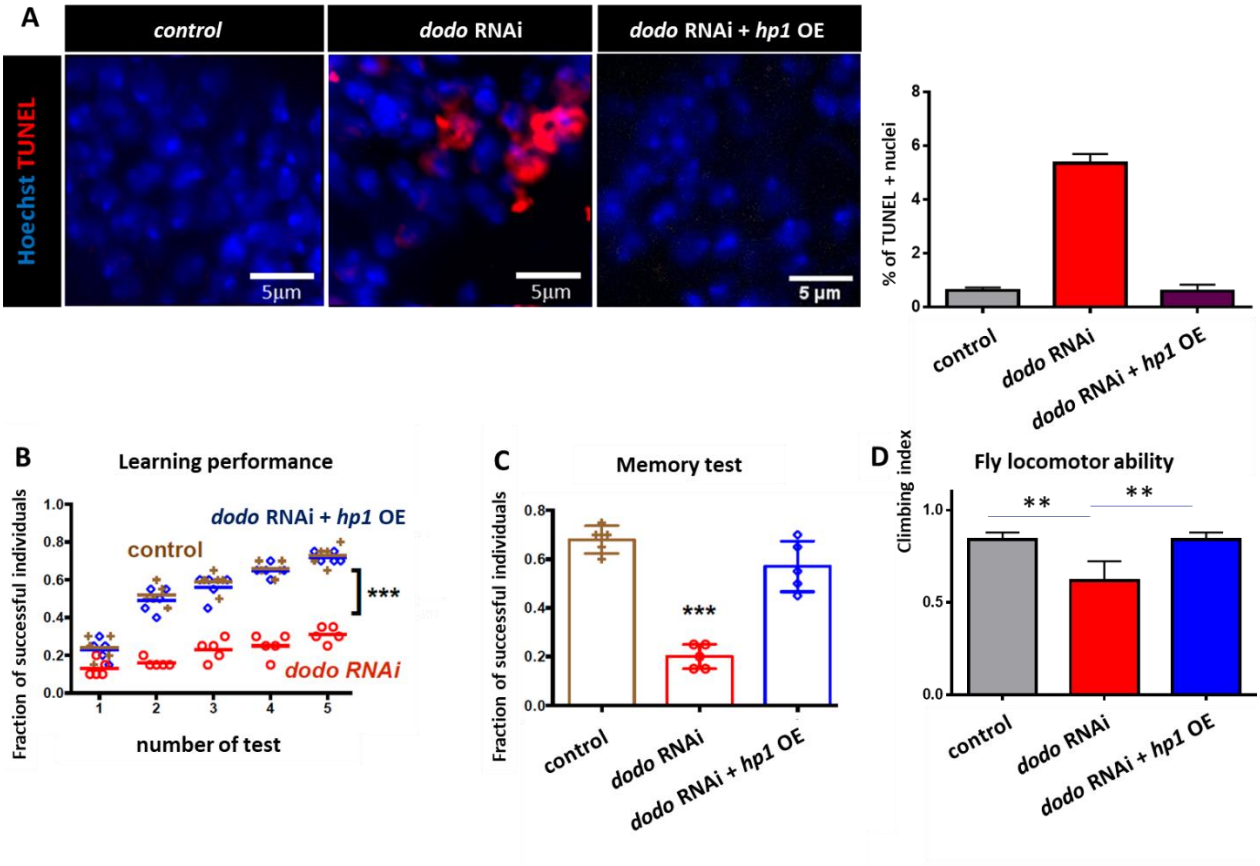


Figure 22. Loss of Dodo is associated with loss of dHP1a-dependent neuronal health.

A) Whole-mount TUNEL staining in the brain of 20 days old control flies (*w*^{1118}*/w*^{1118}*;UAS-dodo*^{RNAi}*/+;*) and flies expressing the indicated constructs with the *Tubulin-GAL4* driver. Nuclei are stained with HOECHST. Images are representative ofof N=6 individuals. Quantification of TUNEL^{+} nuclei is shown in the right panel. Values represent mean ± s.d. ***P value <0.001 by two tailed unpaired Student's t-test.

B) Learning assay in 36 days old control flies (*w*^{1118}*/w*^{1118}*;UAS-dodo*^{RNAi}*/+;*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Values represent mean ± s.d. ***P value <0.001 by two tailed unpaired t-test. Courtesy of Dr. Valeria Specchia, University of Salento, Italy.

C) Memory assay in 36 days old control flies (*w*^{1118}*/w*^{1118}*;UAS-dodo*^{RNAi}*/+;*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Values represent mean ± s.d. ***P value <0.001 by two tailed unpaired t-test. Courtesy of Dr. Valeria Specchia, University of Salento, Italy.

D) Negative geotaxis assay in 14 days old control flies (*w*^{1118}*/w*^{1118}*;UAS-dodo*^{RNAi}*/+;*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Climbing index indicates the fraction of successful individuals. Values represent mean ± s.d. of N=5 biological replicates **P value <0.01 by two-tailed unpaired Sudent's t-test.

3.1.4. Dodo preserves nuclear envelope structure, the tethering to the nuclear lamina and protein stability of dHP1a

Previous results obtained in the lab showed that, in *dodo* mutant fly brain and ovary, loss of Dodo was not associated with any change of *dHP1a* mRNA levels (Voto PhD thesis 2016), suggesting that Dodo regulated dHP1a protein levels post-transcriptionally. Post-transcriptional control of dHP1a protein levels was proposed to rely on the regulation of dHP1a protein stability and turnover by the ubiquitin-proteasome system (UPS) (Chaturvedi et al., 2010; Chaturvedi et al., 2012; Krishnamoorthy et al., 2018). We hypothesized that Dodo could regulate dHP1a protein stability and turnover mediated by the UPS. To test this hypothesis, we evaluated, by western blot analysis, the levels of dHP1a protein in the heads of *dodo* mutant flies, treated with the inhibitor of the proteasome MG132. In these conditions, the reduction of dHP1a protein levels associated with loss of Dodo, compared to control flies, was suppressed by pharmacological inhibition of proteasome activity, supporting the idea that Dodo prevents proteasome-dependent degradation of dHP1a protein (Fig. 23 A). This idea was further supported by the observation that, also in the heads of flies expressing *dodo* RNAi with the Elav-Gal4 driver, the reduction of dHP1a protein levels associated with loss of Dodo, compared to control flies, was suppressed by treatment with MG132 (data not shown).

In line with these results, in fly brains treated with the PIN1 inhibitor KPT-6566, which reduced Dodo (supplementary Fig. S 2 A) and dHP1a protein levels (Fig. 23 B), loss of the *Drosophila* ortholog of the HECW2 ubiquitin ligase (dHecw), which targets HP1α in mammals (Krishnamoorthy et al., 2018), rescued dHP1a protein levels (Fig. 23 B). Furthermore, in both brain and adypocytes of *dodo* mutant flies, we observed an of dHecw protein was enriched in the nucleus, compared to contrl flies (Fig. 24 A, B), supporting the idea that Dodo prevents dHP1a protein ubiquitination by dHecw (Santin A, unpublished data).

Together thiese results indicated that Dodo prevents dHP1a protein degradation mediated by the ubiquitin-proteasome system (UPS).

We sought to understand the molecular mechanism underlying regulation of dHP1a protein by Dodo. In mammalian cells, regulation of HP1α protein stability and turnover was proposed to be dependent on the proper organization of the nuclear lamina (Ye et al 1997, Kourmouli et al 2000, Chaturvedi et al 2012), to which HP1α is anchored by interaction with different proteins, including Lamins and LaminB receptor (LBR) (Ye et al 1997, Kourmouli et al 2000, Chaturvedi et

al 2012). In line with this, in the head of two different *Drosophila* Lamin B (dLamB) mutant strains, *dLamB*^{K2} and *dLamB*$^{04643/+}$, which express low levels of dLamB, we observed a reduction of dHP1a protein (Fig. 25 A), but not mRNA (Fig. 25 B) levels, compared to control flies, independently of Dodo protein levels, which were unaltered in those conditions (Fig. 25 A). Furthermore, in the head of *dLamB*^{K2} flies, the reduction of dHP1a protein levels associated with loss of LamB function was suppressed by knockout of *dHecw* (Fig. 25 C). In contrast, dHP1a protein levels were not affected by loss of *Drosophila* A/C type LaminC (dLamC) or LBR proteins (Supplementary Fig. S 3 A, B).

Previous data obtained in the lab showed that, in *dodo* mutant flies, LamB total protein levels were unchanged, compared to control flies (Fig. 25 D, Ferrari Bravo data previously reported in Voto PhD thesis 2016). This suggested that the reduction of dHP1a protein levels associated with loss of Dodo was independent of dLamB total protein levels. We reasoned that Dodo could regulate nuclear lamina organisation and interaction with dHP1 protein, thus preserving dHP1a protein stability. In line with this idea, we observed that in the head of wild-type flies, dLamB and dHP1a formed a protein complex, and this interaction was lost in *dodo* mutant flies, as shown by co-IP experiments (Fig. 26 A, Ferrari Bravo data previously reported in Voto PhD Thesis 2016), suggesting that Dodo is required for the formation of a protein complex including dLamb and dHP1a, which stabilises and prevents UPS-mediated degradation of dHP1a protein.

We further observed that in the head of wild-type flies, Dodo interacted with both dLamB and dHP1a (Fig. 26 B-C, Ferrari Bravo data previously reported in Voto PhD Thesis 2016), as shown by co-immunoprecipitation (co-IP) assay. These results were in line with a previous report which suggested tha Dodo and dHP1a proteins interact with each other (Ryu et al., 2014), and supported the idea that Dodo directly maintains dLamB/dHP1a interaction. To assess whether this function of Dodo requires its canonical phosphorylation-dependent catalytic activity, we assessed, in the head of wild-type flies, the effect of protein dephosphorylation on the formation of dLamb/dHP1a protein complex. This was impaired also by treatment with phosphatase, as shown by co-IP assay, suggesting that the tethering of dHP1a to the nuclear lamina is regulated by kinase signaling (Fig. 26 D). Then, we evaluated dHP1a protein levels in the brain of flies overexpressing *dodo* with the *Elav-GAL4* driver (supplementary Fig. 2 B), treated with the PIN1 inhibitor KPT-6566. This treatment was associated with a reduction of dHP1a protein levels but not Dodo protein levels (supplementary Fig. 2 C), suggesting that the inhibition of Dodo catalytic

activity was sufficient to impair dHP1a protein stability. In these conditions, the amount of dLamB protein interacting with dHP1a protein was decreased (Fig. 26 E) compared to control flies, as shown by co-IP assay, suggesting that Dodo enzymatic function is required to promote the tethering of dHP1a protein to the NE.

To assess how Dodo preserves dHP1a stability, we evaluated, in *dodo* mutant fly tissues, NE morphology, by LamB immunofluorescence analysis. In *dodo* mutant fly brain and adipocytes, loss of Dodo was associated with nuclear envelope ruffling and invaginations (Fig. 27 A), suggesting that Dodo preserves nuclear lamina organisation.

Taken together, these results suggest that, in *Drosophila*, Dodo maintains dLamB organisation and interaction with dHP1a, preserving dHP1a stability.

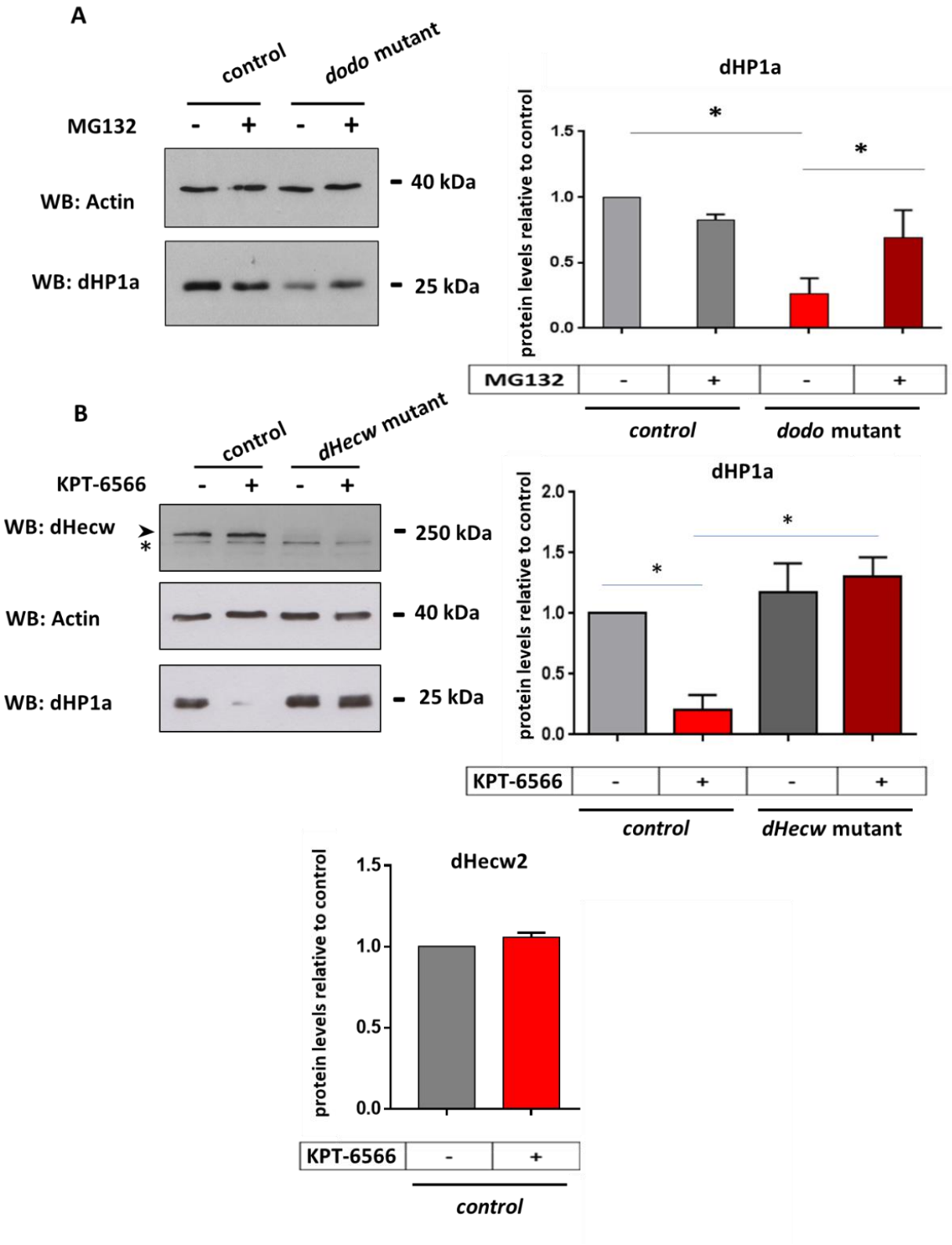


Figure 23. Loss of Dodo is associated with proteasome and dHecw -dependent degradation of dHP1a in the *Drosophila* brain.

A) Western blot analysis in the heads of 4 days old control (w^{1118}) and *dodo* mutant ($dod^{EY03779}$) flies. Actin was used as loading control. The blot is representative of N=3 biological replicates.

Quantification of dHP1a protein level is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.
B) Western blot analysis in the heads of 4 days old control (w^{1118}) and *dHecw* mutant ($dHecw^{KO}$) flies. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of dHP1a protein level is shown in the right panel. Quantification of dHecw protein level is shown in the bottom panel. Values represent mean ± s.d. ***P value <0.001, by two tailed unpaired Student's t-test.

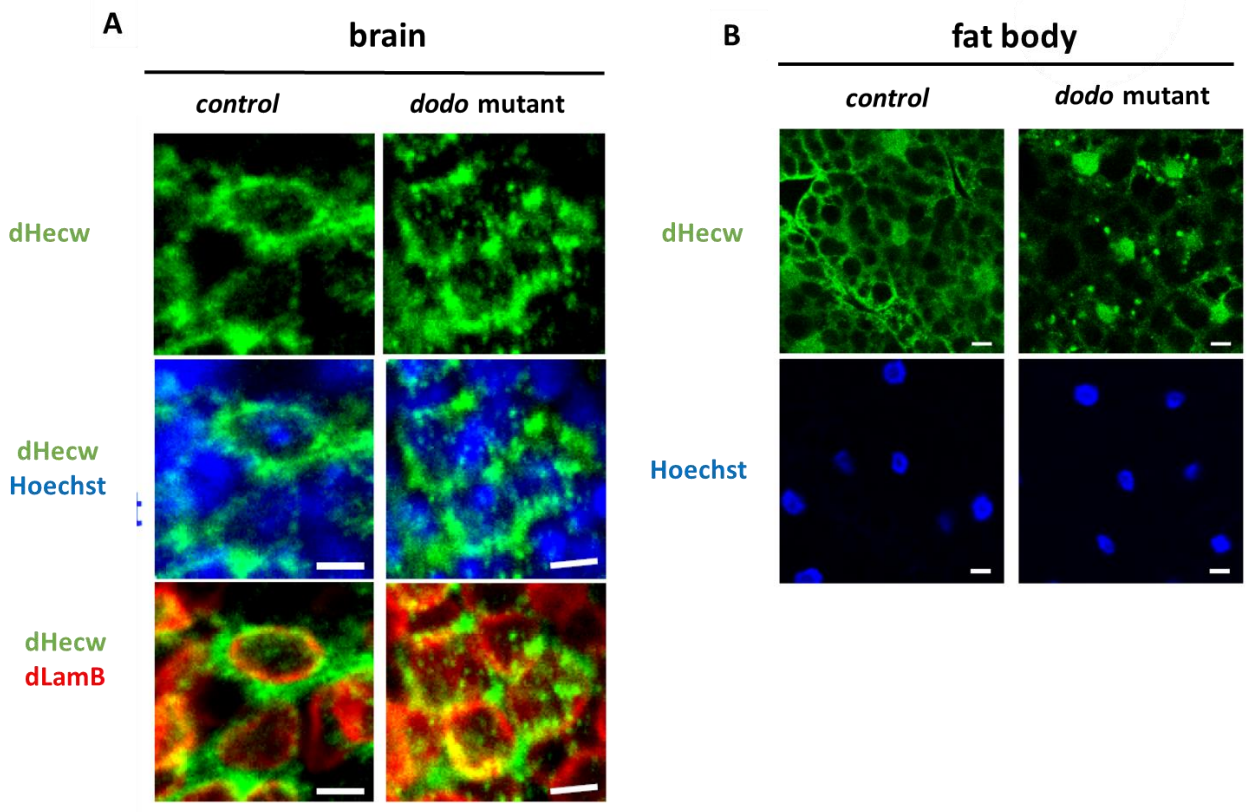


Figure 24. Loss of Dodo is associated with dHecw nuclear enrichment in *Drosophila*.
A) Single confocal section immunofluorescence images in the brain of 4 days old control (w^{1118}) and *dodo* mutant ($dod^{EY03779}$) flies. Images are is representative of N=4 individuals. Nuclei are stained with Hoechst (in blue). Scale bar 2µm. Courtesy of Dr. F. Napoletano.
B) Single confocal section immunofluorescence images in the 3rd instar larval adipocytes (fat bodies) of control (w^{1118}) and *dodo* mutant ($dod^{EY03779}$) flies. Images are representative of N=4 individuals. Nuclei are stained with Hoechst (in blue). Scale bar 5µm. Courtesy of Dr. F. Napoletano.

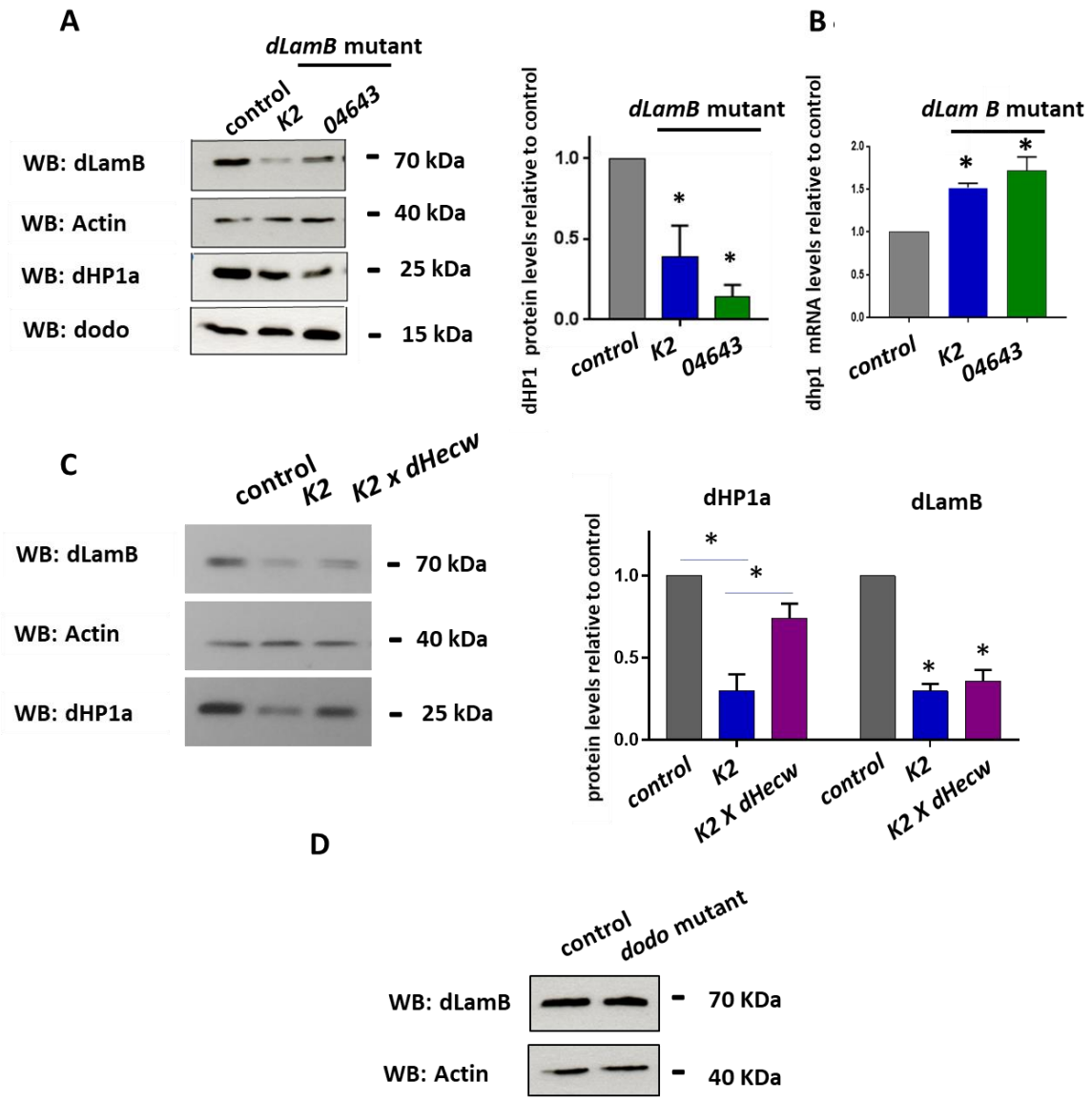


Figure 25. Loss of dLamB function is associated with proteasome and dHecw -dependent degradation of dHP1a in the *Drosophila* brain.

A) Western blot analysis in the heads of 4 days old control (w^{1118}) and *dLamB* mutant flies. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of dHP1a protein level is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.

B) RT-qPCR analysis in the heads of 4 days old control flies (w^{1118}) and *dLamB* mutant flies. *actin* was used as reference for quantification. Values represent mean ± s.d. of N=3 biological replicates.

C) Western blot analysis in the heads of 4 days old control (w^{1118}) and the indicated mutant flies. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of dHP1a and dLamB protein levels is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.

D) Western blot analysis in the heads of 4 days old control (w^{1118}) and *dodo* mutant ($dod^{EY03779}$) flies. Actin was used as loading control. The blot is representative of N=3 biological replicates.

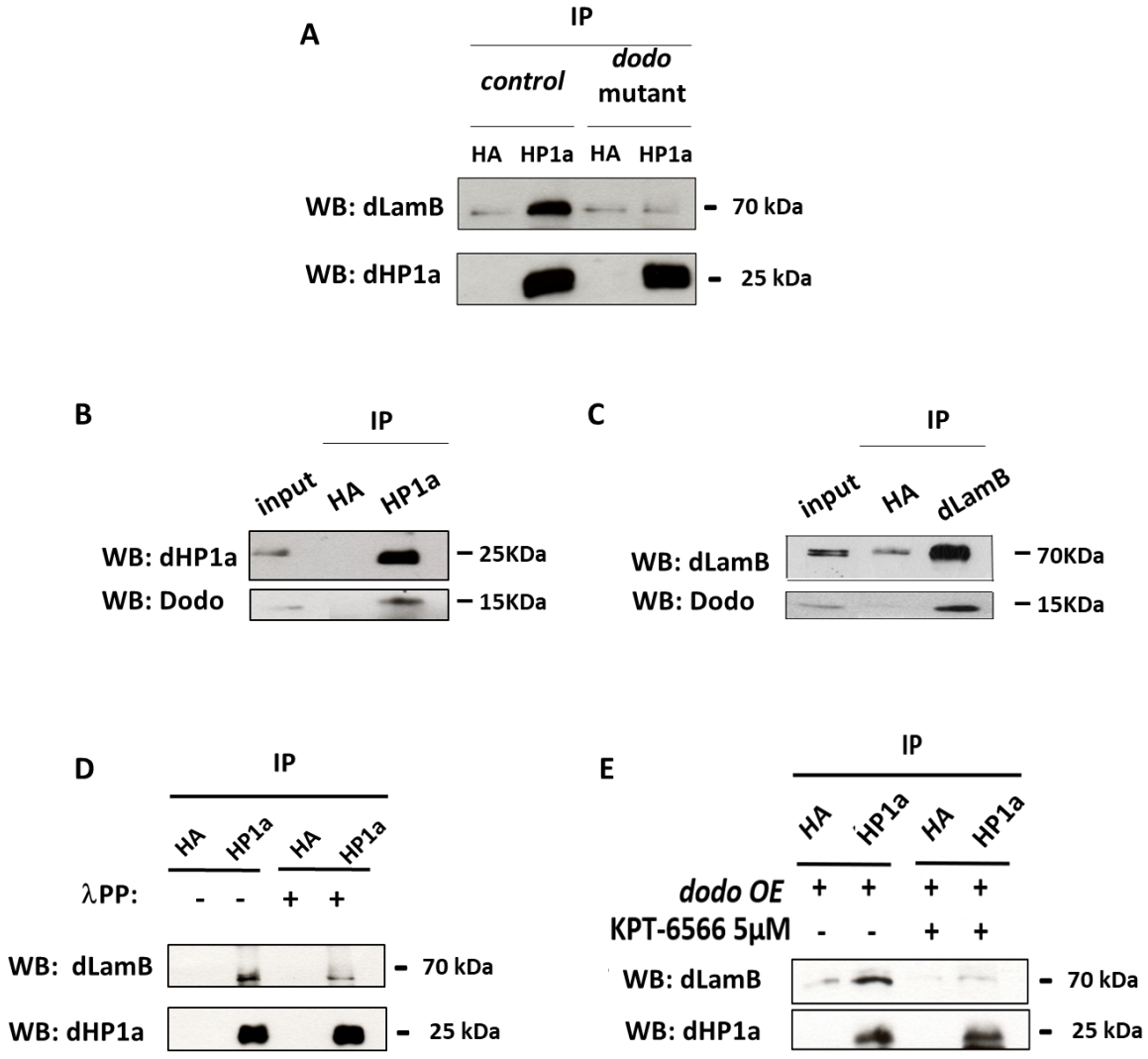


Figure 26. Dodo activity is required for the formation of a protein complex with dHP1a and dLamB in the *Drosophila* brain.

A) Co-immunoprecipitation assay (IP) in the heads of 4 days old control (w^{1118}) and *dodo* mutant ($dod^{EY03779}$) flies. The blot is representative of N=3 biological replicates. Anti-HA antibody was used as negative control.

B-C) Co-immunoprecipitation assay (IP) in the heads of 4 days old wild-type (w^{1118}) flies. Blot are representative of N=3 biological replicates. Anti-HA antibody was used as negative control.

D) Co-immunoprecipitation assay (IP) in the heads of 4 days old wild-type (w^{1118}) flies treated with lambda-phosphatase (λPP). Anti-HP1a (C1A9) antibody was used for immunoprecipitation (IP) reaction in total protein lysat. The blot is representative of N=3 biological replicates. Anti-HA antibody was used as negative control.

E) Co-immunoprecipitation assay (IP) in the heads of 4 days old flies overexpressing *dodo* with the *Elav*-GAL4 driver (*dodo* OE). The blot is representative of N=3 biological replicates. Anti-HA antibody was used as negative control.

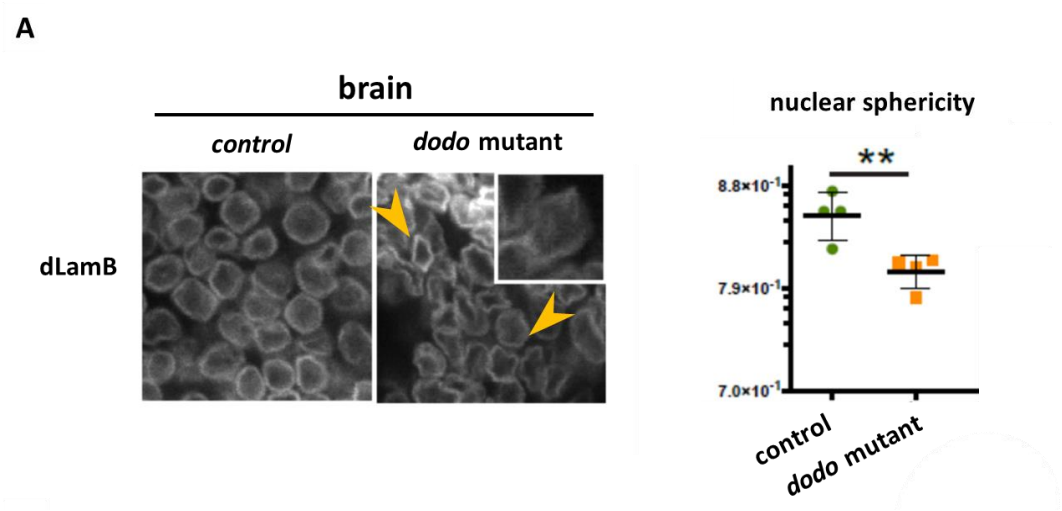


Figure 27. Loss of Dodo is associated with nuclear envelope invagination in *Drosophila*.
A) Single confocal section immunofluorescence images in the brain of 4 days old control (w^{1118}) and *dodo* mutant ($dod^{EY03779}$) flies. Arrowheads indicate nuclear lamina invaginations. Images are representative of N=4 individuals. Quantification of nuclear sphericity is shown in the right panel. Values represent mean ± s.d. **P value <0.01 by two tailed unpaired Student's t-test.

3.2. Dodo maintains heterochromatin and brain neuron health by preserving nuclear envelope mechanics

3.2.1. Dodo preserves nuclear envelope mechanics

Through the NE, chromatin is connected to the cytoskeleton. The cytoskeleton is attached to the nuclear lamina through the LINC protein complex, which includes proteins spanning inner (INM) and outer (ONM) nuclear membrane. Many recent studies have demonstrated that the nuclear envelope can sense and respond to mechanical forces exerted on or by the cytoskeleton (Chancellor et al., 2010; Lovett et al., 2013; Swift et al., 2013; Alam et al., 2015). Because the extracellular matrix (ECM) is linked mechanically to the nucleus via the cytoskeleton, intracellular tension is balanced in part by the nucleus and can induce nuclear shape changes and invagination (Sims, et al. 1992; Maniotis, et al. 1997; Dahl et al., 2008; Khatau et al., 2009; Chancellor, et al. 2010; Lombardi, et al. 2011; Versaevel et al., 2012) besides altering cell morphology (Marganski et al., 2003; Discher et al., 2005). This connection allows that environmental, such as the ones generated by the extracellular matrix (ECM), or intracellular mechanical stimuli are transduced and coupled to the regulation of genome organization and gene expression (Sims, et al. 1992; Maniotis, et al. 1997; Cremer and Cremer, 2001; Dahl et al., 2008 ; Khatau et al., 2009 ; Chancellor, et al. 2010; Lombardi, et al. 2011; Versaevel et al., 2012; Maharana et al., 2016). Alterations in chromatin and gene expression have been proposed to contribute to several aging-related pathologies, including AD. Intriguingly, AD has been also associated with factors, including aberrant fluid pressure and traumatic injury, which cause mechanical stress (see Introduction and Discussion). We reasoned that Dodo function at the NE could be important to preserve the mechanical properties of the nuclear lamina, preventing that mechanical stress negatively impact on chromatin organisation and gene expression. In line with this hypothesis, in wild-type fly brain, exposure to hypertonic environment, which enhances actin polymerisation (Yamamoto JBC 2006), did not affect NE morphology (Fig. 28 A), compared to isotonic conditions. Instead, in these conditions, NE ruffling and invagination in *dodo* mutant brain cells were enhanced (Fig. 28 A). A similar effect was caused by treatment with jasplakinolide, which induces actin polymerisation (Fig. 28 B).

These results suggest that the function of Dodo at the NE is important to preserve nuclear mechanical properties and structure.

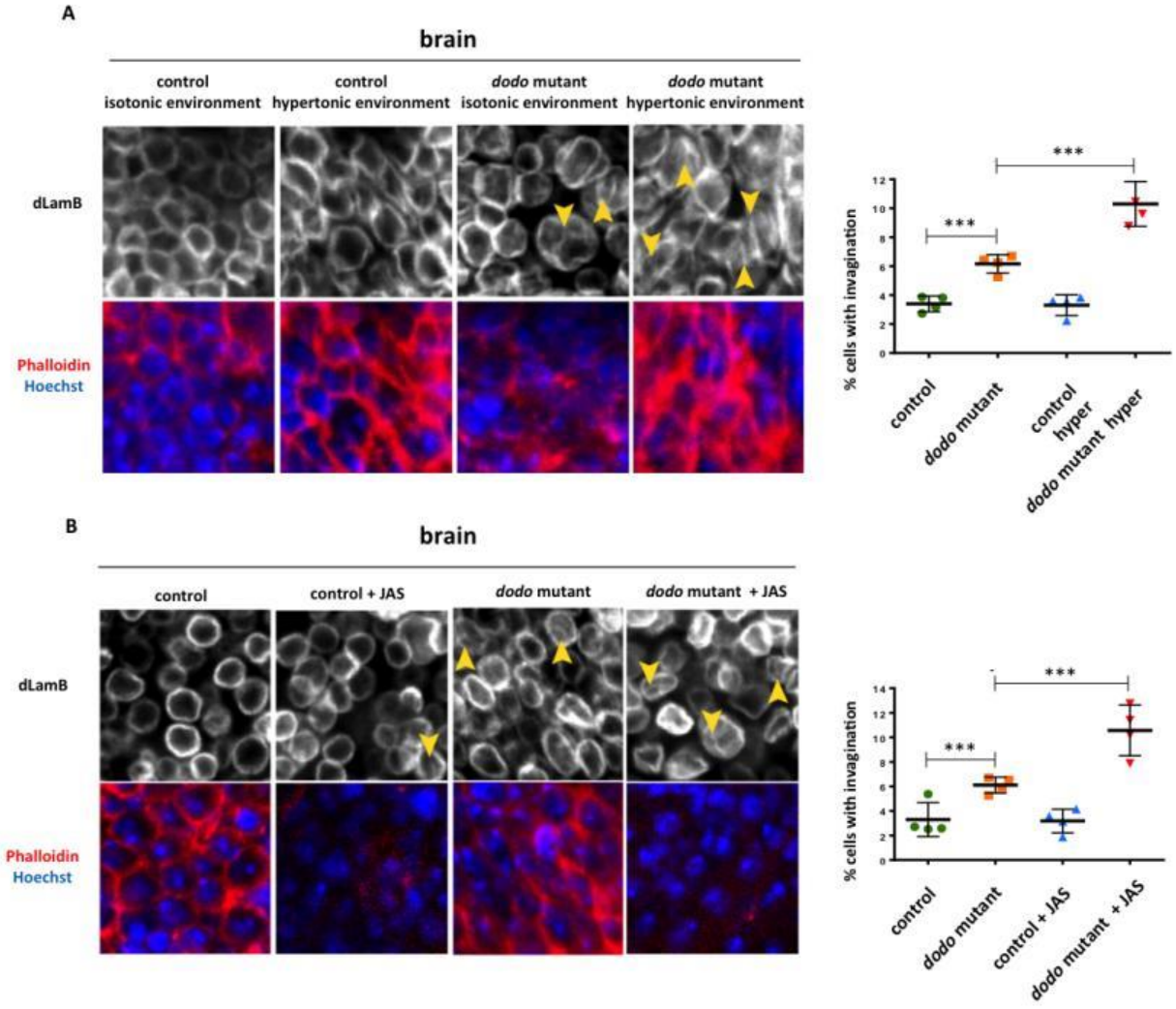


Figure 28. Loss of Dodo sensitizes nuclear envelope structure to mechanical stress in the *Drosophila* brain.

A) Single confocal section immunofluorescence images in the brain of 4 days old control (w^{1118}) and *dodo* mutant ($dod^{EY03779}$) flies. Arrowheads indicate nuclear lamina invaginations. Images are representative of N=4 individuals. Quantification of nuclear lamina invaginations is shown in the right panel. Values represent mean ± s.d. ***P value <0.001 by two tailed unpaired Student's t-test. Courtesy of Dr. F. Napoletano.

B) Single confocal section immunofluorescence images in the brain of 4 days old control (w^{1118}) and *dodo* mutant ($dod^{EY03779}$) flies. Jasplakinolide (JAS) and Phalloidin compete for F-Actin binding. Arrowheads indicate nuclear lamina invaginations. Images are representative of N=4 individuals. Quantification of nuclear lamina invaginations is shown in the right panel. Values represent mean ± s.d. ***P value <0.001 by two tailed unpaired Student's t-test. Courtesy of Dr. F. Napoletano.

3.2.2. Loss of Dodo impairs nuclear mechanics and exposes heterochromatin to mechanical stress in AD pathogenesis

To assess the physiopathological impact of Dodo-dependent regulation of nuclear mechanics, we took advantage of a *Drosophila* model of AD based on ectopic expression of the human TAU (hTAU) protein, which has been shown to recapitulate several hallmarks of AD pathogenesis, including hTAU hyperphosphorylation (Wittmann et al., 2001), and in which mechanical stress impinging on the NE has been shown to contribute to neurodegeneration (Frost et al., 2016). In the heads of transgenic flies ectopically expressing the hTAU protein under the control of the *Elav-GAL4* driver, in which hTAU induces mehcanical stress impinging on the NE by promoting actin hyperpolymerisation (Bardai et al 2018, Frost et al. Curr Biol 2016, Fulga et al. NCB 2006), we observed that age-dependent neurodegeneration, as shown by increased number of $TUNEL^+$ cells (Fig. 29 A), was associated with decreased H3K9me3 protein levels, as shown by western blot analysis, compared to control flies (Fig. 30 B), which indicated relaxation of heterochromatin. This correlated with NE invaginations (Fig. 29 B), reduced dHP1a protein levels (Fig. 30 A), increased TE mRNA levels (Fig. 31 A), and locomotor defects (Fig. 31 C), in line with previous reports (Sun et al. Nat Neurosci 2018, Frost et al. Curr Biol 2016, Frost et al. Nat Neurosci 2014). In the brain of these flies, the protein levels of Dodo were reduced, compared to control flies (Fig. 29 C), suggesting that loss of Dodo could contribute to the phenotypes of hTAU expressing flies. We did not observe any significant decrease of dodo mRNA in the heads of these flies compared to control flies (supplementary Fig. S4 B), suggesting that reduction of the Dodo protein occurred at the post-transcriptional level. Of note, in these conditions, dHP1a protein levels appeared more sensitive to reduction of Dodo protein levels, compared to *dodo* mutant and *dodo* RNAi expressing flies, suggesting that mechanical stress contributed to reduce dHP1a protein levels. Importantly, Dodo overexpression (supplementary Fig. 4 D) suppressed all the phenotypes associated to hTAU expression, NE invagination (Fig. 29 B), reduction of dHP1a and H3K9me3 protein levels (Fig. 30 A, B), TE mRNA increase (Fig. 31 B), neurodegeneration (as shown by reduced the number of $TUNEL^+$ cells, Fig. 29 A) and fly locomotor defects (Fig. 31 C).

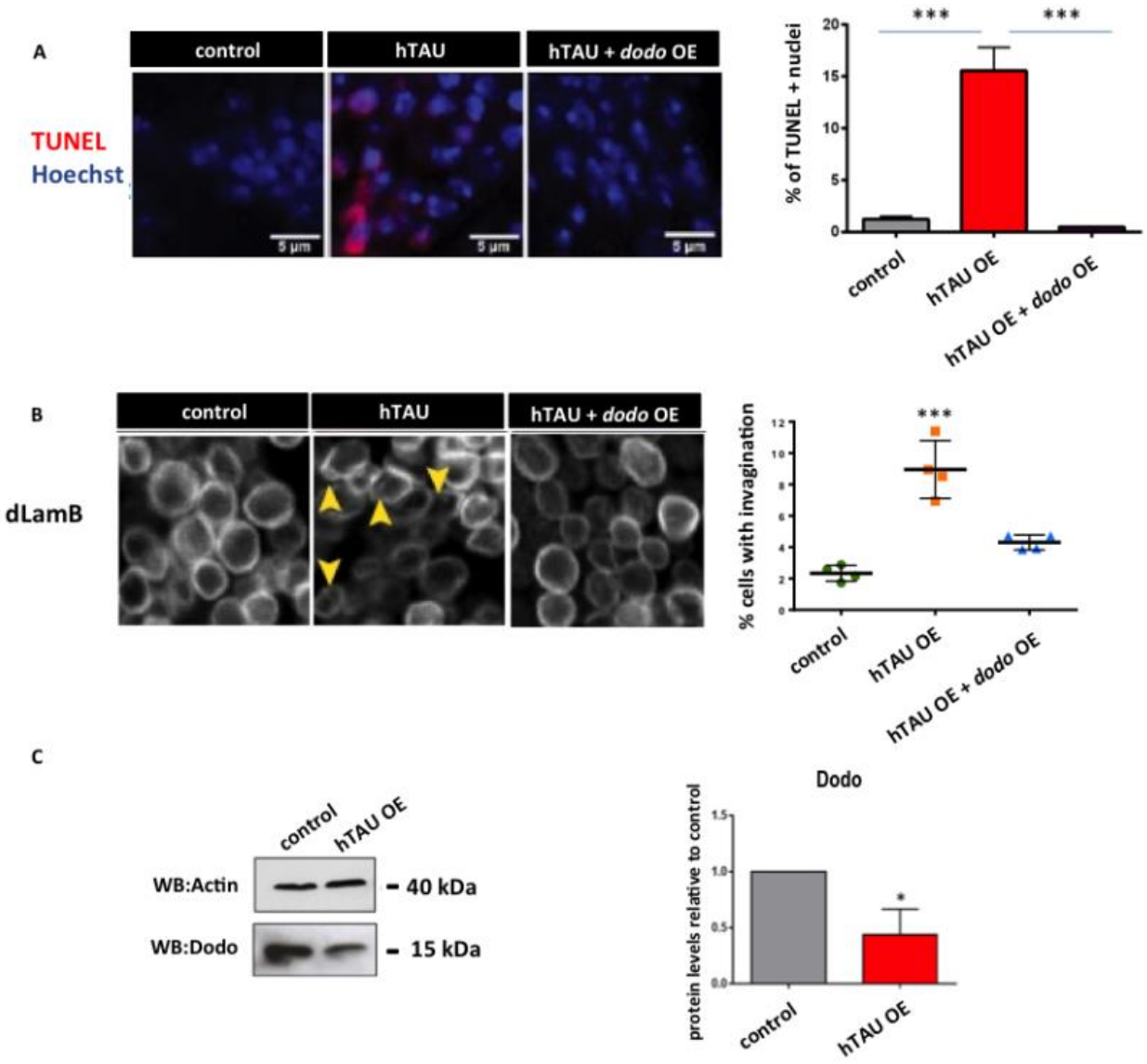


Figure 29. hTAU induced neurodegenration and nuclear lamina invagination by reducing Dodo protein levels in the *Drosophila* brain.

A) Whole-mount TUNEL staining in the brain of 10 days old control flies (*w*^{1118}*/w*^{1118}*;UAS-Dodo/+;UAS-hTAU/+*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Nuclei are stained with HOECHST. Images are representative ofof N=6 individuals. Quantification of TUNEL^{+} nuclei is shown in the right panel. Values represent mean ± s.d. ***P value <0.001 by two tailed unpaired Student's t-test.

B) Single confocal section immunofluorescence in the brain of 7 days old control flies (*w*^{1118}*/w*^{1118}*;UAS-Dodo/+;UAS-hTAU/+*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Arrowheads indicate nuclear lamina invaginations. Images are representative of N=4 individuals. Quantification of nuclear lamina invaginations is shown in the right panel. Values represent mean ± s.d. ***P value <0.001 by two tailed unpaired Student's t-test. Courtesy of Dr. F. Napoletano.

C) Western blot analysis in the heads of 7 days old control flies (*;UAS-hTAU/+*) and flies expressing the indicated construct with the *Elav-GAL4* driver. Actin was used as loading control. The blot is

representative of N=3 biological replicates. Quantification of Dodo protein level is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.

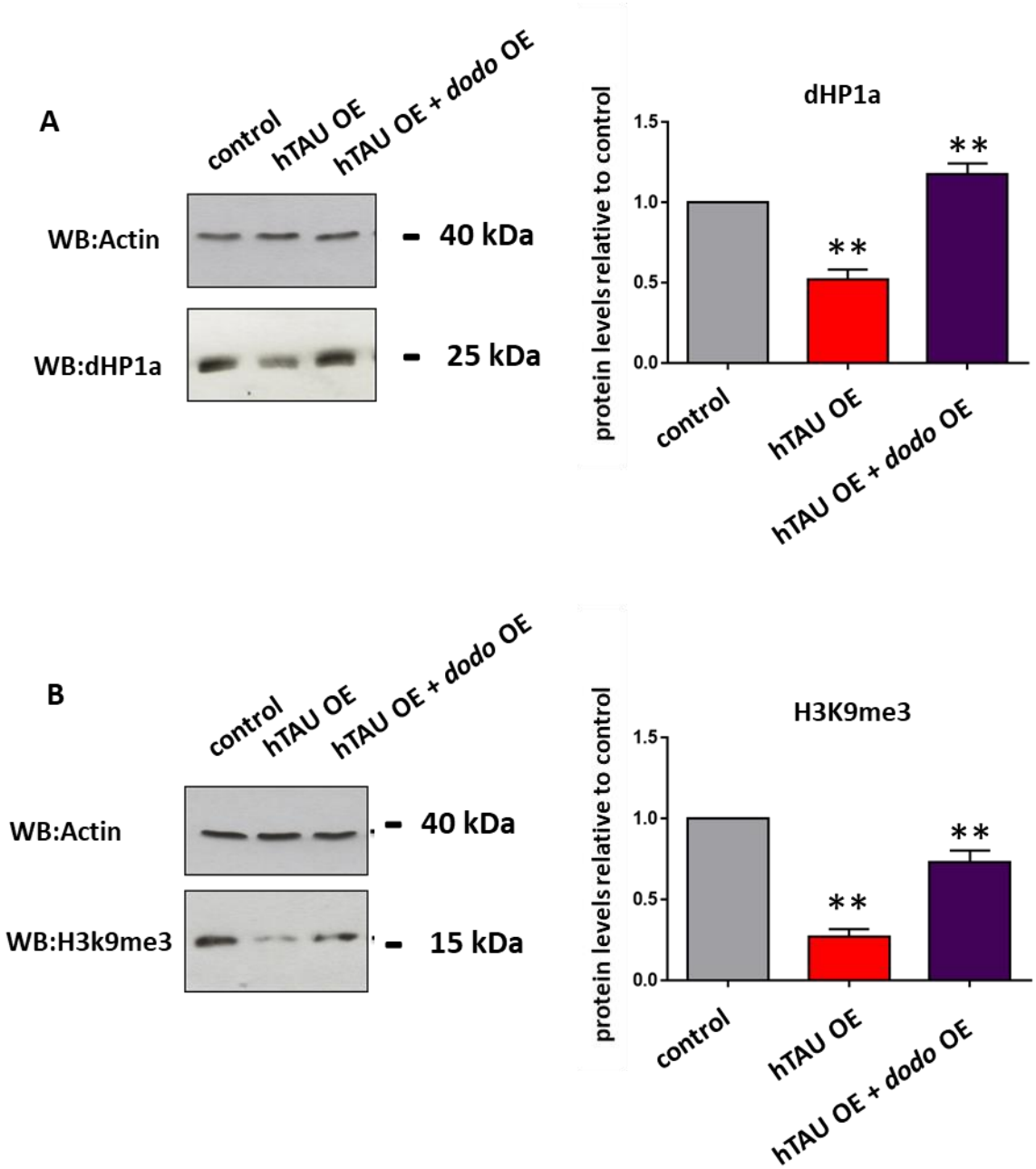
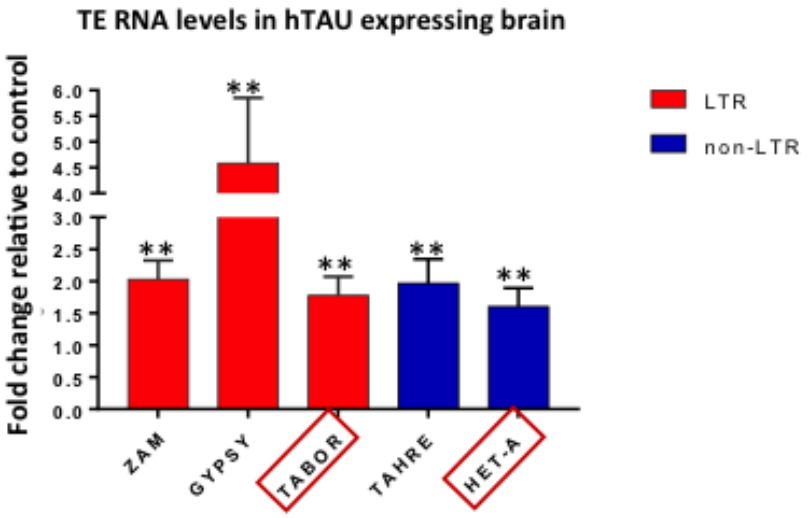


Figure 30. hTAU induces heterochromatin relaxation by reducing Dodo protein levels in the *Drosophila* brain.

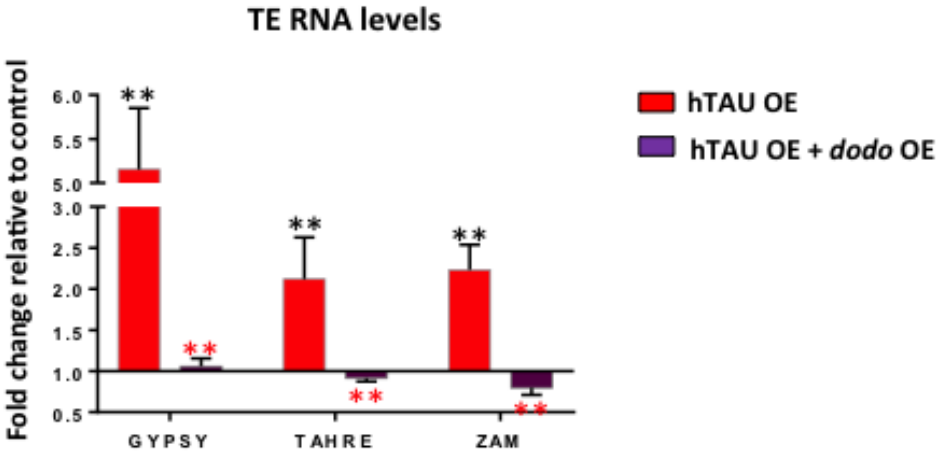
A) Western blot analysis in the heads of 7 days old control flies (*w^{1118}/w^{1118};UAS-Dodo/+;UAS-hTAU/+*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of dHP1a protein level is shown in the right panel. Values represent mean ± s.d. **P value <0.01, by two tailed unpaired Student's t-test.

B) Western blot analysis in the heads of 7 days old control flies (*w^{1118}/w^{1118};UAS-Dodo/+;UAS-hTAU/+*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of H3K9me3 protein level is shown in the right panel. Values represent mean ± s.d. **P value <0.01, by two tailed unpaired Student's t-test.

A


TE RNA levels in hTAU expressing brain
Fold change relative to control
6.0
5.5
5.0
4.5
4.0
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
ZAM
GYPSY
TABOR
TAHRE
HET-A
LTR
non-LTR

B


TE RNA levels
Fold change relative to control
6.0
5.5
5.0
3.0
2.5
2.0
1.5
1.0
0.5
**
GYPSY
TAHRE
ZAM
hTAU OE
hTAU OE + dodo OE

C

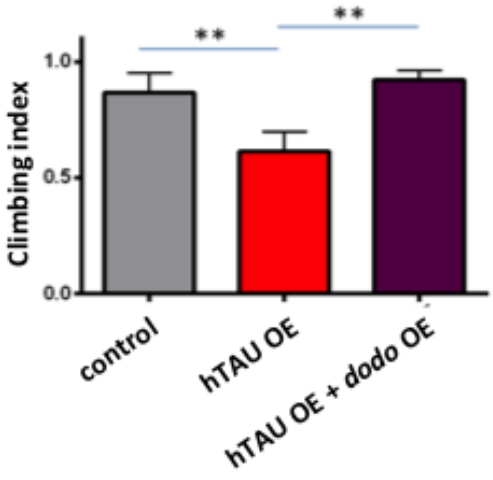

Climbing index
1.0
0.5
0.0
**
**
control
hTAU OE
hTAU OE + dodo OE

Figure 31. hTAU induces TE mRNA expression in the fly brain and fly motor defects by reducing Dodo protein levels.

A) RT-qPCR analysis in the heads of 7 days old control flies (*;UAS-hTAU/+*) and flies expressing the indicated construct with the *Elav-GAL4* driver. Red box indicate TEs that ar not regulated by Dodo (data not shown). *actin* was used as reference for quantification. Values represent mean ± s.d. of N=3 biological replicates. **P value <0.01 by two tailed unpaired Student's t-test.
B) RT-qPCR analysis in the heads of 7 days old flies expressing the indicated constructs with the *Elav-GAL4* driver, relative to control flies (w^{1118}/w^{1118}*;UAS-Dodo/+;UAS-hTAU/+*). *actin* was used as reference for quantification. Values represent mean ± s.d. of N=3 biological replicates. Black and red asterisks indicate P value of *hTAU* OE v_s control and *hTAU* OE *+ Dodo* OE, respectively. **P value <0.01 by two tailed unpaired Student's t-test.
C) Negative Negative geotaxis assay in 10 days old control flies (w^{1118}/w^{1118}*;UAS-Dodo/+;UAS-hTAU/+*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Climbing index indicates the fraction of successful individuals. Values represent mean ± s.d. of N=5 biological replicates **P value <0.01 by two-tailed unpaired Sudent's t-test.

These results indicate that loss of Dodo-dependent NE integrity exposes heterochromatin to pathogenic mechanical stress, and that this mechanisms contributes to TAU toxicity in AD pathogenesis. In line with this, in the brain of flies expressing hTAU with the *Elav-GAL4* driver, the reduction of dHP1a protein levels was not due to decreased levels of *hp1a* mRNA, as shown by RT-qPCR analysis (supplementary Fig. 4), but was dependent on proteasome activity, as suggested by the fact that treatment with the proteasome inhibitor MG132 rescued dHP1a protein levels (Fig. 32 A). Thus, in these conditions, loss of Dodo may promote UPS-mediated degradation of dHP1a.

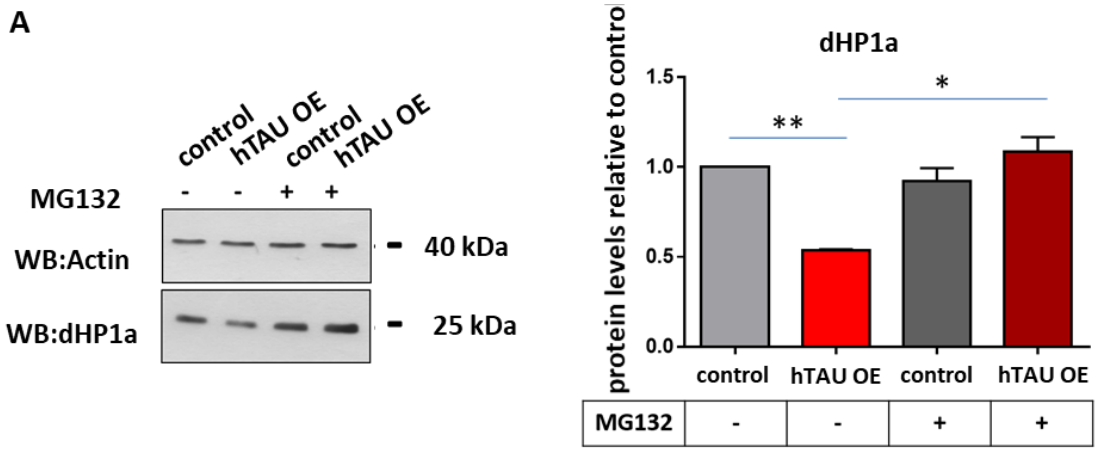


Figure 32. hTAU expression is associated with proteasome-dependent degradation of the dHP1a protein in the Drosophila brain.

Western blot analysis in the heads of 7 days old control flies (*;UAS-hTAU/+*) and flies expressing the indicated construct with the *Elav-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of dHP1a protein level is shown in the right panel. Values represent mean ± s.d. *P value <0.05, **P value <0.01, by two tailed unpaired Student's t-test.

3.3 PIN1 maintains heterochromatin and TE repression in mammals

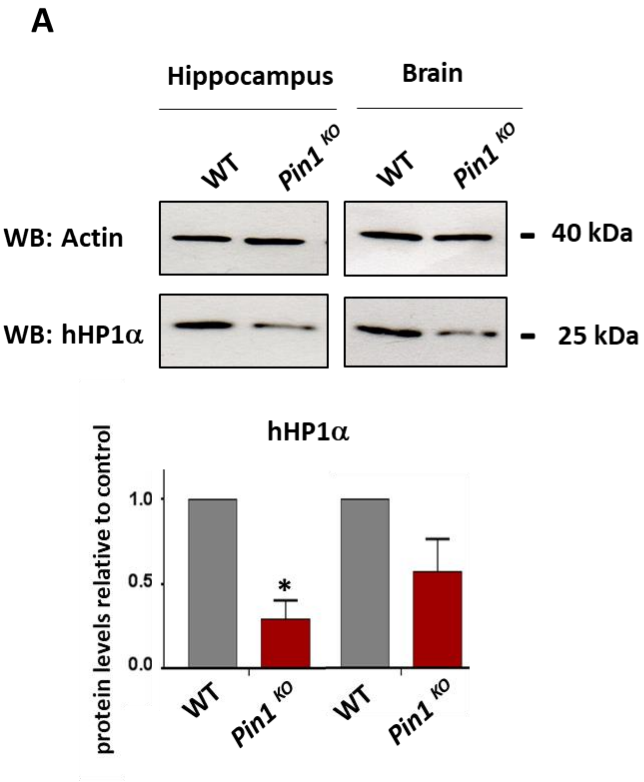
To validate our findings in mammals, we used mouse models and human cell cultures.

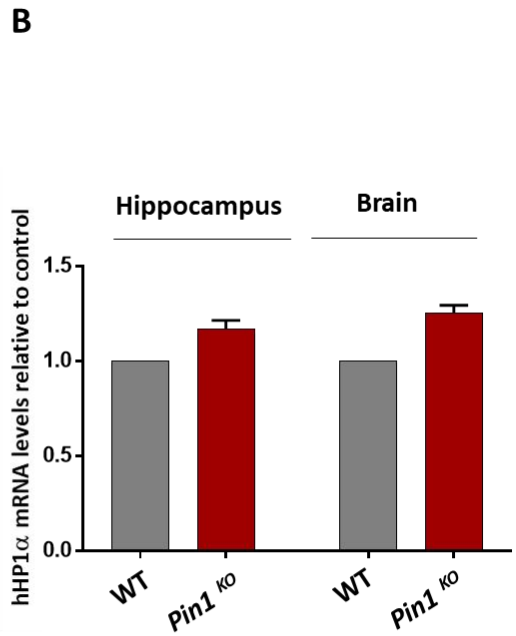
Pin^{KO} mice display progressive age-dependent neurodegeneration in the brain, whose onset is around 9-14 months of age (Liou et al., 2003). We evaluated the expression of HP1α in the hippocampus and the total brain of *Pin*^{KO} mice, compared to wild-type littermates. In both hippocampus and the total brain of aged (12 and 24 months old) *Pin*^{KO} mice, loss of PIN1 was associated with reduced protein (as shown by western blot analysis, Fig. 33 A, C), but not mRNA (as shown by RT-qPCR analysis, Fig. 33 B, D), levels of HP1α. We did not observe any reduction of Hp1α protein levels in young (4 months old) *Pin*^{KO} mice, compared to wild-type littermates (data not shown). Thus, *Pin*^{KO} mice display age-dependent reduction of HP1α protein levels in the brain, whose onset correlates with the onset neurodegeneration. This strongly suggests that the neuroprotective function of Pin1-dependent maintenance of HP1α protein levels is conserved from *Drosophila* to mammals. In line with this, in both hippocampus and total brain of aged (24 months old) *Pin*^{KO} mice, loss of PIN1 was associated with increased TE mRNA levels (as shown by RT-qPCR analysis, Fig. 34 A, B). This further suggests that the role of Pin1 in restraining TE activity, by heterochromatin-mediated silencing, is conserved from *Drosophila* to mammals.

To verify the underlying mechanism, we used isogenic PIN1$^{+/+}$ and PIN1^{KO} human cell clones, previously generated in the lab by A. Rustighi A. By immunoprecipitation analysis In PIN1$^{+/+}$ HEK-293T cells, we found that Lamin B1 (LamB1) formed a protein complex with HP1α (supplementary Fig. 6). In PIN1^{KO} HEK-293T cells, the formation of this protein complex was impaired (supplementary Fig. 6), and HP1α protein levels reduction at post-transcriptional levels (data not shown).

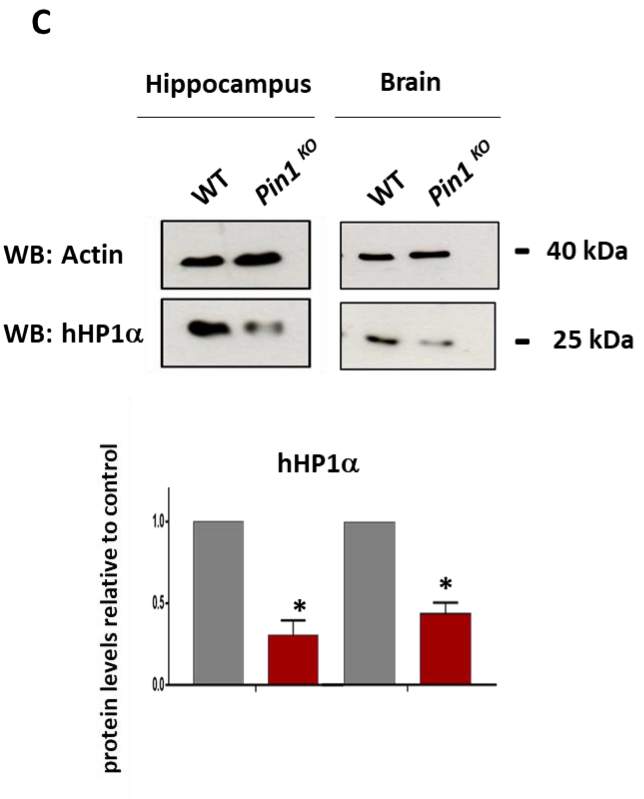
Taken together, these results suggest that PIN1 has a fundamental role conserved from fly to humans, since is required for the formation of a protein complex including Lamb and HP1α, which stabilises HP1α protein.

12 months





24 months



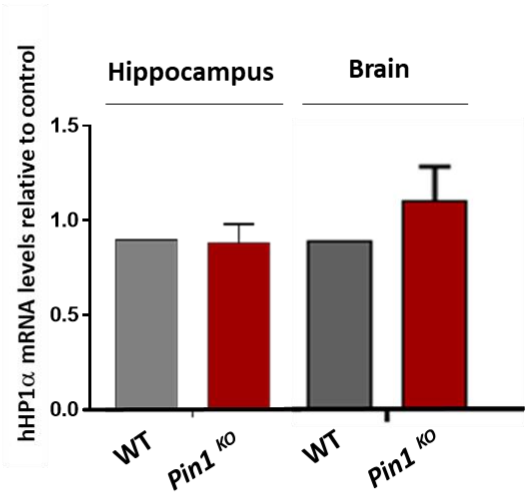


Figure 33. *Pin1* ^{KO} mice show reduction of heterochromatin marks in the brain.
A) Western blot analysis in the indicated tissues of 12 months old *Pin* ^{KO} mice and wild-type (WT) *Pin* $^{+/+}$ littermates. Actin was used as loading control. Blots are representative of N=3 individuals. Quantification of HP1α protein levels in shown in the bottom panel. Values represent mean ± s.d. *P value <0.05 by two tailed Student's t-test.
B) RT-qPCR analysis in the indicated tissues of 12 months old *Pin* ^{KO} mice and wild-type (WT) *Pin* $^{+/+}$ littermates. *actin* was used as reference for quantification. Values represent mean ± s.d of N=3 individuals.
C) Western blot analysis in the indicated tissues of 24 months old *Pin* ^{KO} mice and wild-type (WT) *Pin* $^{+/+}$ littermates. Actin was used as loading control. Blots are representative of N=3 individuals. Quantification of HP1α protein levels in shown in the bottom panel. Values represent mean ± s.d. *P value <0.05 by two tailed Student's t-test.
D) RT-qPCR analysis in the indicated tissues of 24 months old *Pin* ^{KO} mice and wild-type (WT) *Pin* $^{+/+}$ littermates. *actin* was used as reference for quantification. Values represent mean ± s.d of N=3 individuals.

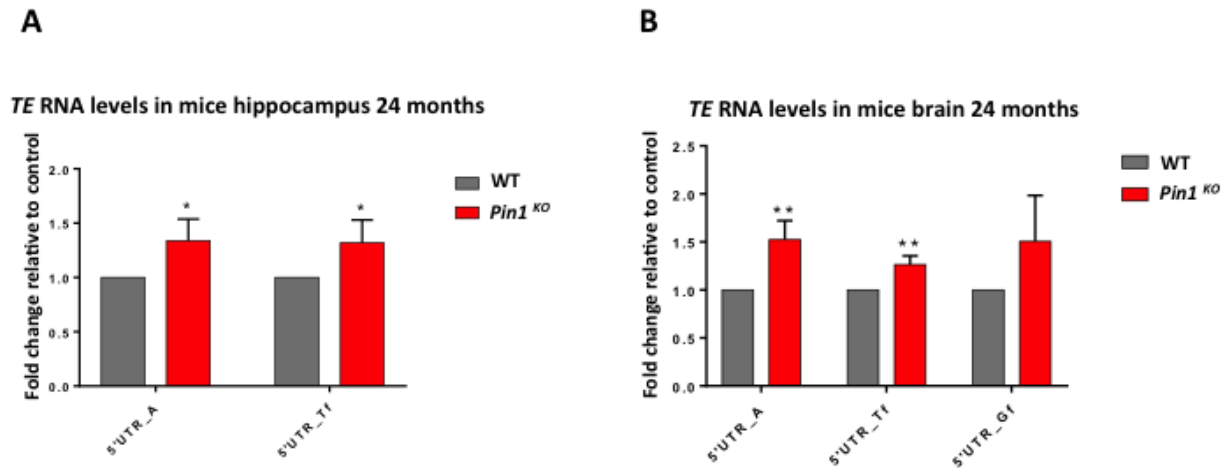


Figure 34. *Pin1* ^{KO} mice show increased mRNA levels of TEs in the brain.
A) RT-qPCR analysis of *LINE-like* TE mRNA levels in the indicated tissues of 24 months old *Pin* ^{KO} mice and wild-type (WT) *Pin* $^{+/+}$ littermates. *actin* was used as reference for quantification. Values represent mean ± s.d of N=3 individuals. **P value <0.01; ***P value <0.001 by two tailed t-test.

4. Discussion

4.1 Mechanisms of heterochromatin maintenance by PIN1

The results presented in this Thesis demonstrate that the prolyl-isomerase PIN1 has an evolutionarily conserved function in preserving the stability of HP1a protein, thus maintaining heterochromatin and restraining TE activity.

We provide evidence that, both in *Drosophila* and in human cells, PIN1 preserves HP1a protein stability by promoting the anchoring of HP1a to the nuclear lamina, through the formation of a protein complex including HP1a and B-type Lamin. Our data also show that, in *Drosophila*, pharmacological inhibition of the catalytic activity of the PIN1 ortholog Dodo is sufficient to impair the formation of dHP1a/dLamB protein complex, suggesting that PIN1 isomerase activity is required for the tethering of HP1a to the nuclear envelope (NE). This raises the question of what is/are the involved Dodo/PIN1 substrate(s). Inspection of dLamB and human Lamin B1 primary sequences highlighted several putative canonical PIN1 binding sites (Ser/Thr-Pro) (Machowska et al., 2015), while no Ser/Thr-Pro sites were found in the aminoacid sequence of dHP1a and hHP1a. However, both dHP1a and hHP1a proteins harbour two Glu-Pro sites, which have been shown to function as PIN1 binding sites, though with low affinity (Yaffe et al. 1997). Alternatively, HP1a protein conformational change could be triggered indirectly by PIN1, via isomerization of a PIN1 protein substrate, as it has been proposed for p53 isomerisation-dependent BAX activation (Follis et al., 2015). Intriguingly, prolyl isomerasation has been suggested to regulate the phase behavior of proteins (Gomes and Shorter 2018), and HP1a conformational change was recently proposed to drive heterochromatin formation by phase separation (Strom et al., 2017; Larson et al., 2017; Tatarakis et al., 2017).

The formation of the HP1a/B-type Lamin protein complex may involve other partners. In human fibroblasts infected with Human Cytomegalovirus (HCMV), PIN1 was reported to interact with phosphorylated Lamin A/C (Milbradt et al. 2010). In this context, PIN1 was shown to promote phosphorylation-dependent Lamin A/C disassembly (Milbradt et al. 2016). However, our data showed that, in fly brain, the *Drosophila* A/C type *LaminC* (*dLamC*) is not required to maintain dHP1a protein levels, suggesting that A/C-type Lamin proteins are dispensable for the tethering of HP1a to the NE. Indeed, brain neurons express high levels of B-type Lamin proteins and low levels of A-type Lamin proteins (Young et al., 2014), supporting the idea that B-type Lamin

proteins play a major role in the regulation of NE structure and function in neurons. Also the Lamin B receptor (LBR) protein, in both *Drosophila* and human, harbours phosphorylable Ser/Thr-Pro sites (Nikolakaki et al., 2017), and has been proposed to tether HP1a to the NE in human cells (Ye & Worman, 1996). However, our results showed that, in the fly brain, *Drosophila LBR* (*dLBR*) is not required for maintaining dHP1a protein levels, suggesting that *dLBR* is dispensable for the anchoring of HP1a to the NE. This is in line with a recent report showing that, in contrast to mammals (Solovei et al., 2013), *dLBR* is not required for the tethering of heterochromatin to the nuclear lamina (Ulianov et al., 2019).

Our biochemical analyses further suggest that phosphorylation of (a) yet to be identified component(s) is necessary for the formation of the protein complex including Dodo/PIN1, B-type Lamin and HP1a. This is in line with the idea that the formation of B-type Lamin/HP1a protein complex requires the canonical phosphorylation-dependent isomerase activity of PIN1. Several kinases that phosphorylate B-type Lamin proteins at Ser/Thr-Pro sites (such as CDK1 and CDK5) (Machowska et al., 2015), and HP1a (e.g. CK2, Pim-1) (Koike et al., 2000; Zhao and Eissenberg, 1999), have been identified. However, phosphorylation of Dodo/PIN1 itself may be also required as an activatory post-translational modification (PTM). PIN1 harbours several evolutionarily conserved residues that can be targeted by inhibitory or activatory kinase activities (Huang et al., 2001; Eckerdt, et al. 2005; Rangasamy et al., 2012). These include Serine residues that have been shown to be phosphorylated by PLK1 (Eckerdt, et al. 2005) and MLK3 (Rangasamy et al., 2012), resulting in stimulation of PIN1 activity. The identification of the kinase signaling pathway(s) and of possible regulatory phosphatase activity involved in the formation of the protein complex including Dodo/PIN1, B-type Lamin and HP1a would provide potential targets to modulate PIN1 function at the NE.

We propose that the protein complex including PIN1, B-type Lamin and HP1a anchors HP1a to the NE, thus preserving HP1a protein stability. Our results suggest that, in *Drosophila*, this mechanism prevents the degradation of dHP1a protein mediated by the E3 ubiquitin ligase dHecw, which has been recently identified as the fly ortholog of HECW2/NEDDL2 (Fajner V. et al., manuscript in preparation), and by the ubiquitin-proteasome system (UPS). Our interpretation is in line with the observation that also *dLamB* is required to prevent the *dHecw*-dependent degradation of dHP1a protein. In human cells, HECW2 has been shown to regulate HP1a protein turnover via the UPS (Krishnamoorthy et al., 2018), suggesting that this mechanism is

evolutionarily conserved from invertebrates to humans. To further support this idea, we are currently assessing whether, in PIN1-depleted mouse brain neurons and human cells, HECW2 is required for UPS-mediated degradation of HP1a (manuscript in preparation). In addition to HP1a, human HECW2 has been proposed to target other nuclear proteins, including Lamin B1 (Krishnamoorthy et al., 2018), HP1b (Krishnamoorthy et al., 2018a), ATR (Muralikrishna et al., 2012) and PCNA (Krishnamoorthy et al., 2018). Our data suggest that, in the *Drosophila* brain, dHecw is not required to regulate dLamB protein levels. This could be due to redundancy with the E3 ubiquitin ligase RNF123/KPC1, which, in human cells, has been shown to target both HP1a (Chaturvedi et al., 2012), HP1b (Krishnamoorthy et al., 2018a), and Lamin B1 (Khanna et al., 2018) for proteasomal degradation.

We provide evidence that, in *Drosophila*, loss of Dodo is associated with ruffling and invagination of the NE and with enrichment of dHecw protein in the nucleus. This suggests that Dodo safeguards NE integrity, thus ensuring proper compartmentalisation of dHecw protein in the cytoplasm, which limits the access of dHecw to nuclear protein targets, including HP1a. In line with this, in human cells harbouring Lamin A mutations that compromise NE integrity (Manju et al., 2006), HECW2 was shown to be retained in the nucleus upon pharmacological inhibition of nucleo-cytoplasmic transport, and to mediate the proteasomal degradation of nuclear protein targets (Krishnamoorthy et al., 2018), suggesting engagement of the UPS system in the nucleus. In this context, HECW2 mRNA and protein levels were found to be upregulated (Chaturvedi & Parnaik, 2010). One possibility is that this is due to relaxation of Lamin A-dependent repressive heterochromatin. However, our results indicate that loss of Dodo does not alter the protein levels of dHecw nor Lamins, further supporting the idea that Dodo-dependent NE integrity is necessary to protect dHP1a from UPS-mediated degradation. To assess whether NE invaginations associated with loss of Dodo can be classified as type-I (involving only the inner nuclear membrane, INM) or -II (involving both INM and outer nuclear membrane, ONM) (Malhas et al., 2011), we are currently performing Transmission Electron Microscopy analysis of *dodo* mutant fly brain (manuscript in preparation).

In summary, our findings are consistent with a model in which PIN1 safeguards NE integrity and the tethering of heterochromatin to the intra-nuclear periphery, thus regulating the stability of nuclear factors involved in gene expression, including HP1a. In this regard, a broad relevance of NE integrity and interaction of chromatin with the nuclear lamina is further supported by the

observation that, in human cells harbouring Lamin A mutations that compromise NE integrity, several proteins that interact directly or indirectly with chromatin and nuclear lamina, including HP1a (Chaturvedi et al., 2012), HP1b (Krishnamoorthy et al., 2018a), Lamin B1 (Khanna et al., 2018), ATR (Muralikrishna et al., 2012), Emerin (Muchir et al., 2006), Nesprins and pRb (Johnson et al., 2004), undergo UPS-dependent degradation. This suggests that our results may have significant implications in the mechanism of laminopathic diseases.

4.2 Role of PIN1 in nuclear mechanics

Our results suggest that the function of PIN1 is important to regulate how mechanical cues transduced by the cytoskeleton impact, through the NE, on chromatin organization and TE activity. In support of this, we provide three lines of evidence: i) in the fly brain, Dodo safeguards NE structure from mechanical stress; ii) in hTAU expressing flies, compared to *dodo* mutant and *dodo RNAi* expressing flies, the extent of NE invagination and dHP1a protein level reduction is higher despite a higher level of Dodo protein. This suggests that, in hTAU expressing flies, mechanical stress due to formation of ectopic actin fibers (Fulga et al., 2007) synergises with the loss of Dodo, exacerbating NE invagination and dHP1a degradation. To further support this interpretation, we are currently assessing, in hTAU expressing flies, to which extent the inhibition of actin polymerization restores dHP1a protein levels (manuscript in preparation). It will be important also to assess if mechanical stress has a more global impact on the organization of chromosome domains, as it has been proposed (Maharana et al., 2016; Cremer and Cremer, 2001); iii) in line with these considerations, in hTAU expressing flies, compared to *dodo* mutant and *dodo RNAi* expressing flies, the increase of TE mRNAs does is stronger despite a higher level of Dodo protein. This suggests that, in hTAU expressing flies, mechanical stress due to formation of ectopic actin fibers (Fulga et al., 2007) synergises with the loss of Dodo, exacerbating the increase of TE mRNAs. To further support this interpretation, we are currently assessing, in hTAU expressing flies, to which extent the inhibition of actin polymerization suppresses the increase of TE mRNAs (manuscript in preparation).

The NE is connected to both Actin filaments and microtubules (MTs) through the LINC complex. Identifying the involved players could provide molecular targets to modulate the function PIN1 in regulating NE organization. We are currently exploring the possibilities that this involves components of the LINC complex and MTs, which would be in line with previously proposed roles

of LINC complex components and of MTs in mediating TAU-dependent NE invagination in *Drosophila* and mammals, respectively (Fulga et al., 2007; Frost et al., 2016).

Based on our results, it is tempting to speculate that PIN1 function at the NE could be part of a cellular response to mechanical stress aimed at preserving NE integrity and restrain TE activity. The regulation of HECW2 activity by PIN1 would be in line with this idea, as HECW2 targets ATR (Muralikrishna et al., 2012), which has been shown to promote a cellular response to mechanical cues aimed at preserving NE integrity (Kumar et al., 2014). We are currently assessing whether PIN1 and ATR activities at the NE are part of the same cellular response. According to this idea, HECW2 activity may be also sensitive to mechanical stress. Thus, the UPS may operate as a key regulator of nuclear mechanotransduction, by controlling the turnover of several nuclear proteins involved in nuclear mechanics, including B-type Lamins, ATR and HP1a itself. Indeed, chromatin has been shown to contribute to nuclear mechanical properties (Stephens et al., 2019; Stephens et al., 2017; Stephens et al., 2017b; Schreiner et al., 2014).

We provide evidence that PIN1 promotes HP1a tethering to the nuclear lamina also in non-neuronal cells, suggesting that PIN1 could have a fundamental role in coupling nuclear mechanics with regulation of heterochromatin. This could have profound implications in diseases that imply a contribution of altered mechanotransduction and genome instability, including cancer. In tumor cells, NE deformation has long been recognized as a pathological hallmark, and PIN1 levels/activity is often upregulated, which has been demonstrated to promote cancer cell proliferation and tumor progression (Rustighi et al., 2014). While NE weakness and genome instability can foster cancer cell heterogeneity, thus favouring adaptation to harsh microenvironment, excessive NE ruptures could be cell lethal. Thus, upregulation of PIN1 levels/activity, besides promoting the function of oncogenes (Rustighi et al., 2014), could serve to buffer NE alterations. In striking similarity, ATR has been proposed to promote tumor invasiveness and tumor progression by preserving NE integrity during cancer cell migration (Kumar et al., 2014). We are currently assessing the relevance of PIN1 function at the NE in cancer cells.

4.3 Impact of loss of PIN1 function at the nuclear envelope

Several lines of evidence suggest that mechanical stress could be an important driver of aging-dependent diseases, including AD (Nogueira et al., 2015): i) the pathogenesis of AD and other brain disorders involves intra and extra-cellular deposits of amyloid (Chiti and Dobson, 2006), which are much stiffer than normal tissue (Fitzpatrick et al., 2013) and could cause mechanical stress. Recent studies reported that integrins bind to amyloid fibrils, mediating signal transmission from extracellular sites of amyloid deposits into the cell and ultimately to the nucleus (Caltagarone et al., 2006); ii) furthermore, altered brain fluid pressure and traumatic brain injury, which cause mechanical stress, have been proposed to contribute to AD pathogenesis (Savolainen et al., 1999; Bech et al., 1999; Golomb et al., 2000; Cabral et al., 2011; Chauhan 2014; Stein et al., 2014); iii) in addition, AD risk loci are enriched in regulators of focal adhesions (FAs), mechanotransduction and cytoskeleton, including Integrins, Src, Rho, CAS, FAK family proteins (Dourlen et al., 2019), some of which have been shown to contribute to AD pathogenesis in *Drosophila* models (Dourlen et al., 2019). This has been proposed to imply an alteration of the function of these proteins that causes synaptic defects (Dourlen et al., 2019), which have been recognised as early events in AD pathogenesis. Based on the results presented in this Thesis, an intriguing possibility is all these factors could also contribute to AD pathogenesis through aberrant nuclear mechanotransduction. Thus, administration of drugs that interfere with mechanotransduction, such as ROCK inhibitors, could suppress AD-related phenotypes. In support of this strategy, the ROCK inihbitor Fasudil has been shown to suppress neurodegeneration in models of tauopathies (Gentry et al., 2016; Gu et al., 2018).

Both reduction of PIN1 function/activity and formation of ectopic actin fibers impinging on the NE have been observed in AD patients (Lu et al., 1999; Chen et al., 2015; Frost 2016). This, together with our evidence that the function of PIN1 at the NE is conserved from invertebrates to humans, suggests that the role of PIN1 in safeguarding heterochromatin from mechanical stress could be important to protect from AD. To further support this idea, we are currently assessing whether, in PIN1-depleted mouse brain neurons, accumulation of TAU correlates with formation of ectopic actin fibers (manuscript in preparation). In AD pathogenesis, as PIN1 prevents both the formation of amyloidogenic peptides and hyperphosphorylation of TAU, loss of PIN1 function and mechanical stress could be part of a pathogenic self-sustaining loop.

How, in *Drosophila*, hTAU could lead to decrease of Dodo protein levels, independently of *dodo* gene expression, remains to be established. One possibility is that a fraction of the Dodo protein becomes insoluble and/or is degraded. In neurons of patients affected by tauopathies such as AD and Frontotemporal dementia (FTD), TAU hyperphosphorylation was proposed to lead to soluble PIN1 protein depletion (Lu et al. 1999). Accumulation of hyperphosphorylated hTAU in fly brain neurons could provide an excess of binding sites for Dodo, and lead to Dodo protein depletion via yet to be identified mechanisms. The use of flies ectopically expressing mutant form of hTAU that are pseudophosphorylated (e.g. $hTAU^{E14}$; Steinhilb et al., 2007) or phosphorylation-incompetent (e.g. TAU^{AP}; Steinhilb et al., 2007) could represent a first strategy to clarify this issue. Even if hTAU does not form NFTs in fly brain neurons, it is possible that Dodo is bound by oligomeric/soluble forms of hyperphosphorylated hTAU. Alternatively, ectopic expression of hTAU in fly brain neurons could lead to depletion of the Dodo protein via indirect mechanisms. Recent evidence in animal models and brains of AD patients suggest that hyperphosphorylated hTAU impairs the nucleocytoplasmic transport of proteins (Eftekharzadeh et al., 2018; Paonessa et al., 2019) and RNA (Cornelison et al., 2018). This mechanism could be responsible for an impaired nuclear import of Dodo/PIN1, making it available for sequestration and degradation in the cytoplasm, and sinergyse with loss of PIN1-dependent nuclear compartimentalisation.

In line with a neuroprotective effect of PIN1 in ensuring proper mechanotransduction, Death-associated protein kinase 1 (DAPK1), which inhibits PIN1 (Lee et al., 2011) and is overexpressed in AD (Kim et al., 2014), was recently shown to be involved in rigidity sensing at FAs (Qin et al., 2018). Cells can sense and respond to a diverse variety of mechanical cues from their environment including matrix rigidity (Discher et al., 2005). In particular, the rigidity of the ECM has emerged as a key parameter for controlling neuronal homeostasis and survival (Lau et al., 2013; Bonneh-Barkay and Wiley, 2009). Several small molecules inhibiting DAP kinases have been identified (Farag & Roh, 2018), and the development of DAPK1 selective inhibitors could represent a strategy to promote PIN1 function in AD (Xu et al., 2019).

Our results support a model in which PIN1-dependent maintenance of heterochromatin is crucial to prevent TE hyperactivity, whose age-dependent accumulation would cause cognitive impairment and neurodegeneration. In line with our data, in AD patients and fly models, heterochromatin relaxation has been shown to correlate with TE neurotoxic hyperactivity (Frost et al., 2014; Frost et al. 2016; Guo et al., 2018; Sun et al., 2018). It is likely that, in this context,

chromatin relaxation both causes TE derepression and generates an environment permissive for TE insertion. Furthermore, TE insertion could alter the expression of genes located in the targeted loci, as well as alter gene expression programs by mobilization of regulatory sequences. TE activity may also contribute to AD etiology, as a recent study showed that neurons of patients affected by sporadic AD have thousands of variants of genomic cDNAs (gencDNA), derived from the *APP* gene through "retroinsertion" of RNA, involving transcription, DNA breaks and reverse transcriptase activity (Lee et al., 2018). We speculate that, among the TEs upregulated in AD, retrotransposons may contribute to AD etiology by providing unscheduled reverse transcriptase (RT) expression and activity.

How TE hyperactivity leads to neurodegeneration remains to be fully elucidated. Our results suggest that de novo TE insertions cause DNA damage. One possibility is that sustained activation of the DNA damage response pathway (DDR) leads to cell death. In line with this hypothesis, the Chk-2 ortholog Loki has been recently shown to mediate neurodegeneration downstream of TE mobilization in a *Drosophila* model of Amyotrophic Lateral Sclerosis based on the ectopic expression of the TDP-43 protein (Krug et al., 2017). In addition, we noted that cognitive defects associated with loss of Dodo occurred early in young flies, preceding neurodegeneration, and were prevented by administration of 3TC (data not shown). This suggests that unscheduled TE mobilisation could be an early event in AD pathogenesis.

Heterochromatin relaxation could directly impact on the expression of both sequences embedded in constitutive heterochromatin, such as TEs, and genes regulated by facultative heterochromatin repression. In postmitotic neurons in the adult *Drosophila* brain, HP1a has been recently shown to target genes required for cell cycle progression (Marshall and Brand, 2017). This suggests that HP1a could contribute to neuron differentiation by repressing genes that promote cell proliferation. We are currently elucidating whether such genes are derepressed in Dodo-depleted and hTAU expressing fly brain. This would be consistent with the proposed re-activation of genes regulating cell-cycle observed in several models of neurodegenerative diseases, including fly AD models (Frost et al., 2014). However, repeated sequences embedded in constitutive heterochromatin may be more sensitive to the reduction of HP1a protein levels. Furthermore, other members of the HP1 protein family could compensate the effects of loss of HP1a in the regulation of euchromatic genes (Hayakawa et al., 2003; Smallwood et al., 2012). In addition to its effects on HP1a and H3K9 methylation, loss of PIN1 may have a more global impact

on epigenetics. In this regard, it will be important also to assess if loss of PIN1 has a global epigenetic impact that recapitulates defects observed in AD patients (Klein et al., 2019).

The relevance of PIN1 function in preventing chromatin alterations and TE hyperactivity may extend beyond AD. Results from our collaborator V. Specchia (University of Salento, Italy) show that *dodo* mutant flies have reduced fertility (manuscript in preparation), suggesting that PIN1 function at the NE could regulate germline cell homeostasis. In line with this hypothesis, the nuclear lamina, ATR and Chk-2 have been recently shown to be involved in a checkpoint controlling stem cell quality in the *Drosophila* germline (Barton et al., 2018), a tissue whose homeostasis is highly sensitive to TE activity.

Our results suggest that PIN1 maintains heterochromatin by stabilizing HP1a protein, thus silencing TEs at the transcriptional level. In this scenario, we identified HECW2 as a potential key regulator of HP1a stability relevant for AD pathogenesis. In support of this idea, we report that, in AD fly models, hTAU expression is associated with pathogenic loss of Dodo function and with UPS-mediated degradation of dHP1a protein. We are currently assessing whether, in AD models, UPS-dependent degradation of HP1a is mediated by HECW2 (manuscript in preparation). This would provide a target whose activity could be modulated to maintain heterochromatin in aging and aging-related diseases such as tauopathies, including AD.

PIN1-dependent TE silencing may involve additional regulators of HP1a function, such as PIWI, which has been shown to interact with HP1a (Brower-Toland et al., 2007) and silence TEs. Two observations are in line with this hypothesis: i) in flies expressing a mutated version of hTAU, associated with fronto-temporal dementia, loss of PIWI contributes to TE hyperactivity (Sun et al., 2018) and ii) *dodo* deficiency leads to dorso-ventral polarity defects in the fly embryo that phenocopy mutations in genes encoding for PIWI proteins (Wang & Elgin, 2011).

Despite several years of intense scrutiny, a promising therapeutic approach for AD is still lacking, while it is estimated that dementia will become epidemic in the coming decades. Taken together, the findings presented in this Thesis may provide the rationale for developing HECW2 inhibitors to be tested in combination with retrotranscriptase inhibitors (e.g. 3TC, AZT; Sun et al., 2018) and/or ROCK inhibitors (Koch et al., 2018), as potential treatment for AD.

5. MATERIAL AND METHODS

5.1 *Drosophila* strains

Drosophila lines were maintained at 25°C in plastic tubes where we added a standard food prepared as follows: 9,2g/L Agar; 83,3g/L brewer yeast; 83,3g/L corn; 4,8ml/L propionic acid; 25ml/L Tegosept. The experiments shown in this thesis were performed using the following fly strains:

- w^{1118} as control strain. It harbors a mutation in the *white* gene, conferring a white eye phenotype (this stock was kindly provided by the laboratory of Dr. V. Specchia, University of Salento);
- $dodo^{EY03779}$ (Bloomington stock center #15677);
- *UAS-*$dodo^{KK108535}$ (VRDC stock center #v110593), as *dodo RNAi*;
- *UAS-dodo* (Kang et al., 2015);
- *UAS-hPIN1* (generated in this work, plasmid microinjection in fly embryos was performed by BestGene Inc., US);
- *UAS-dHP1a* (Xing and Li, 2015);
- *UAS-h0N4RTAU* (Wittmann et al., 2001);
- *Elav-GAL4, UAS-Syt-GFP/FM7* (Bloomington stock center #6923), a driver expressed in all postmitotic neurons;
- *Tubulin-GAL4/CyO; Sb/TM6b*, a ubiquitously expressed driver;
- *Su(var)*205^{05}/CyO, (*dHP1a*^{05}, Bloomington stock center #6234), a null allele of *dHP1a*.

5.2 Negative geotaxis assay

5 flies were placed in a single vial with a horizontal line at 5 cm from the bottom. The flies were gently tapped at the bottom of the vial and then we scored the number of flies that crossed the line in 9 seconds.

5.3 Fly learning and memory assays

For each condition, n=5 groups (N=20 individuals/group) were assayed for the ability to associate light with a negative stimulus (bitter taste obtained through a quinine solution 0.1 M) a negative stimulus that reduces phototaxis. Each 5 group underwent 5 iterative tests (with 30” acclimation

in the dark). 100 µM 3TC (Lamivudine) was added to the food. Fly learning and memory assays was performed by Dr. V. Specchia (University of Salento).

5.4 Tissue dissection, ex-vivo brain culture and drug treatment

Flies and larvae were ice-anesthetized, adult heads and 3rd instar larval fat bodies were dissected in Insect-XPRESS medium (LONZA 12-730), supplemented with 10% heat-inactivated FBS and 10mg/L Insulin. For ex-vivo brain culture, retina was removed and heads incubated in in Insect-XPRESS medium (LONZA 12-730) supplemented with 10% heat-inactivated FBS and 10mg/L Insulin supplemented with DMSO (control vehicle), 0.2 µM PiB (3h, Room Temperature (RT)), 4 µM ATRA (3h, RT), 5 µM KPT-6566 (3h, RT), 25 µM MG132 (3h, RT). .

5.5 Tissue cryosections

Dissected flies heads were 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. Heads were were embedded in OCT medium and then frozen on isopentane/liquid nitrogen bath. The embedded tissue was stored at -80°C. 10 µm slices of head tissue were placed on Superfrot Plus glass slides (Thermo Scientific). The slides were stored at -80°C.

5.6. Whole-mount *Drosophila* tissue immunofluorescence

Drosophila tissues were stained according to a standard whole mount protocol (Bertolio et al., 2019; Napoletano et al., 2017). Briefly, flies and larvae were ice-anesthetized and dissected in PBS. Brains and fat bodies were fixed in 4% paraformaldehyde for 15 minutes at 25°C, rinsed in PBS and permeabilized in 0.1% Triton X-100 PBS, and incubated with primary antibodies in 0.1% Triton X-100 PBS for 14-16 hours at 4°C. Then washed in 0.1% Triton X-100 PBS, incubated with Alexa Fluor secondary antibodies (488, 568, 594, 647- conjugated, Thermofisher, 1:400 dilution) in 0.1% Triton X-100 PBS for 2 hours at 25°C. Then rinsed in 0.1% Triton X-100 PBS and incubated with HOECHST 33342 (Life Technologies, 2µg/ml in PBS) 10 minutes at 25°C. Finally, brains were rinsed in water and mounted on glass slides with Prolog Gold fluorescence anti-fading reagent (Invitrogen, P36930). Rhodamine phalloidin (Thermo Fisher, R415) was used to label F-Actin.

5.7. Whole-mount *Drosophila* brain TUNEL

Freshly dissected brains were fixed in 4% paraformaldehyde for 15 minutes at 25°C, washed in 0.1% Triton X-100 PBS, permeabilized in sodium citrate 100mM, 0.3% Triton X-100 PBS at 65°C for 45 minutes, and incubated with TUNEL reagent (In Situ Cell Death Detection Kit, TMR red, Sigma 12156792910) 14-16 hours at 37°C in dark humid chamber. Then brains were washed in 0.1% Triton X-100 PBS and incubated with HOECHST 33342 (Life Technologies, 2µg/ml in PBS) 10 minutes at 25°C. Finally, brains were rinsed in water and mounted on glass slides with Prolog Gold fluorescence anti-fading reagent (Invitrogen, P36930).

5.8 *Drosophila* frozen tissue Immunofluorescence

Slices of heads tissue were 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 was made 14-16h at 4°C in a humid chamber. After 3 washes of 10 minutes in PBST we added Alexa Fluor secondary antibodies (488, 568, 594, 647- conjugated, Thermofisher, 1:400 dilution) and performed a 2 hour incubation at room temperature in a humid chamber. Then 3 washes in PBST were performed and we stained the nuclei with HOECHST 33342 (Life Technologies, 2µg/ml in PBS) 10 minutes at 25°C. After 3 washes in PBST and one in water, we mounted the slices with Prolog Gold fluorescence anti-fading reagent (Invitrogen, P36930).

5.9. Image acquisition and analysis

Images were acquired with a Nikon ECLIPSE C1si confocal microscope and analysed with Nikon NIS-Elements Imaging (Confocal microscopy facility, University of Trieste) and FIJI Softwares.

5.10 Immunoprecipitation

Dissected fly heads were washed 3 times in PBS; then they were transferred in a buffer containing 20mM Tris-HCl pH=8, 150mM NaCl, 0.2% NP-40, 0.2% Triton X-100, 5mM EDTA and protease and phosphatase inhibitors (NaF 5mM; PMSF 1mM; CLAP 0.1Mm; Na_3VO_4 1mM) and manually lysed using a pestle. To remove the debris the lysates were centrifuged; the supernatant was placed in

a new tube and then sonicated 3 times. The lysates were incubated with 2 µg of anti-Hp1a monoclonal antibody, or with 2 µg of anti-HA antibody as control, on a wheel O.N. at 4°C. The lysates were then transferred on protein G beads and incubated for 1h at 4°C on a speeding wheel. After 3 washes the immunocomplex was eluted using Sample Buffer 2X and the analyzed on SDS-PAGE. 80 *Drosophila* heads were poled for each sample.

5.11 Western Blot

Freshly dissected tissues (N=15 female flies or N=1 mice) were transferred in a buffer containing 20mM Tris-HCl pH=8, 150mM NaCl, 0.2% NP-40, 0.2% Triton X-100, 5mM EDTA, protease and phosphatase inhibitors (NaF 5mM; PMSF 1mM; CLAP 0.1Mm; Na_3VO_4 1mM). Fly samples were manually lysed using a pestle. Mouse samples were homogenized using the gentle MACS™ Octo Dissociator of the Miltery Biotec. Lysates were sonicated and boiled (5 minutes at 95°). SDS-PAGE stacking gel composition was: 5% acrylamide, 0.14% bisacrylamide, 0.125M Tris-HCl pH 6.9, 0.1% SDS. Running gel was composed by 17.5% acrylamide, 0.1% bisacrylamide, 0,374M Tris-HCl pH 8.7, 0.1% SDS. Transfer buffer was composed by Tris 48 mM, glycine 39 mM, SDS 4.13 mM, 20% w/v methanol. Semidry transfer cell (Biorad) was used to transfer proteins on nitrocellulose membrane. After incubation of nitrocellulose membrane for at least 30 minutes in blotto tween (5% milk, 0.2% Tween20 in PBS), membranes were incubated with the primary antibodies diluted in blotto tween for 2h or O/N at 4°C. Membranes were then rinsed 3 times in PBS and incubated with the HRP-conjugated secondary antibodies for 1 hour at 4°C. Membranes were washed 4 times with PBS and signal revealed with ECL plus (Pierce) or ECL (Amersham) reagents.

5.12 Primary antibodies

anti dHP1 (DSHB, C1A9);
anti HA (12CA5 Boehringer Mannheim);
anti dLamB (DSHB, ADL6710);
anti γH2Av (DSHB, UNC93-5.2.1);
anti-Elav (DSHB 9F8A9 and 7E8A10);
anti dHecw (kind gift generated in the lab of Dr. Simona Polo, IFOM, Milan);
anti PIN1 (homemade by Del Sal laboratory);
anti Actin (Sigma, A2066);

anti H3K9me3 (Diagenod, C15410056);

anti hTAU (GeneTex, GTX112981);

anti HP1α (abcam, ab77256);

anti Lamin B1 (abcam, ab16048).

5.13 RNA purification and cDNA synthesis

Freshly dissected tissues (N= 5 pooled female flies individuals or N=1 mice) were transferred in QIAZOL reagent. Fly samples were manually lysed using a pestle. Mouse samples were homogenized using the gentle MACS^{TM} Octo Dissociator (Miltery Biotec). RNA was purified according to the QIAZOL manufacturer's instructions. For cDNA synthesis, Total RNA was used for reverse transcription (RT) with the QuantiTect Reverse Transcription kit (Qiagen).

5.14 Genomic DNA purification

Dissected Drosophila heads (a pool of N=30 female individuals) were transferred in Lysis buffer (Tris HCl 0,1 M (pH 9.0); EDTA 0.1M; SDS 1%), manually lysed using a pestle, and treated with RNAsi A for 30 minutes. Genomic DNA (gDNA) was purified by standard Phenol/chloroform extraction and alcoholic precipitation. Purified gDNA was used for Copy number variation (CNV) Taqman assays.

5.15 Quantitative PCR

cDNA synthesized from total RNA or gDNA (for CNV assays) were used as template for real-time quantitative PCR (qPCR). qPCR was performed using the SsoAdvanced SYBR Green Supermix (BIORAD) or the iQ Multiplex Powermix (BIORAD, for Taqman assays) reagent, and a BIORAD CFX96 Touch™ Real-Time PCR Detection System termocycler. Quantification was based on the $2^{-\Delta\Delta Ct}$ method using *Drosophila rp49*, *Drosophila actin*, *Drosophila DMRT1C* (for CNV assays) or mouse *GAPDH* as reference.

List of primer and Taqman probe sequences:

TARGET	**PRIMER SEQUENCE**
dodo	Fw: GAAAGTTCGGCAGAGGTCAG
	Rev: CTGGGCATTCCGTTTTATTC
dHP1	Fw: CGCAAGGATGAGGAGAAGTCA
	Rev: TCCTGAAACGGGAATGGTGTC
ZAM (*Drosophila*)	Fw: TCGTCGCCGCAGGAAACTCTC
	Rev: GTGGAGCGACGATTGGAAGAA
IVK (*Drosophila*)	Fw: ACTCTGGGTTCCCAGTCATC
	Rev: GGTCCTTGGAGTTAAACGGA
QUASIMODO (*Drosophila*)	Fw: TCTACAGTGCCATCGAGAGG
	Rw: TAGTTCAGCCCAAGTGTTGC
TAHRE (*Drosophila*)	Fw: ATCCAGGCCAAGGATATGAC
	Rw: TCTGATGATGACTCGGAAGC
ROO (*Drosophila*)	Fw: CGTCTGCAATGTACTGGCTCT
	Rw: CGGCACTCCACTAACTTCTCC
ROOA (*Drosophila*)	Fw: CAGAAGATGTTAACTCCAATTT
	Rw: TCAATGAGTGTAGCTGTTTCG
412 (*Drosophila*)	Fw: ACAAGGAGGGCATACAGGCATTAC
	Rw: ATCGTCATTGGAGTCTTTGTGTGC
actin (*Drosophila*)	Fw: GCGTCGGTCAATTCAATCTT
	Rev: AAGCTGCAACCTCTTCGTCA
rp49 (*Drosophila*)	Fw: ATCGGTTACGGATCGAACAA
	Rev: GACAATCACCTTGCGCTTCT
hTAU	Fw: GAAACCCACAAGCTGACCTTC
	Rev: GGAGGAGACATTGCTGAGATG
5'UTR_Tf (Mouse)	Fw: TGAGCACTGAAACTCAGAGGAG
	Rev: GATTGTTCTTCTGGTGATTCTGTTA
	Probe: GAATCTGTCTCCCAGGTCTG
5'UTR_Gf (Mouse)	Fw: CCAAACACCAGATAACTGTACACC

	Rev: CGTGGGAGACAAGCTCTCTT
	Probe: TGAAAGAGGAGAGCTTGCCT
5'UTR_A (Mouse)	Fw: TGCCCACTGAAACTAAGGAGA
	Rev: GCTTGTTCTTCAGGTGACTCTGT
	Probe: TGCTACCCTCCAGGTCTGCT
GAPDH (Mouse)	Fw: CGACCCCTTCATTGACCTC
	Rev: CTCCACGACATACTCAGCACCProbe: CTCCACTCACGGCAAATTC
IVK (*Drosophila*)	Fw: ACCTGCCTGTGTATCATGCA
	Rev: GCTTGTTTTGATGTGGAGCA
	Probe: ACCTTCCCGTGCCATGCACT
ZAM (*Drosophila*)	Fw: CCACCACGCCTACACAAGTA
	Rev: TATGGAATTGTATGCGTTGC
	Probe: CGAACCGGGAAGCTTTGCGA
DMRT1C (*Drosophila*)	Fw: GAACGGAGCTCTCTGGAAAC
	Rev: GACCCTCCTGGCTCTCTC
	Probe: CTGCGGCGAAGCTGTTGAC

5.15 Animal care and handling

To prevent brain damage, mice were killed by cervical dislocation. After brain extraction, Hippocampus was dissected according to standard protocol (Sultan et al., 2012; Sultan, 2013). Mice were housed and used in a specific pathogen-free (SPF) animal facility. Procedures involving animals and their care were performed in conformity with institutional guidelines (D.L. 116/92 and subsequent complementing circulars).

5.16 Cell lines

HEK 293 (Human embryonic kidney 293) is a human cell line originally derived from human embryonic kidney cells grown in tissue culture. HEK 293T cells were cultured in DMEM (LONZA) supplemented with 10% Foetal Bovine Serum (FBS) 100 U/mL penicillin and 10 µg/mL streptomycin. HEK 293 Pin^{KO} was obtened in our lab from Dr. Rustighi A.

6. Supplementary Information

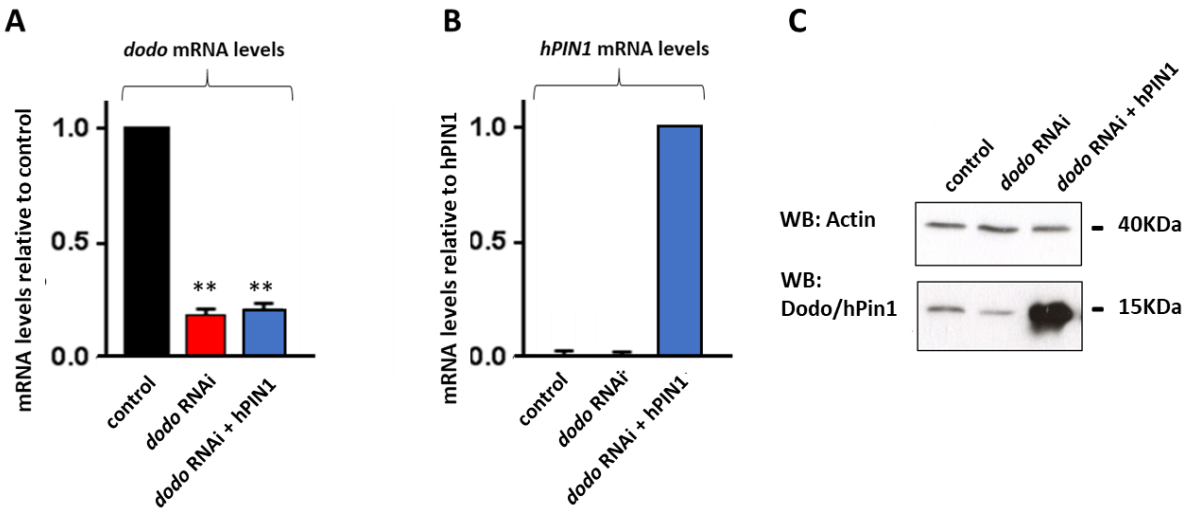


Figure S1. Generation of transgenic flies expressing *hPIN1*.
A-B) RT-qPCR analysis in the heads of 4 days old control flies (*w^{1118}/w^{1118};UAS-dodo^{RNAi}/+UAS-hPIN1/+*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. *actin* was used as reference for quantification. Values represent mean ± s.d. of N=3 biological replicates. **P value <0.01 by two tailed unpaired Student's t-test.
C) Western blot analysis in the heads of 4 days old control flies (*w^{1118}/w^{1118};UAS-dodo^{RNAi}/+UAS-hPIN1/+*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates.

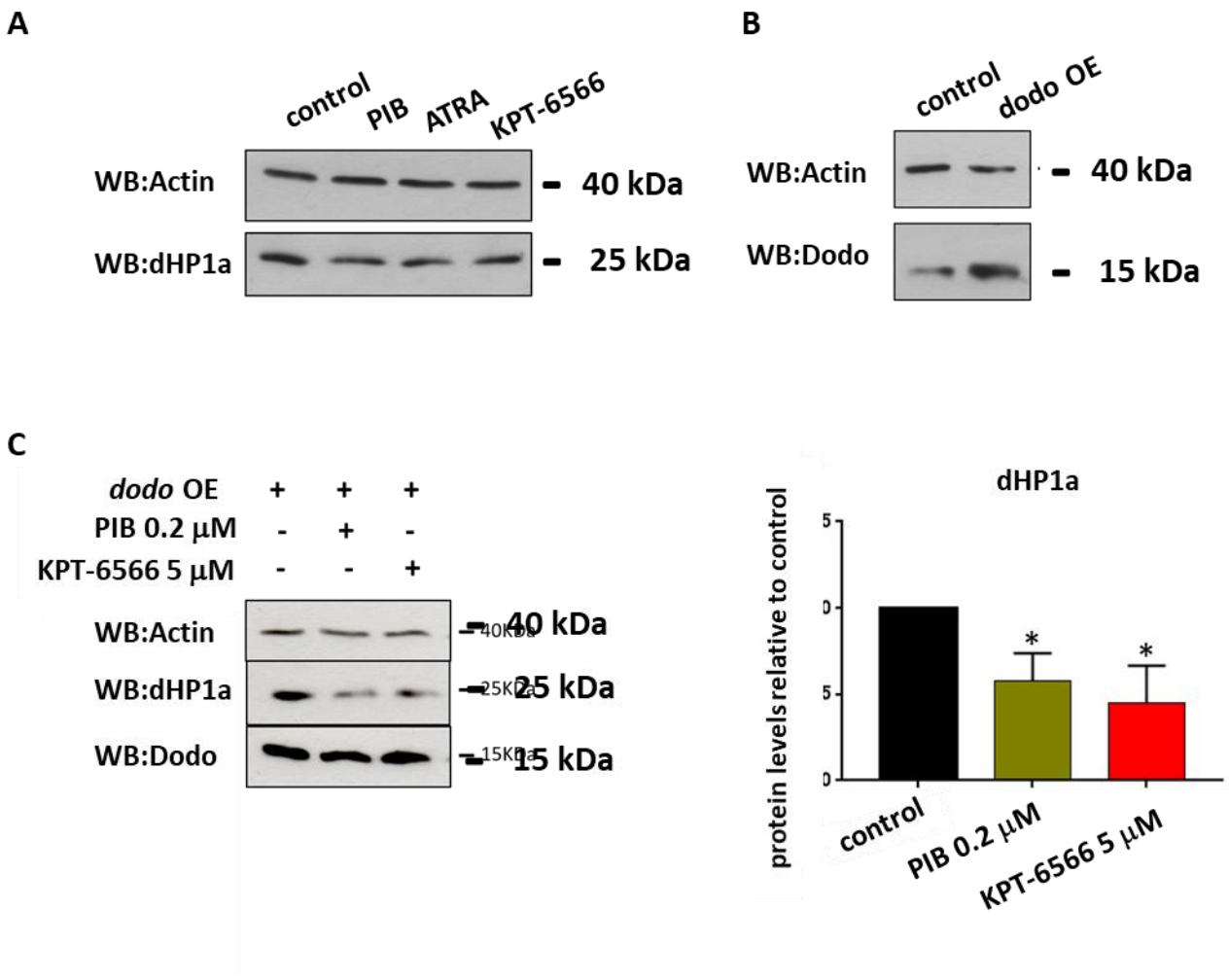


Figure S2. Dodo catalytic activity is required to maintain dHP1a protein levels in the *Drosophila* brain.

A) Western blot analysis in the heads of 4 days old wild-type (w^{1118}) flies treated with vehicle (DMSO, control) or the indicated drugs. The blot is representative of N=3 biological replicates.

B) Western blot analysis in the heads of 4 days old flies overexpressing *dodo* with the *Elav*-GAL4 driver (*dodo* OE). The blot is representative of N=3 biological replicates.

C) Western blot analysis in the heads of 4 days old flies overexpressing *dodo* with the *Elav*-GAL4 driver (*dodo* OE). Blots are representative of N=3 biological replicates. Quantification of dHP1a protein levels is shown in the right panel. Values represent mean ± s.d. *P value <0.05, by two tailed unpaired Student's t-test.

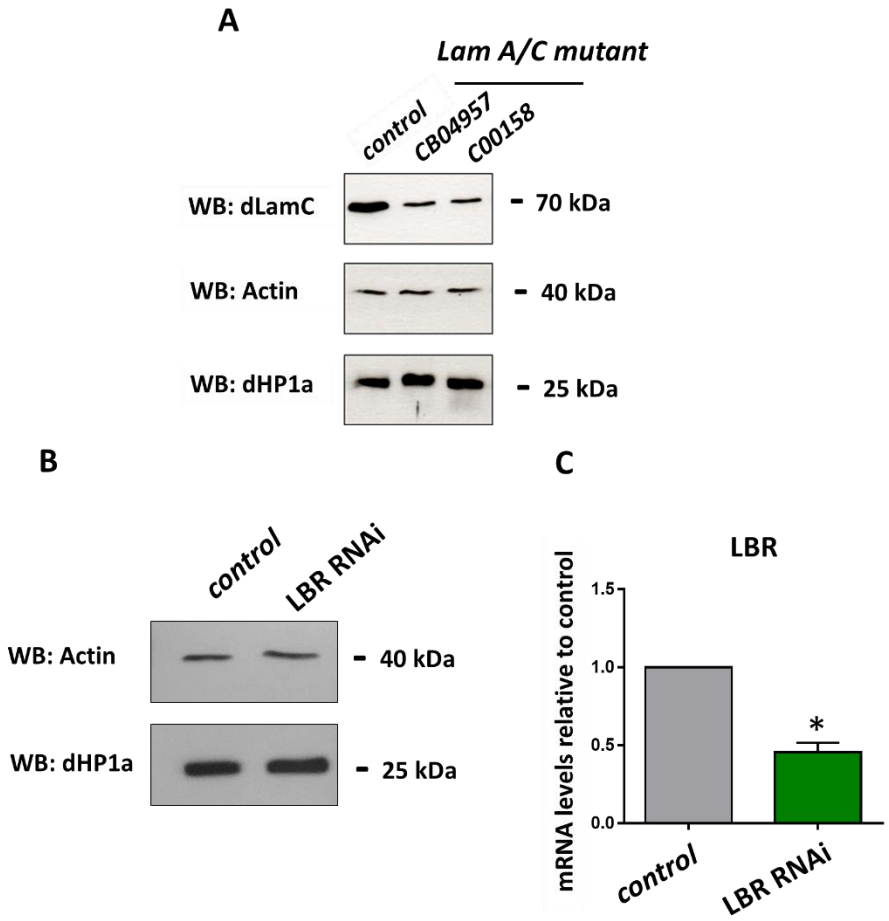


Figure S3. Loss A/C-type Lamin or Lamin B receptor are not associated with alteration of dHP1a protein levels in the *Drosophila* brain.

A) Western blot analysis in the heads of 4 days old control (w^{1118}) and *dLamC* mutant flies. Actin was used as loading control. The blot is representative of N=3 biological replicates.

B) Western blot analysis in the heads of 4 days old control flies (($w^{1118}/w^{1118};UAS\text{-}dLBR^{RNAi}/+;$) and flies expressing *dLBR* RNAi with the *Elav-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates.

C) RT-qPCR analysis in the heads of 10 days old control flies ($w^{1118}/w^{1118};UAS\text{-}dLBR^{RNAi}/+;$) and flies expressing the indicated construct with the *Elav-GAL4* driver. *actin* was used as reference for quantification. Values represent mean ± s.d. of N=3 biological replicates. *P value <0.05 by two tailed unpaired Student's t-test.

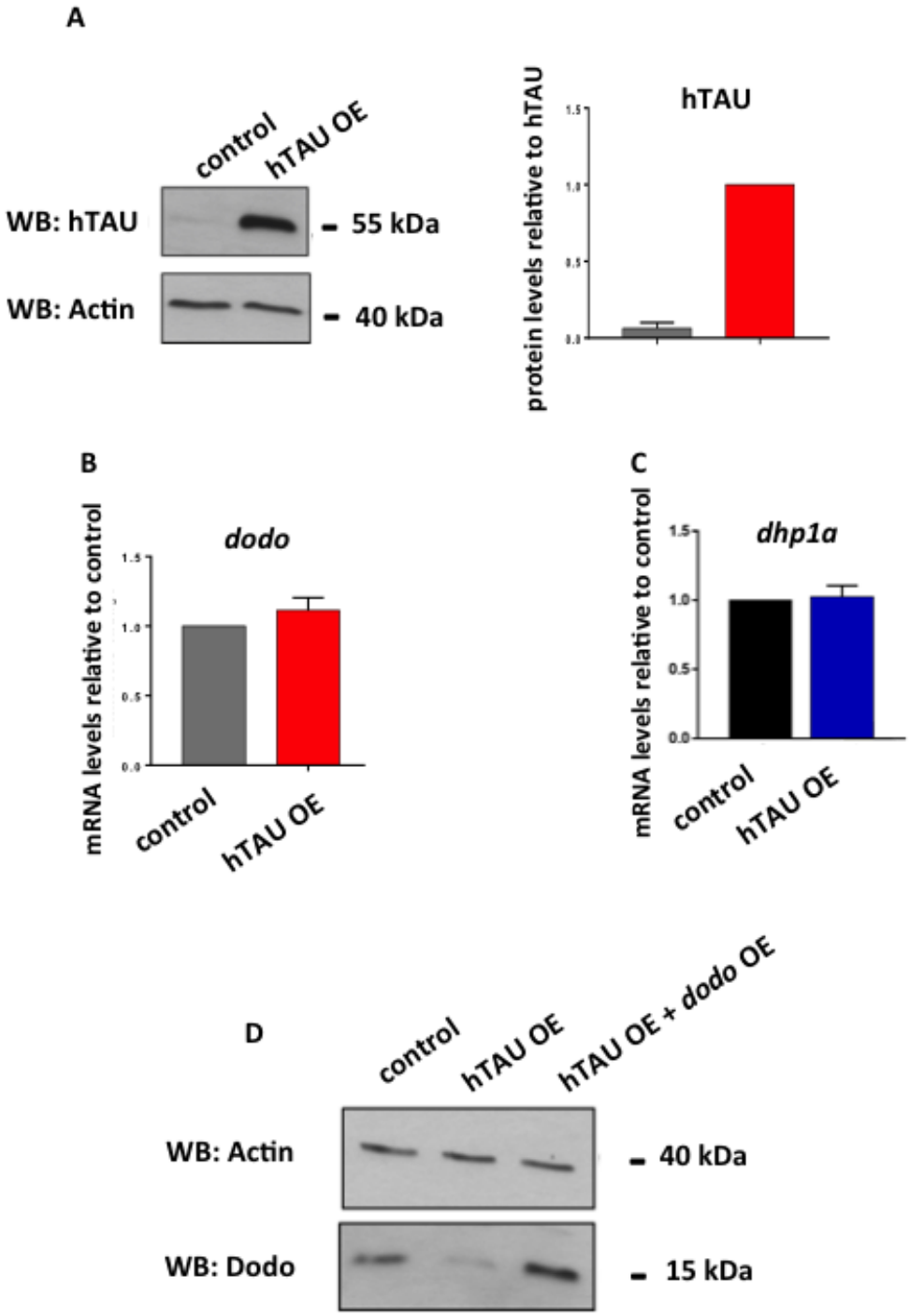


Figure S4. hTAU expression is not associated with alteration of *dodo* and *dHP1a* mRNA levels in the *Drosophila* brain.

A) Western blot analysis in the heads of 7 days old control flies (*;UAS-hTAU/+*) and flies expressing the indicated construct with the *Elav-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates. Quantification of hTAU protein level is shown in the right panel. Values represent mean ± s.d.

B-C) RT-qPCR analyses in the heads of 7 days old control flies (w^{1118}/w^{1118}*;UAS-hTAU/+;*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. *actin* was used as reference for quantification. Values represent mean ± s.d. of N=3 biological replicates.

D) Western blot analysis in the heads of 7 days old control flies (*w*^{1118}*/w*^{1118}*;UAS-Dodo/+;UAS-hTAU/+*) and flies expressing the indicated constructs with the *Elav-GAL4* driver. Actin was used as loading control. The blot is representative of N=3 biological replicates.

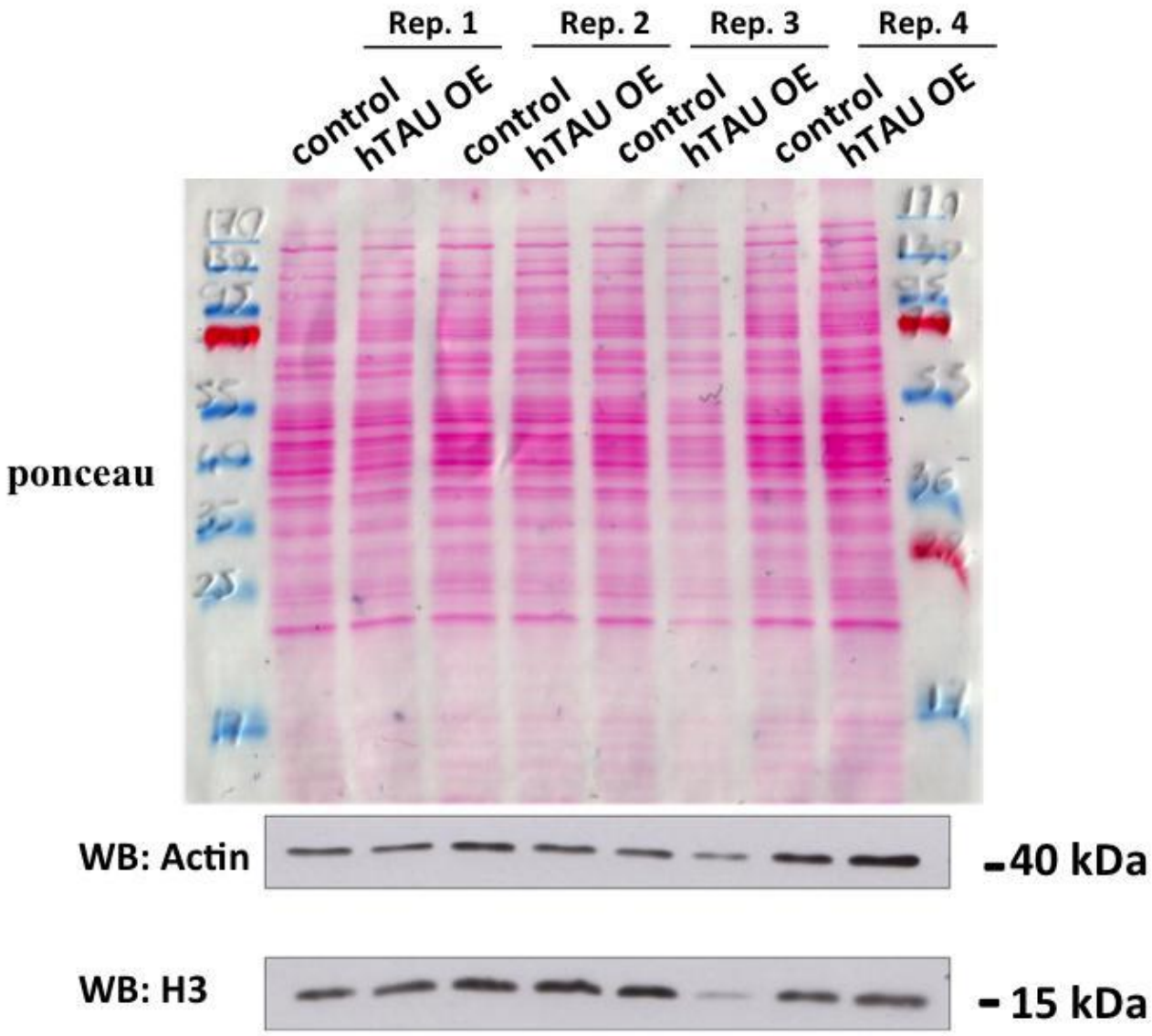


Figure S5. hTAU expression is not associated with alteration of Actin protein levels in the *Drosophila* brain.

Ponceau staining and western blot analysis in 7 days old control flies (*;UAS-hTAU/+*) and flies expressing the indicated construct with the *Elav-GAL4* driver. N=4 biological replicates.

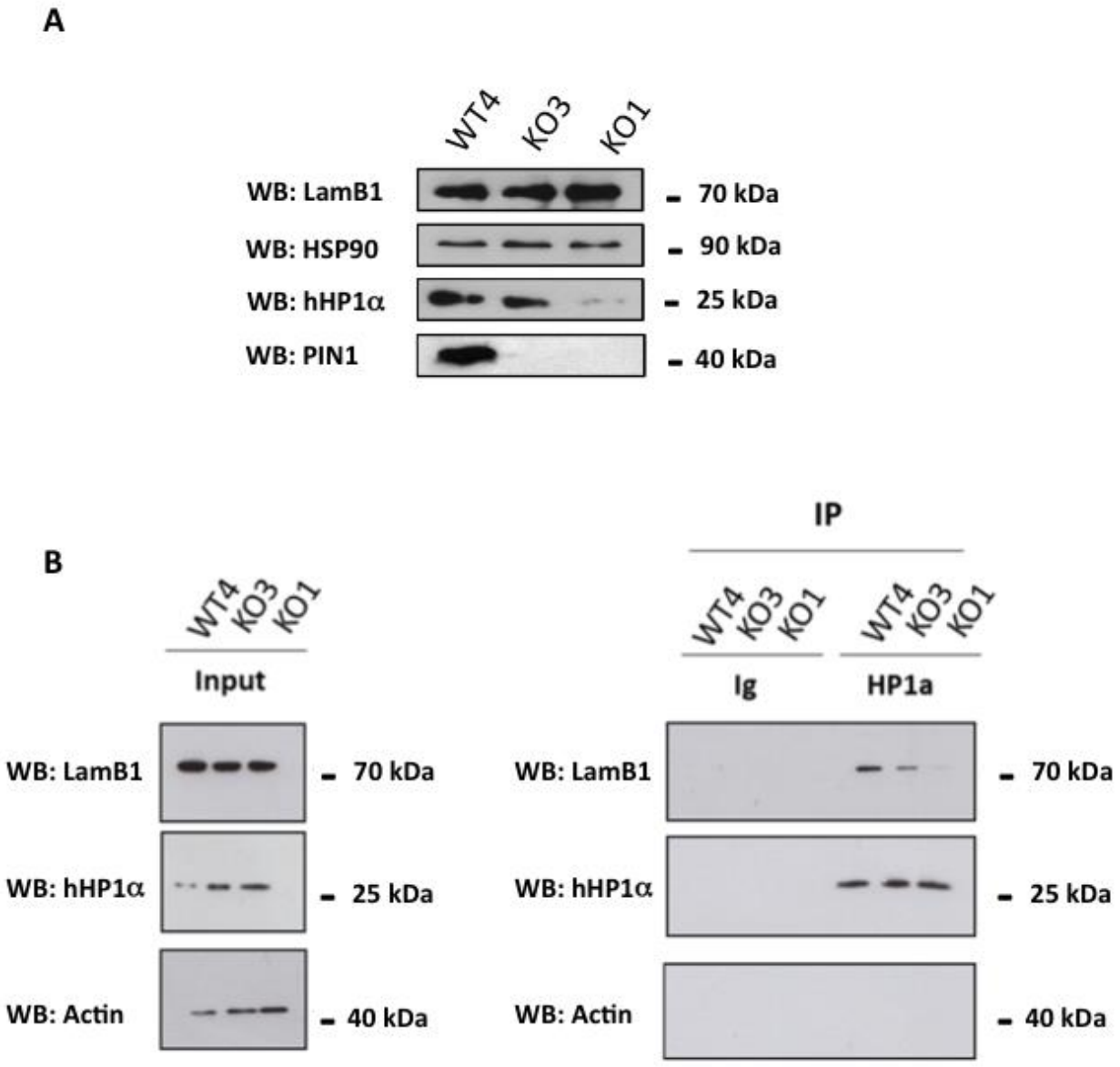


Figure S6. HP1a and Lamin B1 form a complex in human cells.

A) Western blot analysis in *PIN*^{KO} wild-type (*WT*) *PIN*$^{+/+}$ isogenic HEK-293T cell clones. HSP90 was used as loading control.

B) Co-immunoprecipitation (IP) assay in *PIN* ^{KO} wild-type (*WT*) *PIN* $^{+/+}$ isogenic HEK-293T cell clones. HSP90 was used as loading control. Anti-IgG antibody was used as negative control.

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Acknowledgements

I am thankful to Prof. Giannino Del Sal for allowing me to work in his highly formative and challenging laboratory, for his suggestions, scientific discussions and enthusiastic help on this project.

This research project has been performed within an ongoing and long lasting collaboration with Dr. Valeria Specchia (University of Salento). Dr. Specchia is a geneticist with great expertise in the study of genes and molecular pathways involved in TE regulation in Drosophila. Our research group and I are deeply indebted with Dr. Specchia for constantly providing scientific inputs, generously sharing results and supplying Drosophila strains and reagents, which have represented invaluable contributions to sustain the research work.

I owe much of my improvement as a scientist to Dr. Fiamma Mantovani, whose insightful comments and suggestions have helped me in these years. She helped me understand how to carry on research.

I would like to thank Dr. Francesco Napoletano. He has been greatly tolerant and supportive in teaching me everything I know about Drosophila stabulation. Thanks also for guiding me step-by-step in the assembly of figures I have shown in this thesis. Moreover, I am particularly grateful for the huge work in proofreading this thesis.

I thank also the PhD student Ilaria Voto for the encouragement in getting better as a PhD student and master's degree students Evelina Poletto, Amalia Perna, Davide Viotto, Lucia Celora and Aurora Satin who actively contributed to this work.

I wish to thank all the colleagues at LNCIB for their helpful and productive discussions and for all the good and bad times shared in the lab.

Thanks to all the special people who have been close to me during these years. None of this would have been possible without you.