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Drosophila melanogaster as a model to study *in vivo* the functional role of Transposable Elements in Huntington Disease pathogenesis

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“Del domani mi ripeto che:
andrà tutto bene!”

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ABSTRACT

Huntington's disease (HD) is a late-onset, autosomal dominant disorder characterized by progressive motor dysfunction, early death, cognitive decline and psychiatric disturbances. The disease is caused by a CAG repeat expansion in the IT15 gene, which elongates a stretch of polyglutamine (polyQ) at the amino-terminus of the HD protein, huntingtin (Htt). Despite the impressive data that have been accumulated on the molecular basis of neurodegeneration, no cure is still available.

Transposable elements (TEs) are mobile genetic elements that constitute a large fraction of eukaryotic genomes. Retrotransposons replicate through an RNA intermediate and represent approximately 40% and 30% of the human and *Drosophila* genomes. Mounting evidence suggests mammalian L1 elements are normally active during neurogenesis. Interestingly, recent reports show that a deregulated TE activation is associated with neurodegenerative diseases.

Drosophila melanogaster is an excellent model for studying HD pathology and disease mechanisms, because the majority of pathological features can be recapitulated in transgenic fly models. Our experimental results show that retrotransposons are up-regulated in HD fly brains and that their genetic or pharmacological inhibition determines the block of polyQ-dependent neurodegeneration; moreover, HD brains present a high level of TE-related genomic instability. Additionally, Position-Effect Variegation (PEV) assay allow us to assess that mutant Htt acts as a dominant modifier of heterochromatic gene silencing. Taken together, these data suggest that TEs represent an important piece in the complicated puzzle of polyQ-induced neurotoxicity.

INTRODUCTION

Huntington Disease (HD)

Genetic determinants and clinical manifestations

Huntington Disease (OMIM #143100) is a late-onset, autosomal, dominant, progressive disorder caused by the expansion of a CAG trinucleotide repeat, translated into a polyglutamine (polyQ) stretch at the amino-terminus of the *huntingtin* protein (Htt) (Huntington's Disease Collaborative Research Group, 1993).
The wild-type HTT gene, spanning 180 kilobases and located on the short arm of the fourth chromosome (4p16.3), has 67 exons and in the first one normally may present from 6 to 35 CAG repetitions (with an average median between 17 and 20 repeats (Kremer et al., 1994), while in pathological conditions the triplet can be repeated from 36 to 150 times: longer is the CAG stretch above the threshold and earlier is the onset of the disease (Duyao et al., 1993) (Figure 1). Individuals having from 36 to 40 CAGs may never develop HD symptoms, or may manifest the pathology late in life with reduced penetrance (Gusella and MacDonald, 2000).
Recent reports show the evidence that the timing of HD onset depends only on uninterrupted CAGs repetition size, and not on polyQ stretch size; CAA triplet, encoding for glutamine too, does not contribute to the length of pathogenic polyQ tract (Genetic Modifiers of Huntington’s Disease (GeM-HD) Consortium, 2019).
The common symptoms of HD are unintentional and uncontrolled movements (that is why HD is often called *Còrea*, from the Greek word that means “dance”), cognitive decline, psychiatric disorders, emotional deficits and early death (generally 15-20 years after the

onset) (Folstein, 1989). Additionally, HD is often linked to depression, delirium mania, suicidal tendency, apathy and social disinhibition (Vonsattel and Di Figlia, 1998; Saudou et al., 2016).
A very long polyQ stretch derived from more than 50 CAG repeats determines a more severe phenotype and a very early onset: the so called "Juvenile" HD (JHD), characterized, in addition to the common symptoms, by bradykinesia and seizures (Nance and Myers, 2001). Although neuronal damage is the most prominent symptom of the pathology, HD patients manifest metabolic and immune disturbances, weight loss, osteoporosis and testicular atrophy too (van der Burg et al., 2009).

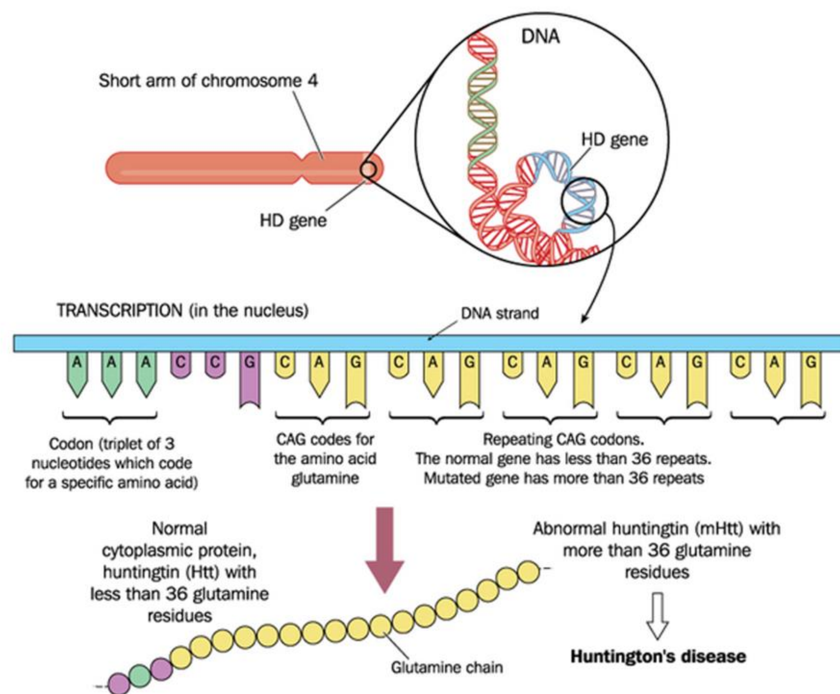

Fig. 1 – Wild-type huntingtin gene may contain in its first exon from 6 to 35 CAG triplet repeats, while in pathological condition the trinucleotide can be repeated from 36 to 150 times, leading to the production of an abnormal protein. Adapted from: https://ghr.nlm.nih.gov/condition/huntington-disease#genes

The district of the brain mainly damaged by HD is the striatum (caudate and putamen) and in particular its medium-sized spiny neurons (MSSNs) expressing the D2-dopamine receptors (Augood et al., 1996); other affected regions are the cerebral cortex (Rosas et al., 2005) and the cerebellum (Vonsattel et al., 1985). In JHD, neurodegeneration is more widespread, interesting a higher population of neurons (Myers et al., 1988).
The pathologically elongated tract of HTT gene is characterized by genomic instability both in gametogenesis (especially in spermatogenesis) and in somatic tissues (Wheeler et al., 2007); the proposed mechanism to justify this instability is the replication machinery "slippage" (Tautz et al., 1994): regions containing repeated nucleotide sequences promote the formation of DNA loops on template strand, making DNA polymerase lose the orientation and continue the synthesis from an incorrect position (Klintschar et al., 2004).
Curiously, the inheritance of the mutant allele through the male germ line is linked to a more severe symptomatology than the maternal inheritance. Furthermore, the change in repeat length with paternal transmission is significantly correlated with the change in age onset between the father and the progeny: this evidence, in HD, correlates with the phenomenon of genetic anticipation; in fact, on average, children show HD clinical manifestations 8 years earlier than their fathers (Ranen et al., 1995).

Wild-type Huntingtin (Htt)

Huntingtin (Htt) is a protein of 348 kDa and 3.144 amino acids, well conserved from flies to mammals and ubiquitously expressed in humans, in particular in CNS neurons and testes (Cattaneo et al., 2005). In the most part of the cells, Htt is localized in the cytoplasm and associated to various organelles, such as mitochondria, Golgi apparatus and endoplasmic reticulum, but a small fraction may be found even in the nucleus (Di Figlia et al., 1995; Trottier et al., 1995).

Huntingtin gene may produce two different mRNA transcripts (10.3 kb and 13.7 kb) and the longer one, carrying an additional 3'UTR sequence, is enriched in the brain (Lin et al., 1993). Recent reports show the presence of alternative splicing leading to the production of Htt variants that may be involved in protein-protein interaction and development (Hughes et al., 2014; Ruzo et al., 2015).

The Htt N-terminal region is the only well characterized part of the protein, because of the presence of the HD-related polyQ stretch, which is preceded by a 17 amino acids regions (N17) and followed by a proline-rich domain (PRD). The remaining large part of the protein (corresponding to 66 exons of 67) is not well-known.

The polyQ region is well conserved but larger in mammals and particularly in humans, where is polymorphic; its functions in wild-type conditions are not understood, but its deletion in mice leads to increase of autophagy and higher longevity (Zheng et al., 2010). This region is very flexible and may assume different secondary conformation, such as α-helix, random coil or extended loop (Kim et al., 2009).

The N17 domain is an amphipathic α-helix (Atwai et al., 2017) that is involved in retention in the endoplasmic reticulum; it is

subjected to different post-translational modifications and works as nuclear export signal (NES) (Maiuri et al., 2013; Rockabrand et al., 2007).

The PRD-domain is a prerogative of the human huntingtin, suggesting its recent evolution (Tartari et al., 2008), and seems to be involved in protein-protein interaction, in particular with the ones containing tryptophans or SH3 domains (Harjes and Wanker, 2003). Its proline-proline (PP) helix is a very rigid structure that is crucial to stabilize polyQ stretch and has a role in the aggregation property of mutant huntingtin.

Two domains that are extensively represented on the entire huntingtin protein are the HEAT and the PEST domains (Figure 2): the first seems to be a scaffold for protein interactions and the second is a proteolysis signal (Palidwor et al., 2009; Warby et al., 2008). Lots of proteases, such as caspases, calpain, cathepsins and metalloproteases (Goldberg et al., 1996; Miller et al., 2010; Tebbenkamp et al., 2012), cleave huntingtin on PEST domains, that are highly represented both in wild-type and mutant huntingtin.

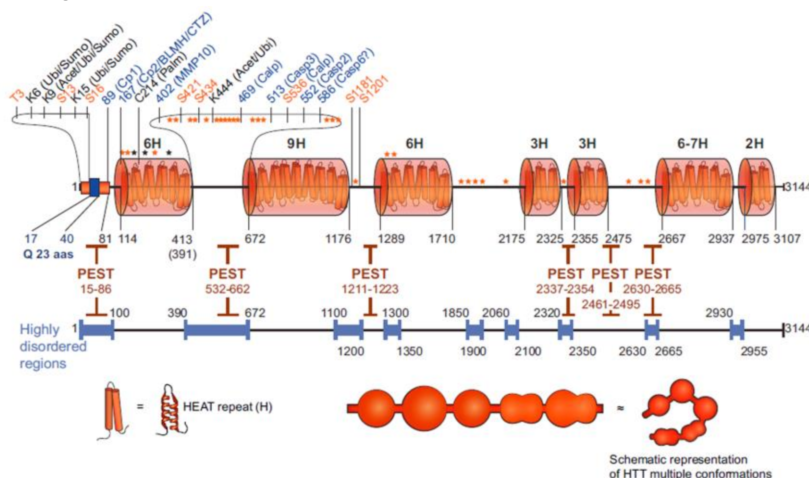

Fig. 2 - Representation of the human Huntingtin domains. Saudou et al., 2016.

For long time, all research efforts focused on mutant huntingtin and its deleterious role in HD pathogenesis, considering the wild-type protein having no clear function. In the last two decades a change in this approach led to discover that normal huntingtin is important for a lot of physiological functions and that its lack could be dysfunctional both in healthy and in HD conditions. In particular, among the various functions that have been discovered, huntingtin controls the transport of various organelles in the cell (Zala et al. 2013; Caviston et al. 2011), is abundant in proliferating cells where interacts with dynein at mitotic spindle coordinating cell division (Elias et al., 2014) and is essential for embryonic development and neurogenesis (Duyao et al., 1995; Godin et al., 2010). Additionally, Htt acts as transcriptional regulator for BDNF (Brain-Derived Neurotrophic Factor), inhibiting, in the cytoplasm, its repressor REST/NRSF factor and so stimulating BDNF expression (Zuccato et al., 2003). Moreover, Htt has important roles not only in the cytoplasm, but also in the nucleus, where it acts as transcription factor, interacting with the proteins EZH2 and SUZ12 of Polycomb PRC2 complex and leading to the deposition of the epigenetic marker H3K27me3 (Seong et al., 2010). Htt binds different transcription factors including CBP and p53 (Steffan et al., 2000), potentially influencing apoptosis, cell-cycle control and DNA repair (Bae et al., 2005).
As expected, the functions above described become altered in the presence of the abnormal polyQ expansion in mutant Htt (mHtt), supporting thus the hypothesis that part of the pathological events could be due to the loss (or modification) of the normal function of wild-type Htt.

Mutant Huntingtin (mHtt) and HD pathogenesis

HD phenotype is mainly caused by a proteinopathy: CAG repeats above the threshold lead to the formation of the long polyQ stretch that characterizes the mutant protein.

The cleavage of N-ter-mHtt by proteases generates small fragments containing the polyQ expansion that form insoluble aggregates (Scherzinger et al., 1997), accumulate in the nucleus and become toxic (Benn et al., 2005; Graham et al., 2006). In addition, proteolysis of C-ter-mHtt produce fragments that do not contain the polyQ stretch but in the same way accumulate in endoplasmic reticulum inducing stress and impairing dynamin 1 functions (El-Daher et al., 2015). Furthermore, all kind of aggregates present abnormal conformations that make them be resistant to the machineries involved in cellular clearance, such as the ubiquitin-proteasome complex (Gusella and MacDonald, 2002) and the autophagy system (Ross and Tabrizi, 2011).

In target neurons, the event that triggers intracellular accumulation happens several years before the manifestation of the first symptoms of neurodegeneration and, at the same time, other cellular issues, such as glutamate-mediated excitotoxicity, mitochondrial dysfunction and apoptotic insults, participate to neuronal cell death (Ross and Tabrizi, 2011). Moreover, recent reports show that mHtt inclusions at synaptic terminals are able to spread throughout the brain, to be internalized in other neurons and to accumulate (Babcock and Ganetzky, 2015). Although the mechanism of the mHtt production is clear, the molecular basis of the correlation between mutant protein and strong neurodegeneration remains poorly understood. It is always more evident that the so dramatic consequences of HD are the effect of a huge plethora of molecular alterations that act in a synergic and

consequential way, impairing different crucial processes of the cell (Figure 3).

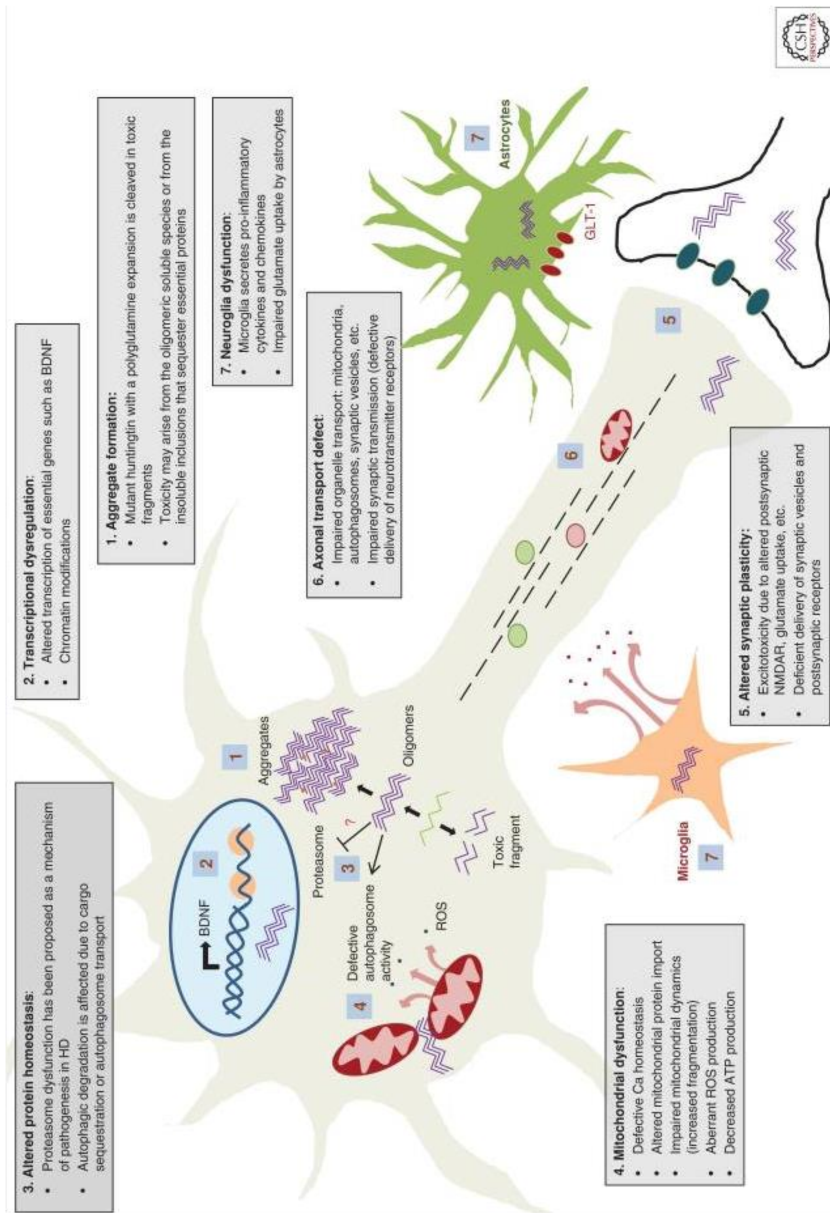

Fig. 3 – Neuronal pathogenic mechanisms in HD. Jimenez-Sanchez et al., 2017.

For instance, recent evidences correlate HD and nuclear pore complex (NPC) dysfunctions. Perinuclear mHtt inclusions seem to disrupt the nuclear membrane in HD murine model (Liu et al., 2015) and cytoplasmic aggregates seem to interrupt nucleo-cytoplasmic trafficking of proteins and RNA (Woerner et al., 2016). Various NPC-associated proteins co-localize with mHtt intracellular aggregates both in murine HD model and in post-mortem human HD brains (Grima et al, 2017). Additionally, several nucleoporins are mislocalized in a *Drosophila* HD model and nucleocytoplasmic transport is compromised; strikingly, targeting the nucleocytoplasmic transport with specific molecules, HD neurodegeneration is blocked (Grima et al., 2017), underlining that NPC pathology contribute to HD pathogenesis.
As probably expected, transcriptional deregulation is one of the earliest alterations in HD: through a microarray approach has been proved that expression patterns of both coding and non-coding RNAs are altered in HD (Luthi-Carter et al., 2002). Data obtained from various polyQ murine models show that in HD brains the expression of neuropeptides and proteins involved in signal transduction is decreased, while are up-regulated genes related to cellular stress (Luthi-Carter et al., 2002). The mechanism provided to justify transcriptional dysfunction in HD is that polyQ stretch may sequestrate some proteins, in particular transcription factors, into both nuclear and cytoplasmic aggregates leading thus to a functional disruption of the process (Preisinger et al., 1999).
Not surprisingly, the deregulated gene expression in HD finds its reasons in a quite different epigenetic landscape. Several studies have correlated changes in DNA methylation with the expression of Htt protein in different HD model systems, as well as in human HD brain (Thomas et al., 2016). For instance, the analysis of DNA methylation status of striatal cells from $Hdh^{Q111/Q111}$ mice showed

an altered methylation pattern at promoter and regulatory regions level, in particular genes involved in neurogenesis and neuronal differentiation (*Sox2, Pax6, Ap-1*) were found hyper-methylated and so with a lower level of expression (Ng et al., 2013). These findings, showing that the pathological alterations might start during neurogenesis, are consistent with the ascription of HD as a neurodevelopmental disorder (Kerschbamer et al., 2015). Additionally, 7-methylguanine (7mG) levels have been found highly different in HD human brains and HD models, probably meaning that the methylation machinery in HD may be totally deregulated (Thomas et al., 2013).

Chromatin status and gene expression are strongly dependent on post-transcriptional modifications of histone tails, whose levels have been found completely altered in HD models. For instance, mHtt seems to interact with the histone acetyltransferase (HAT) CBP, sequestering it in nuclear inclusions and determining a global hypoacetylation of histone H3, thus causing the down-regulation of some genes relevant for neuronal maintenance (Glajch et al., 2015). As mentioned in the former paragraph, Htt has the capability to interact with PRC2 complex and has a specific role in histone methylation process; the extension of the polyQ tract in mHtt facilitates the binding with the core components of PRC2, leading to a higher level of methylation and consequently a higher silencing of chromatin (Seong et al., 2010). Furthermore, the presence of a longer polyQ tract is also related to a decreased level of H3K4me3, determining a lower RNA expression (Biagioli et al., 2015). Taken together, these data support the role of huntingtin as a core regulator of chromatin status and confirm that altered transcription plays an important role in HD pathogenesis.

Lately, mHtt expression has been related to a novel pathogenic mechanism that involves also the translation process. In particular, the protein 4E-BP, that inhibits translation binding the initiation factor eIF4E, is inactivated in HD striatum, leading to an increased level of protein synthesis. Interestingly, the treatment of HD mice with a translation inhibitor, determines a normalized translation level (Creus-Muncunill et al., 2019). Therefore, this result proves an alteration of protein synthesis in HD pathogenesis.
Furthermore, recent studies on the molecular basis of neurodegenerative diseases caused by trinucleotide repeat expansions led to the discovery of a new mechanism of translation linked to the repetition of RNA motifs and non-dependent from the AUG start codon: Repeated Associated Non-AUG (RAN) translation (Zu et al., 2011) (Figure 4). RAN translation determines the production, in both sense and anti-sense directions and in all the reading frames, of small homo-peptides that accumulate in the cytoplasm and become cytotoxic. Firstly discovered in a Spinocerebellar ataxia type 8 (SCA8) model (Zu et al., 2011), RAN translation has been characterized in other pathologies, including HD (Bañez-Coronel et al., 2015). Remarkably, in addition to mHtt, in HD post-mortem brains have been found inclusions of RAN-Alanin, RAN-Serin, RAN-Leucin and RAN-Cystein not only in striatum, cerebral cortex and cerebellum, the brain districts principally involved in HD pathogenesis, but also in different regions where there is no polyQ presence. Interestingly, RAN-peptides aggregation is dependent on the length of CAG tract (Bañez-Coronel et al., 2015). This data assess RAN translation as a novel pathogenic HD mechanism, going to increase the complexity of the already intricate scenario of HD pathogenesis.

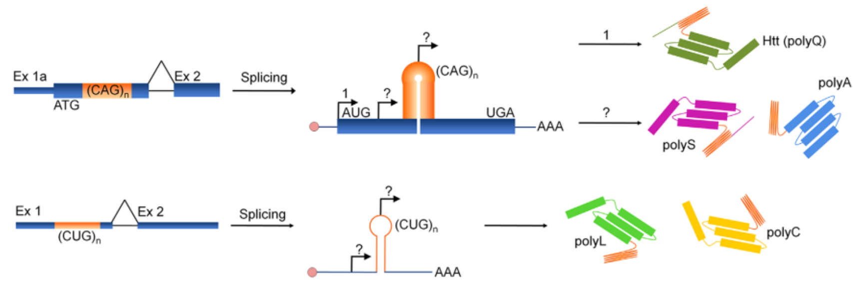

Fig. 4 - RAN translation mechanisms in both sense and anti-sense directions. Adapted from Green et al., 2016.

Drosophila melanogaster as HD model organism

To investigate mHtt interactions and to deepen our understanding on HD pathogenesis, many HD animal models have been generated: the first were four lines of transgenic knock-in murine models expressing the exon 1 of human HTT gene carrying from 116 to 150 CAG expansions (Mangiarini et al., 1996). Additional mouse models have been generated together with HD models in yeast (Krobitsch and Lindquist, 2000), in *Caenorabtidis elegans* (Faber et al., 2003) and in *Drosophila melanogaster* (Jackson et al., 1998; Romero et al., 2008).
Regarding *Drosophila*, it is well-known that the fruit fly has always been a very useful tool to perform genetics studies and that it has all the characteristics that define a "model organism", but, more notably and perhaps unexpectedly, *Drosophila melanogaster* is an excellent model also to study HD and other neurodegenerative disease. The first important similarity is that human basal ganglia and arthropod central complex are homologous brain structures that share the same embryological derivation, functionality and gene expression programs (Strausfeld et al., 2013; Martin-Pena et al., 2014). Moreover, the depletion of dopamine in *Drosophila* determines locomotive deficits, reduced activity and behaviour abnormalities, which are common symptoms of HD pathogenesis (Strausfeld et al., 2013; Martin-Pena et al., 2014).
Additionally, through the use of transgenic flies expressing the human HTT gene, it is possible to summarize the most part of HD symptoms, such as neurotoxicity, motor impairment, nuclear inclusions, progressive neurodegeneration and early death (Chan et al., 2002).

The existing models of HD flies might be divided in two categories: the “exon 1” models and the “full-length” models; the first ones contain only the first exon of HTT gene with the polyQ region, whereas the second ones are characterized by the whole human HTT gene.
Thanks to the creation of an “exon 1” model, expressing 20 or 93 CAGs repeats, Steffan and collaborators showed that the pan-neuronal expression of 93Q construct determined lethality in the 70% of cases and early death in the remaining 30%, if compared to the expression of 20Q wild-type construct (Steffan et al., 2001).
Other “exon 1” models contain 2, 75 or 120 glutamine repetitions, inserted among the first 17 and the following 135 amino acids of the protein. Pushing the expression of these constructs in the eye of the fly, through an eye-specific driver, Jackson and collaborators observed progressive photoreceptor degeneration, whose impact was related to the polyQ fragment length (Jackson et al., 1998).
Although “exon 1” models are very useful to speculate on many aspects of the pathology, this kind of transgenic constructs are not provided of many sites, belonging to other exons of the gene, that are crucial for protein-protein interactions. For instance, they do not present HEAT domains, which are thought to be very important for the interaction between huntingtin and the proteins HIP1 (Huntingtin-Interacting-Protein) and HAP1 (Huntingtin-Associated-Protein) (Harjes and Wanker, 2003). Another important site of Htt, absent in “exon 1” models, is a caspase-6 cleavage site in the amino acid 586: a murine model caspase-6-cleavage-resistent showed reduced neurotoxicity (Graham et al., 2006).
In addition to the models containing only the first exon, transgenic models have been generated that contain a longer fragment, but not

the entire gene. One of these is made up of 336 amino acids of human Htt protein with 128 glutamine repeats and its expression determines an abnormal eye phenotype (Kaltenbach et al., 2007). Lee and collaborators, instead, have created a fly model carrying a construct of 548 amino acids with 128Q: this fragment was appositely generated to have the same dimension of a huntingtin fragment cleaved by caspase-3. Flies expressing this construct show photoreceptor degeneration, motor impairments, early death and cytoplasmic inclusions (Lee et al., 2004).

Drosophila HD model used to perform the experiments described in this thesis was generated by Prof. Botas and carries a transgenic construct containing the entire human huntingtin gene with 128 ($128Qhtt^{FL}$) CAGs (Romero et al., 2008). $128Qhtt^{FL}$ cDNA was obtained replacing the N-terminal fragment of a $16Qhtt^{FL}$ with the corresponding tract with the expansion (Kaltenbach et al., 2007); the construct was cloned in a pUAST vector (Brand and Perrimon, 1993) and injected in an $y^{1}w^{1118}$ genetic background.

Pan-neuronal expression of $128Qhtt^{FL}$ construct determines progressive motor defects, reduced fly and climbing activity and impairments in synaptic release in neurons controlling fly muscles. Eye-driven expression, instead, induces progressive ommatidia degeneration (Romero et al., 2008).

The spatial and temporal control of the expression of $128Qhtt^{FL}$ pathogenic construct was realized thanks to a specific and very useful tool: the UAS-Gal4 system (Brand and Perrimon, 1993). It is characterized by the presence of two transgenic strains of flies, one expressing the yeast transcription factor Gal4 placed downstream a tissue-specific promoter, and another one expressing the GAL4 binding sites, the UAS sequences, placed upstream the transgene of interest. Crossing these two lines, the offspring will receive both the constructs and only in the specific tissue where

the promoter activates its expression, the Gal4 will bind UAS sequences and the transgene will be expressed.
The driver used to push the expression of mutant Htt in the whole brain was the pan-neuronal *Elav* (*embryonic lethal, abnormal vision*), which enhances the Gal4 production in each nervous system cell from the very early stages of development (Robinow and White, 1998). *GMR* (*glass multimer reporter*) lets the Gal4 express only in developing fly eye (Ellis et al., 1993); the expression of polyQ proteins guided by GMR-Gal4 is associated to a wide ommatidia disorganization and degeneration, while the transgene expression Elav-Gal4-mediated does not determine any morphological alteration of the eye (Green and Giorgini, 2012).

Genetic and pharmacological modifiers of HD neurotoxicity in Drosophila melanogaster

One of the main pathological features of HD is the presence of intracellular aggregates composed of mutant huntingtin N-terminal fragments (Scherzinger et al., 1997): it is quite interesting that *in vivo* and *in vitro* analyses have demonstrated that longer is the polyQ tract of mHtt and higher is the propensity to form aggregates (Penney et al., 1997).
Considering that the molecular mechanisms underlying the neurotoxicity of mutant huntingtin protein can be easily recapitulated in *Drosophila*, transgenic fly models have been widely used to screen genetic modifiers of mHtt neurotoxicity (Marsh et al., 2004).
As probably expected, some modifiers of polyQ-induced toxicity belong to the class of chaperones and co-chaperones, whose primary function is to regulate the protein folding and the refolding of misfolded peptides: for example, the over-expression of the human chaperone HSP70 or of the *Drosophila* co-chaperone HSP40 (*DnaJ-1*) suppresses *in vivo* the HD toxicity by reducing polyglutamine aggregation (Warrick et al., 1999; Chan et al., 2000). Interestingly, in order to isolate genetic modifiers of eye degeneration in a 127Q HD *Drosophila* model, Kazemi-Esfarjani and collaborators performed a P-element insertional mutagenesis screening that led to the identification of two polyQ-neurotoxicity suppressors: the gene *DnaJ-1* homologous to the human HSP40 and the gene *dTPR2*, which is homologous to the human tetratricopeptide repeat protein 2. Both these proteins contain a chaperone-related J domain (Kazemi-Esfarjani and Benzer, 2000).
Like genetic screening, a genome wide RNA interference screen allowed Zhang and collaborators to identify 126 genes that

suppress or enhance aggregates formation in a *Drosophila* HD model expressing a eGFP-tagged first exon of human huntingtin gene carrying from 25 to 103 CAG repeats (Zhang et al., 2010). The candidates they found belong to different cellular classes or pathways, such as chaperones (*Hop*, *DnaJ-1*, *HSP83*), chromatin modifiers (*Sin3A*, *lilli*, *Z4*), kinases (*Tor*, *spaghetti*), protein biogenesis (*NelfE*, *Th1*, *Ald*) and cytoskeleton (*Rab1*, *Rab11*, *Atx2*). Among the others, it is interesting to underline that all the isolated genes involved in cytoskeleton and protein trafficking, if functionally inactivated, induce a decreased formation of the aggregates, thus suggesting that interfering with the cellular transport machinery disrupts the aggregates formation process (Zhang et al., 2010). Moreover, recent evidence indicates that the over-expression of the *Drosophila* Junctophilin (jp), a protein that is placed between plasma membrane and endoplasmic reticulum, rescues the HD-induced neurodegeneration (Calpena et al., 2018).

Remarkably, some candidate modifiers of protein aggregation have been identified also in screening for polyQ toxicity modifiers: for example, *smt3* and *uba2* genes, both involved in sumoylation process, modify the polyQ-toxicity (Chan et al., 2002; Steffan et al., 2004) and partially inhibit the formation of aggregates (Zhang et al., 2010). The same goes for genes *sin3A* and *rpd3* that encode two components of the Sin3 chromatin-remodeling complex (Fernandez-Funez et al., 2000; Steffan et al., 2001). Although the mechanisms by which these genes operate the modulation may be different in the two processes, this overlap creates a strong correlation between misfolded protein aggregation and neuronal toxicity in HD pathogenic scenario.

Together with the isolation of genetic modifiers, research efforts are focusing on the identification of substances and compounds

that may exert a protective role in HD pathogenesis, reducing the clinical manifestations or delaying the disease onset.
Encouraging recent studies showed the neuroprotective effect of some polyphenolic compound in HD pathogenesis. Curcumin, for example, is a commonly used spice well-known for its anti-oxidant and anti-inflammatory potential; interestingly it has been demonstrated that a curcumin-supplemented diet rescues, in a dose-dependent manner, the eye degeneration and reduces the cell death rate in a *Drosophila* HD model carrying a 93Q pathogenic construct (Chongtham et al., 2016). Remarkably, curcumin-treated HD mice show better motor activity and neurodegeneration and gastrointestinal disorders are both rescued (Elifani et al., 2019).
Additionally, among other plant-derived polyphenols, it has been recently showed that grape seed polyphenolic extract (GSPE) treatment significantly extends lifespan of 93Q HD flies (Wang et al., 2010) and that green-tea-derived polyphenols block mHtt aggregates formation in a dose-dependent manner (Ehrnhoefer et al., 2006). Moreover, green tea infusion modulates in a *Drosophila* model the reduced lifespan and neurodegeneration mHtt-induced (Varga et al., 2018).
As previously said, mutant huntingtin aggregates are resistant to all cellular clearance mechanisms, including autophagy system (Ross and Tabrizi, 2011): strikingly Ravikumar and collaborators showed that the inhibition of mTOR pathway through rapamycin treatment induces high rate of autophagy and determines the reduction of polyQ toxicity in a 120Q HD fly model (Ravikumar et al., 2004).
Although the huge diversity of toxicity modifiers that have been found, no therapeutic treatment is still available for Huntington Disease: for this reason, and also to better characterize the molecular pathways influenced by mHtt expression, the

suppression of the polyQ-induced toxicity remains a fundamental target of HD research.

Transposable Elements (TEs)

General characteristics and classification

Transposable elements (or jumping genes) are ubiquitous genetic elements, discovered in *Zea mais* by Barabara McClintock in 1948 (McClintock, 1950). They are capable to transfer throughout the genome, moving from a chromosomal locus to another one. Transposons have been found in a great number of organisms, from prokaryotes to eukaryotes and, in particular, they constitute the 44% of human genome and the 20% of the *Drosophila* genome (Hoskins et al., 2002; Kaminker et al., 2002; Kazazian and Moran, 1998). Such abundance is coherent with the hypothesis that TEs played, and are playing, a very important role in genome evolution and plasticity (Cordaux and Batzer, 2009). Nevertheless, all organisms evolved complex and fine-tuned mechanisms to avoid random TE mobilization, particularly in germ line, because their casual reinsertion might determine mutations of gene-encoding or regulatory sequences and chromosomal rearrangements (Levin and Moran, 2011; Waddell et al., 2014).

Generally, TEs may be distinguished in two classes depending on the transposition mechanism they use (Levin and Moran, 2011) (Figure 5):

- DNA transposons: their position in the genome change according to a “cut and paste” mechanism; in other words, they excide from the position they are and re-localize in another insertion site in a non-replicative way (Greenblatt et al., 1963; Rubin et al., 1982). The enzyme that allows excision and reinsertion is the Transposase. A particular subclass of DNA transposons, the *Helitrons*, mobilizes using a rolling-circle replication with a ssDNA intermediate (Kapitonov and Jurka, 2007).

- Retrotransposons: the mRNA of these elements encodes for a reverse transcriptase that, using the transcript as a template, produces a cDNA sequence that reinsert randomly in a new genomic site, generating a new insertion through a “copy and paste” mechanism (Boeke et al., 1985). Retrotransposons may be further subdivided in: LTR-transposons and non-LTR-transposons, depending on they are flanked or not by long terminal repeats (LTR). LTR-retrotransposons are able to insert in the genome thanks to an integrase, as many retroviruses (Brown et al., 1987), whereas non-LTR-retrotransposons integrate through the so-called target-primed reverse transcription (TPRT), a process where reverse transcription and chromosomal integration are coupled (Lual et al., 1993).

Transposable elements might also be distinguished in autonomous or non-autonomous, depending on whether they move independently or need the transposition machinery of autonomous TEs. Additonally, beyond these general classifications, TEs are accurately classified in different subgroups depending on their phylogenetic origin and genetic organization.

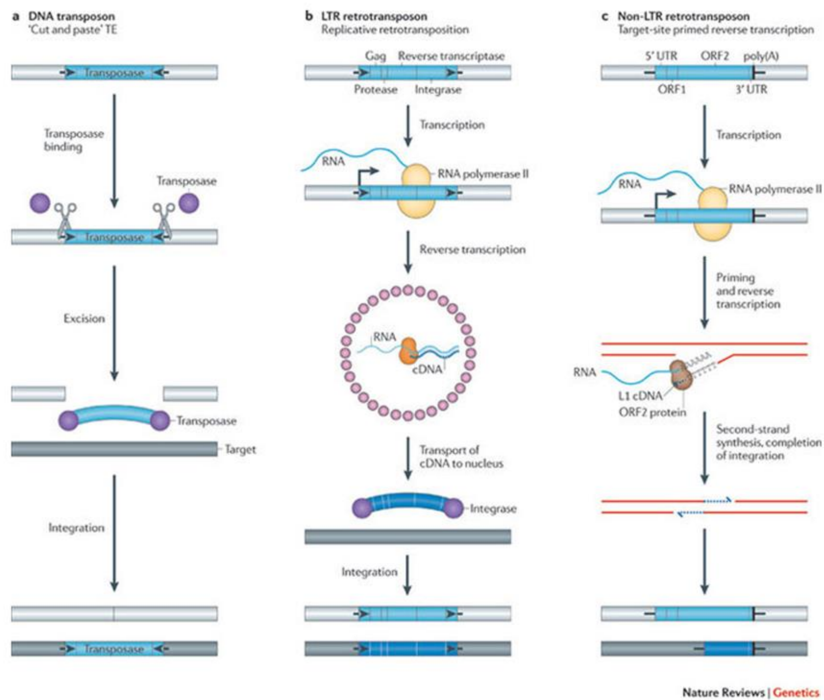

Fig. 5 - The different mechanisms of transposon mobilization. Levin and Moran, 2011.

TEs in Drosophila melanogaster

Drosophila melanogaster presents approximately 100 different TE classes both in single and multiple copies (Hoskins et al., 2002). Among 1500 transposons, only the 30% is potentially active in the genome (Waddell et al., 2014).
In humans, due to the absence of functional transposases, there are no active DNA transposons (McCullers and Steiniger, 2017), while in *Drosophila* at least the 16% of TEs belonging to this class is full-length and active, in particular *1360*, *hobo*, *Bari1* and *P* (Muñoz-López and Garcìa-Pérez, 2010; Palazzo et al., 2013).
P elements are the most well-known TEs of fruit fly genome; they are highly represented in *Drosophila* germline because they preferentially insert in genomic regions that function as replication origins (Spradling et al., 2011). Interestingly, a similar human transposon, *THAP9*, has the ability to move *P* elements both in *Drosophila* and in human cell lines (Majmundar et al., 2013). The main mechanism that regulates *P* element transposition involves the alternative splicing of transposase mRNA: in somatic cells, the retention of an intron determines the presence of an early stop-codon, leading to the production of a truncated and inactive transposase (Laski et al., 1986; Misra et al., 1990). Curiously, the most abundant DNA transposon in *Drosophila* euchromatin, *1360* (or *Hoppel*), is functionally and structurally similar to *P*, but lacks of introns, thus preventing the regulation by alternative splicing (Reiss et al., 2003).
With regards to LTR-retrotransposons, their DNA sequence is quite similar to a host-integrated provirus sequence after a retroviral infection, thus suggesting the phylogenetic origin of this class of TEs (Waddell et al., 2014)

In *Drosophila* have been recognized more than 20 varieties of LTR-retrotransposons, among which *gypsy* is the more complex, because is the only presenting all three retroviral genes *gag*, *pol* and *env* (Kim et al., 1994). *Gag* is the main component of virus-like nucleocapsid particles, *env* encodes for the retroviral envelope, necessary for *gypsy* mobilization outside of the host cell (Kim et al., 2004), and *pol* produces a polyprotein consisting of a protease, an integrase and a reverse transcriptase (Kim et al., 1994; Engelman et al., 2014). In addition, there are a lot of *gypsy-like* elements, lacking of one or more sequences, such as *opus* and *rover*, presenting a truncated *env* gene, or *accord* and *McClintock*, where *env* is completely absent. Other *Drosophila* LTR-transposons belong to the *copia* and *roo* subgroups: *copia* exists in a circular extra-chromosomal form (Flavell et al., 1983), encodes for a capsid protein and lacks of *env*, whereas *roo*, that is the most abundant transposon in fruit fly genome, presents a non-functional *env* gene (Frame et al., 2001). The above-mentioned LTR-TEs and other transposons, such as *Idefix*, *gypsy2*, *Burdock* and *Beagle*, actively transpose in fly brain (Perrat et al., 2013).
Drosophila non-LTR-retrotransposons resemble to the most common mammalian TE *LINE-1* (Long Interspersed Nuclear Element 1), encoding for two peptides: Orf1p, a RNA binding protein, and Orf2p, that works as reverse transcriptase and endonuclease (Cordaux and Batzer, 2009). *LINE-like Drosophila* elements are many and various, such as *R2*, *Het-A*, *I element*, *Doc*, *Doc2*, *Doc3* and *Ivk* and, for this class too, has been observed retrotransposition in the brain (Hoskins et al., 2002; Kaminker et al., 2002). *R2* and *Het-A* belong to two interesting subgroups that have very specific insertion sites: *R2* transposes exclusively in 28S rRNA locus (Jackubzac et al., 1991), while *Het-A* only in telomeric DNA (Pardue et al., 2011). With regards to telomeres, it

is interesting to notice that the role played by mammalian telomerase, in *Drosophila* belongs to the transposons *Het-A*, *TART* and *TAHRE*: they are targeted by their *gag* proteins to telomeres and determine the generation of telomeric tandem repeats to protect chromosomes extremities (Fuller et al., 2010).

TE inhibition in Drosophila melanogaster

TE insertion may alter gene expression and cellular functions in various ways: it can disrupt coding sequences preventing the production of a functional protein or alter the regulation of gene expression if insert in a regulatory element. Moreover, they can transpose in an intronic sequence determining alternative or aberrant splicing (Han et al., 2004) or induce epigenetic regulation, causing ectopic expression or silencing, of genes posed downstream their insertion site (Shpiz et al., 2014) (Figure 6).

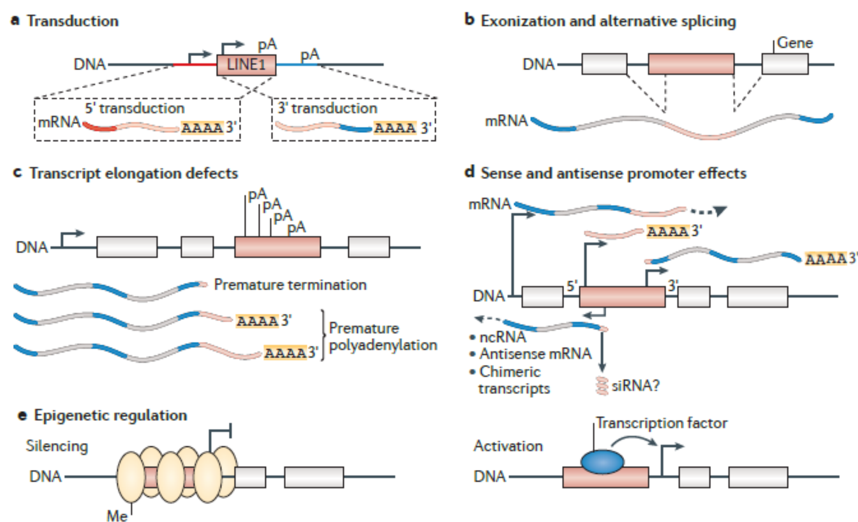

Fig. 6 - Effects of TE random insertion. Erwin et al., 2014.

These are only few of the reasons why all organisms had to evolve fine-regulated mechanisms to inhibit random transposition and insertion of TEs, in particular in germline. In *Drosophila*, this role belongs to the Piwi-piRNA pathway (Malone and Hannon, 2009); piRNAs (Piwi-interacting RNAs) are a class of small non coding

RNAs of 23-29 nucleotides, produced with a Dicer-independent maturation process. piRNA precursors are transcribed in different chromosomal heterochromatic regions, where are located different truncated transposons copies that act as template. After maturation, piRNAs interact with Piwi protein, if its precursor derives from a sense transcript, or with Aubergine (Aub), if originates from an anti-sense transcript: the complex Piwi-piRNA is related to nuclear transcriptional silencing, while the interaction Aub-piRNA is involved in cytoplasmic post-transcriptional silencing. Once in the nucleus, Piwi binds the chromatin, guided by piRNA pairing to its complementary genome sequence, recruits repressive complexes and determines histone tails methylation and transposon silencing (Peng and Lin, 2013). For instance, Piwi interacts with HP1a, which leads to the enrichment of H3K9me3, a well-known histone marker of transcription down-regulation (Lin and Yin, 2008). On the other hand, in the cytoplasm, the post-transcriptional silencing is due to homology-directed cleavage of transcripts by the complex Aub-piRNA (Li et al., 2009).
The mechanisms of transposon silencing in somatic line remain unclear: however, is sure the involvement of Aub and Argonauta3 (Ago3), because mutant flies lacking one or another show very high levels of transposon transcripts (Perrat et al., 2013). Endo-siRNAs (endogenous small interfering RNA), small RNAs produced through a process that requires Dicer2 (Dcr-2) and Argonauta2 (Ago2), are recently considered as new players in TE silencing (Cernilogar et al., 2011): in fly heads transposons are up-regulated when Dcr-2 or Ago2 genes are deleted (Li et al., 2013; Perrat et al., 2013). It is reasonable to hypothesize that piRNA and endo-siRNA pathways cooperate to preserve a fine-tuned regulation of TE activity (Waddell et al., 2014). Speaking of that, transcriptome analysis of *piwi*, *dcr-2* and *piwi/dcr-2* mutants fly

heads showed that TE transcripts increase only in double mutants' heads. In the light of that, a new proposed model hypothesizes the existence of a dual layer for TE repression in adult somatic tissues: a first layer is constituted by Piwi-mediated silencing during embryogenesis and a second layer is determined by Dicer-2-mediated silencing, that has the aim to repress transposons that escape from Piwi action (van den Beek et al., 2018).

TEs and brain pathologies

Until few years ago, retrotransposition was believed as a prerogative of germ cells, cancer tissues and pluripotent cells and that it was completely absent in the brain. In contrast, recent studies showed that retrotransposition occurs in some districts of adult brain, such as in mammalian hippocampus neurons (Baillie et al., 2011; Muotri et al., 2005) and *Drosophila* mushrooms bodies αβ neurons (Perrat et al., 2013). Coherently with this evidence, Aub and Ago3 expression levels are very low in mushroom bodies, allowing thus a high transposable activity (Perrat et al., 2013). Through the analysis of post-mortem hippocampal neurons, LINE-1 somatic retrotransposition has been shown to occur when the donor lacks of an intact binding sequence for Yin Yang 1 (YY1) transcription factor, which is a mediator of DNA methylation; so, when YY1 binding sites are hypo-methylated LINE-1 can retrotranspose (Sanchez-Luque et al., 2019).
The combination of retrotransposition assays, qPCR and genome sequencing demonstrated that neurons are more prone to somatic retrotransposition than other kinds of neural cell types (Coufal et al., 2009; Bundo et al., 2014). Retrotransposition reporter assays indicate if in a cell takes place a complete transposition cycle; for instance, an active LINE-1 may be engineered to contain an enhanced green fluorescent protein (EGFP) reporter that is functional only when TE genomic integration has completed (Moran et al., 1996; Muotri et al., 2005). A typical transposition assay in *Drosophila*, instead, takes advantage of the tendency of *gypsy* to insert into the *ovo* locus: using engineered *ovo* sequences that activate a GFP report when disrupted by TE insertion, endogenous *gypsy* mobilization can be visualized (Labrador et al.,

2008). Using this approach, somatic *gypsy* transposition has been shown to occur in aged *Drosophila* neurons (Li et al., 2013).
Interestingly, TE mobilization in the brain can generate a stochastic mosaicism of gene expression that determines neuronal cellular diversity (Muotri and Gage, 2006; Singer et al., 2010) (Figure 7): the most significant notion is that each neuron in the same brain has a unique genome, leading to distinct transcriptomes. Probably, this neuronal variety may be the reason of different individual behaviour and susceptibility to neuronal and neurodegenerative diseases (Waddel et al., 2014). At cellular level, a moderate level of somatic transposition may determine an expansion of the coding potential of the genome and can generate diversity between neuronal subtypes, whereas, at organismal level, with no doubt it contributes to phenotypical and behavioural variability observed between genetically identical twins (Singer et al., 2010; Muotri and Gage, 2006).

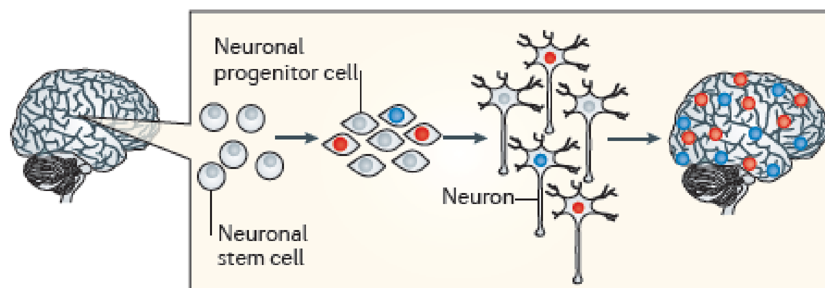

Fig. 7 - Neural transposition in the adult nervous system can generate cellular diversity in the same brain. Erwin et al., 2014.

In contrast, an uncontrolled up-regulation of TE expression and somatic reinsertion in the brain may have deleterious consequences on cognitive functions and may be associated to a lot of neuronal and neurodegenerative pathologies (Muotri et al.,

2010). For instance, Rett syndrome, an autism-spectrum-disorder caused by a mutation in the X-linked MeCP2 gene (Amir et al., 1999), has been recently linked to increased somatic *LINE-1* insertion. It is interesting to notice that MeCP2 knockout mice show increased L1 and other elements expression (Muotri et al., 2010). Moreover, it was observed that also individuals with schizophrenia show high levels of somatic *LINE-1* retrotransposition in neurons (Bundo et al., 2014).
In general, transposons misregulation is nowadays continuously linked to the pathogenesis of a lot of neurodegenerative disease, among others, Amyotrophic Lateral Sclerosis (Douville et al., 2011, Krug et al., 2017), Parkinson Disease (Abrusàn, 2012) and Alzheimer Disease (Guo et al., 2018).
Given the increasing evidences that there is a correlation between transposition and neurodegeneration, it is worth exploring whether other brain diseases may involve transpositional events in the brain.

AIM OF THE RESEARCH

Despite many years of investigation about HD pathogenesis, there are lots of open questions concerning the molecular mechanisms underlying this severe disease and, above all, no cure is still available.
Starting from the evidences that there is active transposition in human and *Drosophila* adult brain (Muotri et al., 2005; Perrat et al., 2013) and that many neuronal and neurodegenerative diseases, such as schizophrenia, amyotrophic lateral sclerosis and Alzheimer disease (Bundo et al., 2014; Douville et al., 2011; Guo et al., 2018) have been recently linked to transposable elements up-regulation, the aim of the work described in this thesis is to verify and then characterize the potential involvement of TEs in Huntington disease pathogenesis, using a transgenic *Drosophila* HD as model organism.
Taking advantage of qRT-PCR technique, we firstly demonstrate the up-regulation of various TE transcripts in HD flies brain and then, through a qChIP approach, we show how TE genes are transcriptionally regulated in HD. Moreover, we want to verify if the inhibition of TE expression leads to the block of polyQ-dependent neurodegeneration. Additionally, considering the connection between HD and genomic instability (Ruzo et al., 2018) and the intrinsic TE characteristic to determine genomic rearrangements, we evaluate, through cytological analysis and immunofluorescence assay, the rate of chromosomal aberrations and DNA damage in HD fly brains. Furthermore, we investigate if TE up-regulation might be related to a global heterochromatin relaxation, taking advantage of Position Effect Variegation (PEV) analysis and studying the consequences of Heterochromatin Protein 1 (HP1) overexpression in HD background.

RESULTS AND DISCUSSION

TE expression is induced in HD fly model

Our *Drosophila* HD experimental model was generated by Romero and collaborators (Romero et al., 2008) and is based on the expression of full-length human Huntingtin (Htt) protein with 128 glutamines (128QHtt^{FL}, pathogenic HD construct) under the control of the UAS-Gal4 system (Brand and Perrimon, 1993). In this bipartite system, the expression of the HD pathogenic construct downstream of UAS sequences depends on the spatial and temporal expression of the yeast transcriptional activator Gal4. In order to induce a pan-neuronal expression of the pathogenic HD construct, we crossed flies carrying the UAS-128Qhtt^{FL} transgene to flies elav-Gal4 driving the expression of the pathogenic construct in every cell of the nervous system, starting from very early stages of development (Robinow and White, 1998). To test the induction levels of the pathogenic UAS-128Qhtt^{FL} construct by the pan-neuronal driver elav-Gal4, we performed a semi-quantitative RT-PCR on cDNA reverse-transcribed from total RNA purified from both elav-Gal4/+ (control samples) and elav-Gal4>UAS-128Qhtt^{FL} fly heads. As showed in Figure 8, the HD pathogenic construct results correctly expressed by the elav-Gal4 driver in HD fly heads.

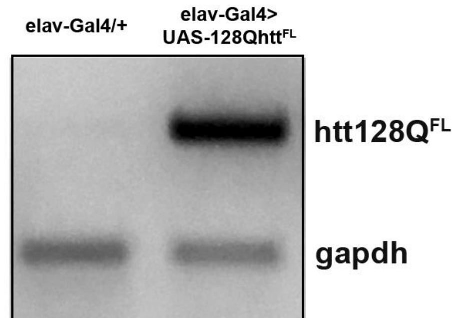

Fig. 8 – Semi-quantitative RT-PCR to check the expression levels of the pathogenic construct by the pan-neuronal driver elav-Gal4 in HD heads (elav-Gal4>UAS-128Qhtt^{FL}) and relative control (elav-Gal4/+). The housekeeping gene *gapdh* was used as endogenous control.

We then analysed different transposon transcripts by qRT-PCR experiments on reverse-transcribed total RNA extracted from HD (elav-Gal4>UAS-128Qhtt^{FL}) and control (elav-Gal4/+) fly brains. Taking into account that during metamorphosis the *Drosophila* nervous system goes through a considerable remodeling that determines cell death and formation of new connections, we decided to check the expression level of TEs both at larval and adult stage. Additionally, adult brains were analyzed at both young (0-2 days) and aged (10-12 days) time points, considering that ageing and neurodegeneration are strongly involved in HD pathogenesis and may alter TE regulation. We found that different classes of TE transcripts are significantly up-regulated in HD neurons at both larval and adult stages (Figure 9), suggesting that their derepression may play a role in disease pathogenesis Moreover, TE up-regulation is correlated with age-dependent neurodegenerative characteristic of the pathology.

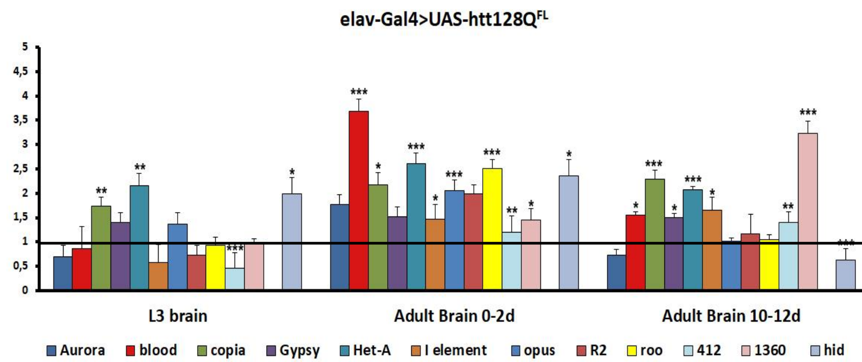

Fig. 9 – qRT-PCR showing TE transcript levels in elav-Gal4>UAS-128Qhtt^{FL} brains dissected from third instar larvae, adult flies 0-2 days old and adult flies 10-12 days old. Expression levels were normalized to rp49 housekeeping gene and represented as fold change relative to wild-type brains (elav-Gal4/+). The expression level of the pro-apoptotic gene hid was used as a marker of neuronal cell death. Statistical significance was determined by unpaired t-test (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

Pan-neuronal expression of the pathogenic construct 128Qhtt^{FL} leads to progressive neurodegeneration

To provide a molecular evidence of the occurring neurodegeneration caused by the expression of HD construct, we performed a western blot analysis on HD larval and adult brains (elav-Gal4>UAS-128Qhtt^{FL}) to check the levels of the protein Elav, a specific neuronal marker (Robinow and White, 1988).

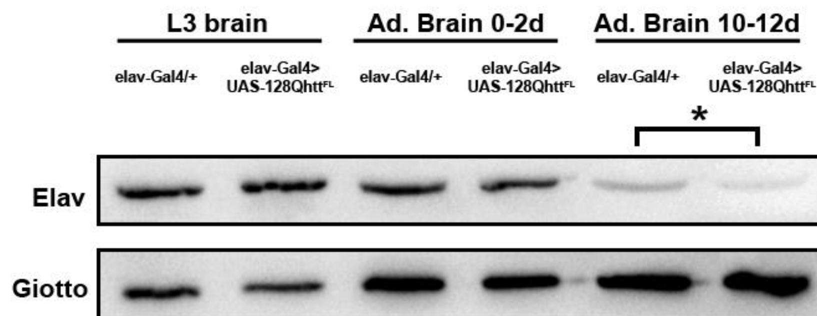

Fig. 10 - Western blot of *Elav* protein in elav-Gal4/+ and elav-Gal4>UAS-128Qhtt^{FL} brains isolated from third instar larvae, adult flies 0-2 days old and adult flies 10-12 days old. Giotto protein was used as loading control. Result is expressed as means of three independent biological replicates. $*p < 0.05$.

As expected, by way of a physiological neuronal depletion, Elav expression decreases with aging both in control and HD flies, but remarkably, at the same age, HD samples show a further reduction of Elav, because of the progressive neurodegenerative effect of huntingtin neuronal expression (Figure 10).

Gypsy-Env protein is overexpressed in HD brains

Drosophila Gypsy retroelement is quite similar in its structure and organization to the proviral form of vertebrate retroviruses (Kim et al., 1994); it contains three open reading frames (ORFs) encoding respectively for the proteins gag, pol and env (Kim et al., 1994). Using a specific monoclonal antibody against env protein (Song et al., 1997) and in order to provide another independent molecular evidence of TE up-regulation in HD neurons, we performed a western blot analysis on brain protein extracts obtained from third instar larvae, young adult (0-2 days) and old adult (10-12 days) flies (Figure 11).

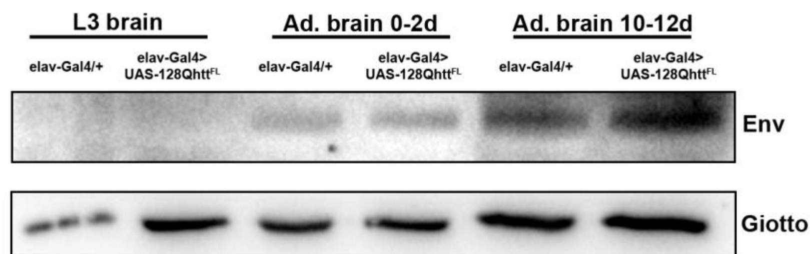

Fig. 11 - Western blot of *Gypsy-Env* protein in elav-Gal4/+ and elav-Gal4>UAS-128Qhtt^{FL} brains isolated from third instar larvae, adult flies 0-2 days old and adult flies 10-12 days old. Giotto protein was used as loading control.

Consistently with the Gypsy transcript levels, we found a significant age-dependent increase of the Gypsy-Env protein (Env) protein levels in adult brain expressing pan-neuronal 128Qhtt^{FL}.

TEs are transcriptionally regulated in HD

To investigate the molecular mechanisms by which HD might contribute to transposons activation, we evaluated through ChIP combined with qPCR the enrichment of H3K9me3 (trimethylation of lysine 9 of histone H3) on promoters and coding regions of different up-regulated TEs in elav-Gal4/+ and elav-Gal4>UAS-128Qhtt^{FL} fly heads isolated at 10-12 days.

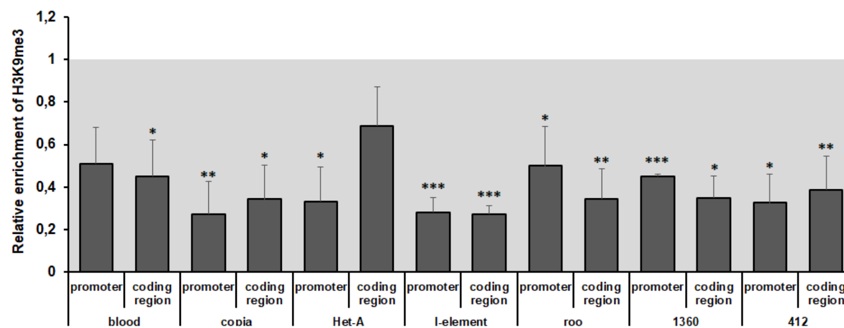

Fig. 12 - The relative fold enrichment, normalized to the heterochromatic F22 control region, was calculated by dividing the amount of DNA immune-precipitated from elav-Gal4> UAS-128Qhtt^{FL} heads by that from the control (elav-Gal4/+). Bars represent mean ± SEM of three independent experiments performed in duplicate. Significance is shown relative to control conditions. *p < 0.05; **p < 0.01; ***p < 0.005. The grey area represents the control value set to 1.

As represented in the histograms above (Figure 12), the histone modification H3K9me3 levels strongly decrease in almost all evaluated sequences. This loss of H3K9me3 perfectly fits with TE up-regulation found in our previous experiments and, moreover, suggests that transcriptional regulation could play an important role in HD pathogenesis.

Overexpression and functional inactivation of DmPIWI protein act as dominant modifiers of eye phenotype produced by the expression of 128Qhtt^{FL}

PIWI, as previously described, is a nuclear protein involved in transcriptional TE epigenetic silencing through the interaction with the small non-coding piRNAs. Its loss is linked, both in germinal and somatic tissues, to a decrease of H3K9me3 repressive modification and to an up-regulation of TE transcripts (Sienski et al., 2012).

Drosophila compound eye is structurally organized in hundreds of ommatidia each of which contains eight photoreceptor neurons, accessory cells and lens apparatus. Thus, any perturbation of this complex ordered structure is easily detectable through light microscopy. In addition, the eye of the fly is an ideal tool to study the genetic control of the neurodegeneration because the eye is not essential for viability (Mishra et al., 2013). The expression of mutant huntingtin in the eyes of the fly determines a wide and progressive depigmentation and strong ommatidia disorganization (Romero et al., 2008).

Starting from these assumptions, to further characterize TE involvement in HD pathogenesis, we observed the phenotype of flies expressing the pathogenic construct in the eye (GMR-Gal4>UAS-128Qhtt^{FL}) that, at the same time, overexpress or are silenced for PIWI protein.

As showed in Figure 13, the ectopic expression of PIWI protein induces a strong suppression of HD eye phenotype, re-establishing ommatidia organization and pigmentation, while the functional inactivation of PIWI causes an enhancement of HD phenotype, characterized by a worsening of ommatidia depigmentation associated with the presence of discrete necrotic lesions.

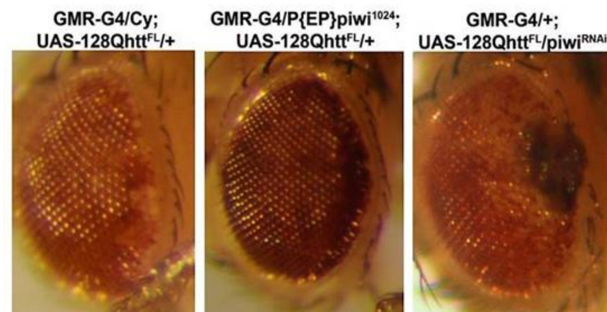

Fig. 13 - Eye phenotype of flies expressing the HD pathogenic construct under the control of the eye-specific GMR-Gal4 driver. PIWI overexpression leads to a suppression of HD phenotype (picture in the middle), whereas its functional inactivation through RNA interference determines a worsening of the HD phenotype (picture on the right), than the control eye (picture on the left).

This result not only provides the evidence that PIWI is a HD genetic modifier, but also confirms that TE activity represents an important element of HD pathogenesis.

HD eye phenotype is rescued by Nucleoside Reverse Transcriptase inhibitors (NRTIs)

Nucleoside Reverse Transcriptase Inhibitors (NRTIs) are antiretroviral drugs commonly used to inhibit the replication process of retroviruses (for instance HIV and HBV) blocking the reverse transcriptase enzymatic function (Fischl et al., 1991; Kewn et al., 1997).

All TEs analyzed for this study, except *1360*, belong to the class of retrotransposons and thus they need to be retro-transcribed to become active and move towards the genome.

From these premises and in order to provide another evidence of the involvement of TEs in HD, we decided to feed flies expressing mHtt in the developing eye (GMR-Gal4>UAS-128Qhtt^{FL}) with a medium supplemented with NRTIs and then verify the effects of these pharmacological treatments on HD eye phenotype.

The NRTIs used were Lamivudine (3TC) to a final concentration of 1 mg/mL and Zidovudine (AZT) to a final concentration of 5 mg/mL, a cytidine and a thymidine analogue respectively. As showed in Figure 14, both the pharmacological treatments administered to flies over the entire developmental period, are capable to induce a strong suppression of HD eye phenotype when compared to the untreated control flies.

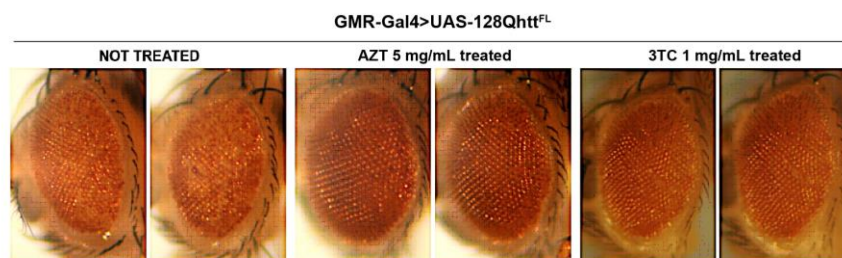

Fig. 14 - Eye phenotype of flies expressing the HD pathogenic construct under the control of the eye-specific GMR-Gal4 driver. Both AZT and 3TC treatment rescue the altered HD eye phenotype.

In particular, both 3TC and AZT suppressed the polyQ-induced phenotype of all the flies carrying one copy of the pathogenic construct (GMR-Gal4/+; UAS-128Qhtt^{FL}/+), whereas only AZT was useful to rescue the HD phenotype in the HD flies with two copies of the mHtt transgene (GMR-Gal4/+; UAS-128Qhtt^{FL}/UAS-128Qhtt^{FL}). For this reason, all successive experiments were performed using AZT.

Pharmacological inhibition by NRTIs rescues lifespan in HD flies

Given the result obtained with the preliminary phenotypical screening and in order to study a systemic effect of NRTI treatment, we performed a survival assay for HD flies fed on standard medium supplemented with 5mg/mL Zidovudine. We crossed parental lines on normal medium and transferred the offspring (elav-Gal4>UAS-128Qhtt^{FL}) on AZT supplemented medium, starting the treatment or at 0-2 days or at 4-5 days after the eclosion. The analysis of the survival curves by log-rank test demonstrated that starting the treatment at 0-2 days determines a higher median lifespan of HD flies than the untreated ones, while starting at 4-5 days does not cause any difference among treated and untreated flies (Figure 15).
This result suggests that the treatment is beneficial only in the early stage of pathogenesis, when is able to inhibit the first transpositional events, while has no effect if disease is already at an advanced stage.

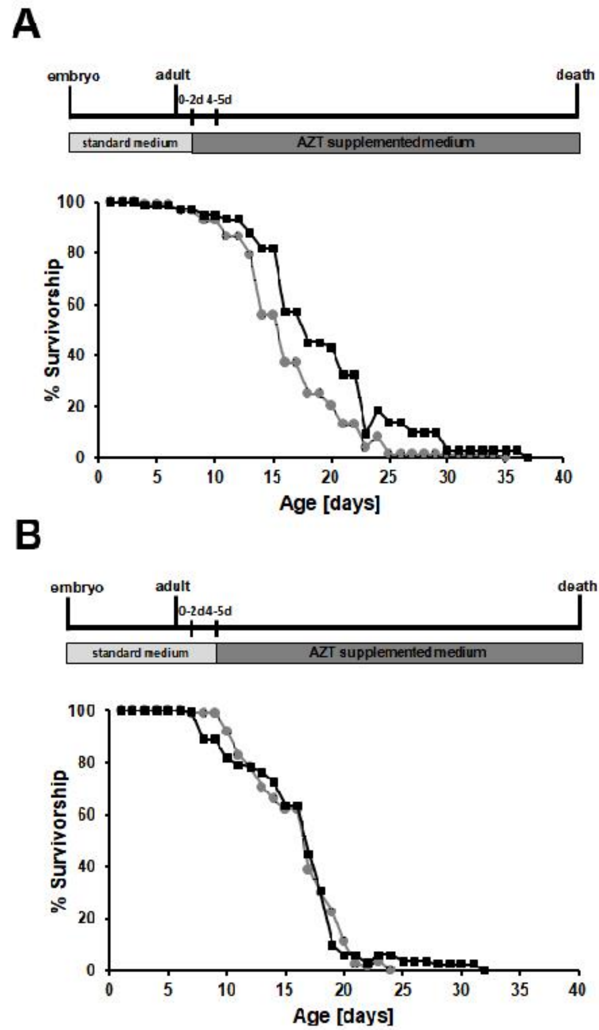

Fig. 15 - Survival curves of elav-Gal4>UAS-128Qhtt^{FL} flies untreated (grey line) or AZT-treated (black line) starting at 0-2 days (A) or at 4-5 days (B) after eclosion. The scheme at the top is an outline of experimental strategy. A) The log rank test shows that the two curves are significantly different (****p < 0,0001). B) The log rank test shows that the two curves are not significantly different (ns, p=0.68).

Transposon activation induces genomic instability in HD

It is well-known that TE mobilization may alter the normal chromosomal structure causing rearrangements and breaks; moreover, recent reports correlate HD and genomic instability in cell lines (Ruzo et al., 2018). For this reasons, we analyzed metaphase chromosomes obtained from brains dissected from elav-Gal4 and elav-Gal4>UAS-128Qhtt^{FL} larvae.
As showed below, HD metaphases present abnormal chromosome configurations (indicated by arrows) such as breakages, fusions, chromosomal rearrangements and a higher degree of chromatin decondensation (Figure 16).

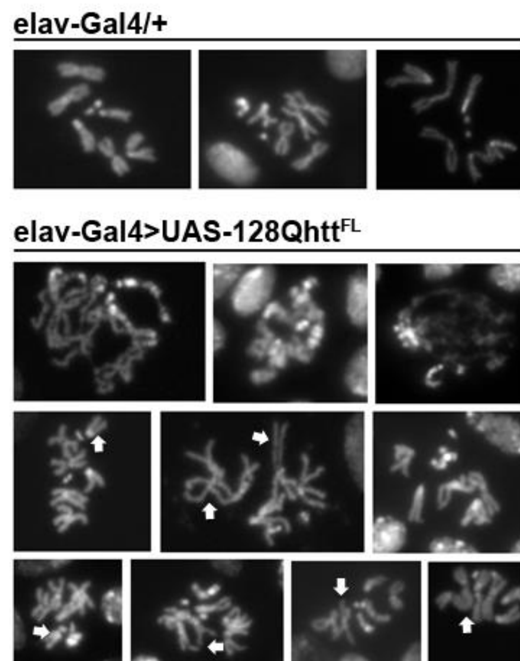

Fig. 16 - Mitotic chromosomes of elav-Gal4/+ and elav-Gal4>UAS-128Qhtt^{FL}. HD chromosomes show a higher degree of chromatin decondensation and structural rearrangements (indicated by arrows) when compared to the control karyotype.

Interestingly, as reported in the graph, the frequencies of chromosomal abnormalities observed in HD larval brains were significantly decreased after AZT treatment (Figure 17).

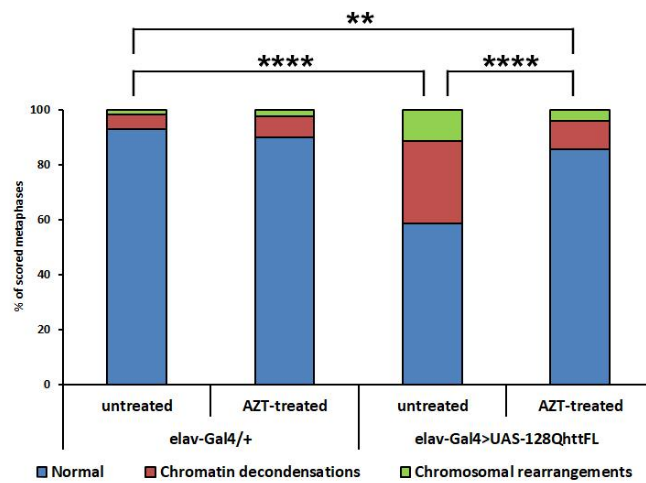

Fig. 17 – Quantification of chromosomal aberrations in elav-Gal4/+ and elav-Gal4>UAS-128Qhtt^{FL} before and after the treatment with AZT. AZT treatment induces a significant reduction of chromosomal structural abnormalities.

Taken together, these results not only confirm the general association between HD and genomic instability, but provide also a strong evidence of a specific TE-driven-instability, that may represent an important piece in the HD pathogenesis scenario.

To confirm the presence of DNA damage in HD brains, we performed an immunofluorescence assay against the histone variant γ-H2AV, a specific marker for DNA double-strand breaks, both in larval and adult HD brains (elav-Gal4>UAS-128Qhtt^{FL}), comparing them to the relative control brains (elav-Gal4/+).

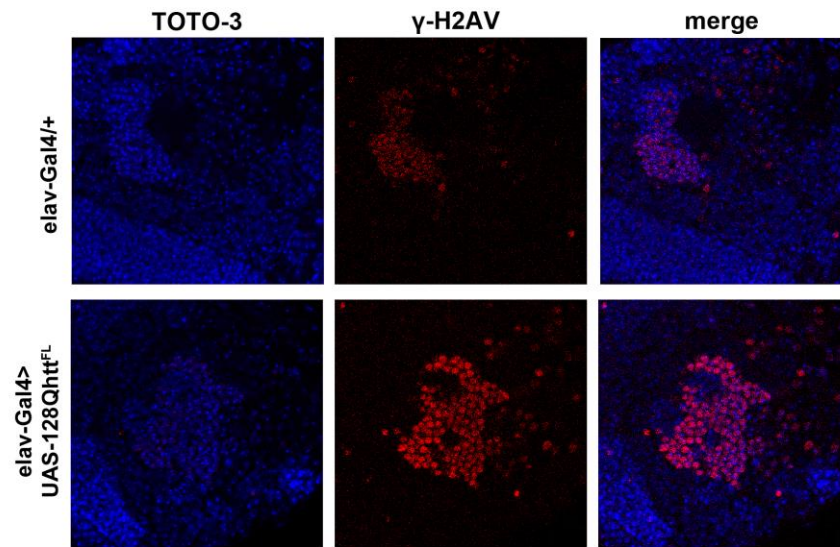

Fig. 18 – Confocal microscopy images of the immunofluorescence against γH2AV on HD (elav-Gal4>UAS-128Qhtt^{FL}) and control (elav-Gal4/+) larval brains. Immunostaining revealed a higher rate of DNA damage in HD brains than in control ones. Images were taken at 63X magnification.

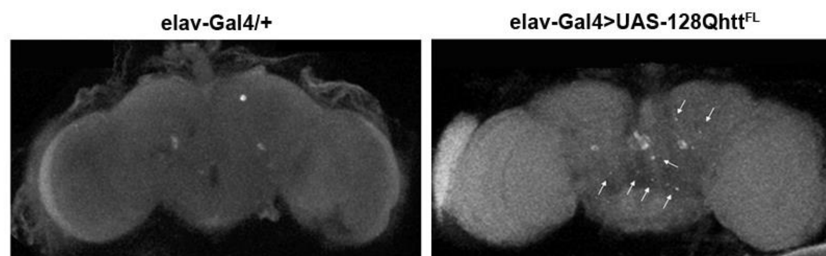

Fig. 19 - Confocal microscopy images of the immunofluorescence against γH2AV on HD (elav-Gal4>UAS-128Qhtt^{FL}) and control (elav-Gal4/+) adult brains. Immunostaining revealed a higher rate of DNA damage foci (indicated by the arrows) in HD brains than in control ones.

Results shown in the Figures 18 and 19 clearly assess the presence of DNA damage foci both in larval and in adult HD brain, thus confirming that genomic instability is a component of pathogenic mechanisms involved in Huntington Disease.

Mitotic Index of HD metaphases

Previous studies on Alzheimer Disease skin fibroblasts cultured cells associated the pathology to a reduction of the mitotic index if compared with healthy individuals (Jenkins et al., 1988).
To gain insights into this aspect of HD pathology, we calculated the mitotic rate (ratio between the numbers of nuclei undergoing mitosis to the total number of nuclei) of metaphases obtained from HD larval brains.

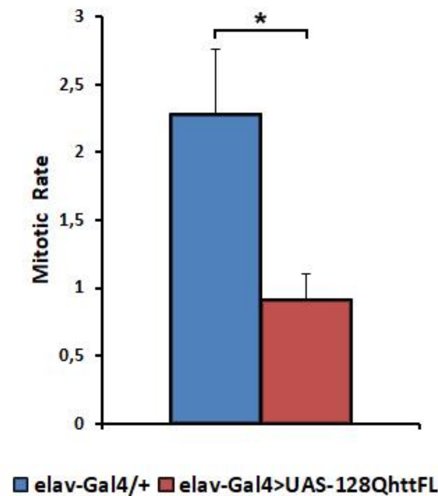

Fig. 20 - elav-Gal4>UAS-128Qhtt^{FL} larval brains show a lower mitotic rate than elav-Gal4/+ brains. Mitotic index was obtained observing five slides for each genotype and the statistical significance was calculated with Mann-Whitney U test. (*, $p < 0,05$).

As showed in Figure 20, HD metaphases have a lower mitotic rate respect to the control ones. This result potentially suggests that HD neurotoxicity pattern may influence mitotic entry or, more in general, cell cycle progression.

The overexpression of Heterochromatin Protein 1 (HP1) suppresses neurotoxicity in HD eye

Recent studies on tau-associated pathologies identified a global heterochromatin decondensation as a source of TE dysregulation (Frost et al., 2014). To verify if heterochromatin relaxation plays a role also in HD pathogenic mechanism, we performed specific genetic crosses to ectopically express or functionally inactivate the protein HP1, a core component of silenced heterochromatin, in the altered eye HD background.

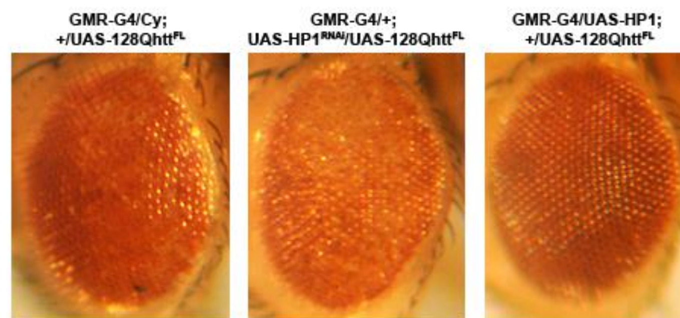

Fig. 21 - Eye phenotype of flies expressing the HD pathogenic construct under the control of the eye-specific GMR-Gal4 driver. HP1 functional inactivation determines a worsening of the HD phenotype (picture in the middle), while its overexpression leads to a suppression of HD phenotype (picture on the right), respect to the control HD eye (picture on the left).

As reported in Figure 21, the overexpression of HP1 in the pathogenic background ameliorates the HD phenotype, re-establishing the ommatidia pigmentation and organization; otherwise, HP1 depletion determines a significant worsening of HD eye phenotype. This preliminary screening, let us hypothesize that HP1, and in general heterochromatin status, may be involved in HD toxicity modulation.

mHtt acts as a dominant modifier of Position-Effect Variegation (PEV)

A common tool to verify the involvement of a gene in the heterochromatin formation process is to analyze its effect on Position-Effect Variegation (PEV), an epigenetic phenomenon that determines a heterochromatic-induced gene silencing that occurs when a euchromatic gene, juxtaposed with heterochromatin by rearrangement or transposition, is transcriptionally silenced and show a variegating phenotype (Elgin and Reuter, 2013).

If a gene has a role in heterochromatin formation and organization it may modify the PEV pattern: it can suppress the variegation if induces a higher expression rate of the euchromatic gene juxtaposed to the heterochromatin, or it can enhance the variegation when the euchromatic gene is less express.

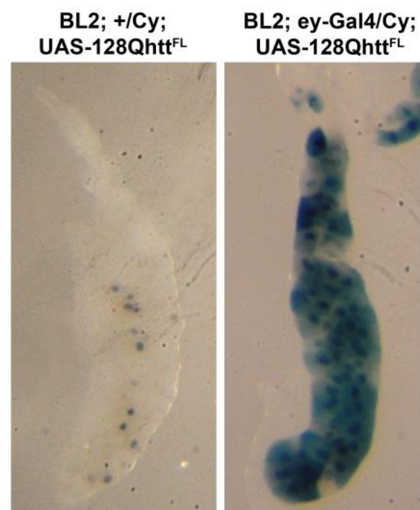

Fig. 22 – Histochemical localization of β-galactosidase on salivary glands isolated from HD male larvae (BL2; ey-Gal4/Cy; UAS-128Qhtt^{FL}) and relative control samples (BL2; +/Cy; UAS-128Qhtt^{FL}).

To carry out this experiment, we performed a genetic cross between females expressing mHtt in larval salivary glands under the control of the eyeless-Gal4 driver (ey-Gal4/Cy; UAS-128Qhtt^{FL}) and males Tp(3;Y)BL2, carrying on their Y chromosome a rearrangement with a Hsp70-lacZ-inducible transgene inserted in the pericentromeric region (Lu et al., 1996). Salivary glands were isolated from the male progeny of this cross and stained with X-gal. X-gal is cleaved by β-galactosidase, yielding galactose and 5-bromo-4-chloro-3-hydroxyindole, that is oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble and blue product. Its presence is used to test the β-galactosidase expression rate.
As showed in Figure 22, β-galactosidase is higher expressed in salivary glands where mHtt is produced than in the control samples, thus remarkably suggesting that the HD pathogenic scenario acts in an unknown way on the heterochromatization process, determining a global relaxation of the heterochromatin and, consequently, a higher rate of expression of heterochromatic sequences, TEs included.

CONCLUSIONS

Our results let us confirm the involvement of Transposable Elements in Huntington Disease pathogenesis.
In particular, we found, thanks to different molecular approaches, an up-regulation of many classes of TE transcripts in HD brains, both at larval and adult stage, suggesting thus their participation to the disease progression. Moreover, this up-regulation increases in an age-dependent manner, perfectly fitting with the neurodegenerative profile of HD. Additionally, through qChIP analysis we observed the decrease of the occupancy of H3K9me3 on transposon sequences, both on coding regions and promoters, thus confirming that TE in HD are finely transcriptionally regulated.
PIWI protein over-expression in HD background and pharmacological treatment with NRTIs Lamivudine and Zidovudine determined the suppression of the altered HD eye phenotype. Moreover, the early AZT-treatment on young HD adult flies led to an extension of the median lifespan. Taken together these data strongly confirms that the inhibition of the early transpositional events ameliorate HD phenotype.
Cytological analysis of HD metaphases showed the presence of chromosomal aberrations and rearrangements, whereas immunofluorescence assay against γ-H2AV on HD larval and adult brain revealed a high rate of dsDNA damage: these results provide the evidence of a strong connection between HD and genomic instability. Remarkably, the level of chromosomal aberration is considerably decreased by AZT-treatment, suggesting thus the occurrence of TE-induced DNA instability in HD pathogenesis.

Interestingly, PEV analysis on HD larvae let us to define mHtt as a suppressor of Position Effect Variegation and thus linked to a global relaxation of the chromatin, probably leading to a general up-regulation of heterochromatic sequences.
With no doubt, in the next future, the interaction between HD background and heterochromatin organization will be better characterized: we want to understand if it is a result of a direct interaction between mHtt and any nuclear component or is the general pathological condition that inhibits the standard heterochromatin formation.
Additionally, it will be challenging to speculate on the effect that mHtt expression has on TE in glial tissues. Our preliminary results let us to hypothesize that TEs are induced also in HD glial cells; furthermore, we believe that glial HD toxicity determines not only glial cells depletion and severe systemic consequences in HD flies, but also, taking into account the important role of glia in neuronal maintenance, that is linked to a strong neuronal damage.
Definitely, our findings confirm the complexity of HD pathogenic scenario and provide the evidence that transposable elements are an important (and new) piece in the puzzle of HD-induced neurotoxicity.

MATERIALS AND METHODS

Drosophila strains

The Drosophila stocks used in this study were obtained from Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN) and are listed below:

w^{1118}; P{UAS-HTT.128Q.FL}f27b - BL33808;
w^{1118}; P{UAS-HTT.16Q.FL}F24/CyO - BL33810;
y^{1}, sc^{*},v^{1}; P{TRiP.HMS00185}attP2/TM3, Sb^{1} - BL34866;
$piwi^{EP}$, ep(2)1024/CyO; hsGal4/hsGal4 - (a gift from H. Lin);
$y^{1}w^{*}$Dp(3;Y)BL2, P{HS-lacZ.scs}65E - BL57371;
w^{1118}; P{GD12524}v31994;
elav-Gal4: (P{GawB}$elav^{C155}$) - BL458;
GMR-Gal4: (w^{*}; P{GAL4-ninaE.GMR}12) - BL1104;
eyeless-Gal4: (y^{1} w^{1118}; P{ey3.5-GAL4.Exel}2) - BL8220

The UAS-HP1a/Cy strain overexpressing HP1a under the control of UAS sequence was generated in our lab.

The Oregon-R stock and balancer stocks, used to balance inserts on the X, second, and third chromosomes, respectively, have been kept in our laboratory for many years. All flies were raised at 24 °C on standard cornmeal-sucrose-yeast-agar medium.

Total RNA extraction, reverse transcription and qPCR

Larval brains, adult brains or heads were collected in 1.5 mL tubes and total RNA was extracted in Qiazol reagent (Qiagen) according to manufacturer' s protocol.

4 μg of total RNA was reverse transcribed using oligo dT or random hexamers and SuperScript Reverse Transcriptase III (Invitrogen). The qPCR experiments were realized with QuantiFast SYBR Green PCR Kit (Qiagen) according to manufacturer's protocol. Relative quantification of different transcripts was determined using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001) using rp49 transcript as control. qRT-PCR experiments were performed in three independent biological replicates each with three technical replicates. Statistical significance was determined by unpaired t test using GraphPad Prism Software. A p value ≤ 0.05 was considered statistically significant. All primer used are listed below:

412	**F:** AGTTTTTGGTAAATGCAGGGAATACAA
	R: TGGAACTGGGTAGTTCTGAATGACA
1360	**F:** TCGTGCAAGACAATGAGAGG
	R: GCAACTGGATCCCTTAGCAA
Aurora	**F:** GAAGGAACTGAGCGTGTTCCA
	R: CGTCTACCGCAACTAATGCAAA
blood	**F:** TGCCACAGTACCTGATTTCG
	R: GATTCGCCTTTTACGTTTGC
copia	**F:** CGTCTACCGTTCGGCTTGAG
	R: CACATCGTCTAGGGCTCTTTG
Gapdh	**F:** CAGCCCCGACATGAAGGT
	R: CGATCTCGAAGTTGTCATTGATG
gypsy	**F:** CTTCACGTTCTGCGAGCGGTCT
	R: CGCTGCAAGGTTACCAGGTAGGTTC
Het-A	**F:** ACTGCTGAAGCTCGGATTCC
	R: TGTAGCCGGATTCGTCATATTTC

hid	**F:** ATCCAGTCTGCCATACCGATAG
	R: AACAGTTGGCCAAGTGAAGCTC
128Qhtt	**F:** CACCGACCAAAGAAAGAACTTTCA
	R: TTTAATTTCCTTATAGAGCTCGAGCTGTAA
I element	**F:** CAATCACAACAACAAAATCC
	R: GGTGTTGGTGTGGTTGGTTG
opus	**F:** CGAGGAGTGGGGAGAGATTG
	R: TGCGAAAATCTGCCTGAACC
R2	**F:** ATGCTCCCGAAACAACAAAC
	R: GCACTGCAGACTTGGTTCAA
roo	**F:** CGTCTGCAATGTACTGGCTCT
	R: CGGCACTCCACTAACTTCTCC
rp49	**F:** GCGCACCAAGCACTTCATC
	R: TTGGGCTTGCGCCATT

NRTI food preparation

Zidovudine (AZT; PHR-1292 Sigma-Aldrich) and Lamivudine (3TC; PHR-1365 Sigma-Aldrich) were dissolved in 95% ethanol and water respectively. NRTI solutions were added in standard medium to give a final concentration of 5 mg/mL for AZT and 1 mg/mL for 3TC.

Mitotic chromosomes preparation

Third instar *Drosophila* larval brains were dissected in 1X PBS (Phosphate Buffered Saline) and kept 8 minutes in hypotonic solution (sodium citrate 0.5%). Brains were placed on a siliconized coverglass and fixed for 10 minutes in 5% formaldehyde – 45% acetic acid solution. Fixed brains were squashed for a minute, frozen in liquid nitrogen and washed in ethanol for 10 minutes. Slides were stained with DAPI (4,6-diamidino-2-phenilindole, 0.01 mg/ml) to visualize DNA, mounted in antifading medium

(23,3 mg/mL of DABCO (1,4-Diazobicyclo-(2,2,2) octane) in 90% glycerol – 10% 1X PBS) and sealed with nail polish.
All images were acquired on Ellipse Epifluorescence microscope (E1000 Nikon) equipped with a CCD camera (Coolsnap) and analysed and further processed using Adobe Photoshop CS6.
Mitotic index (ratio between the number of nuclei undergoing mitosis to the total number of nuclei) was obtained observing five slides for each genotype and statistical significance was calculated with Mann-Whitney U test.

Immunofluorescent staining of *Drosophila* larval and adult brains

The immunofluorescent staining of larval or adult brains was performed according to Wu (Wu et al., 2006) with minor modifications. Briefly, adult *Drosophila* brains were dissected in 0.1 M PB solution (100 mM Na_2HPO_4/NaH_2PO_4, pH 7.2), washed in 0.1 M PB 0.1% Triton (PBT) solution and fixed for 30 minutes in 4% formaldehyde PBT solution. Blocking was made in 5% NGS (Normal Goat Serum) PBT solution for 40 minutes. Primary and secondary antibodies were diluted in a 5% NGS PBT solution and incubated for two nights each one at 4 °C. Brains were stained with TOTO-3 Iodide (Thermo Fisher Scientific) (1 µM) to visualize DNA and mounted in antifading medium. Primary antibody used was rabbit anti-γH2AV (1:50, Rockland). Fluorescent labelled secondary antibody raised in goat was Alexa Fluor 555 conjugated anti-rabbit (1:300, Invitrogen). Confocal observations were performed using a Leica DMIRE (Leica Microsystems, Hiedelberg, Germany) and a Zeiss LSM 780 (Zeiss, Berlin, Germany). Images were analysed and further processed using Zen Software and Adobe Photoshop CS6.

Drosophila eye imaging

Flies were ether-anesthetized and eyes images were taken with a stereoscopic microscope equipped with a Nikon camera D5000. Images were further processed using Adobe Photoshop CS6.

Lifespan analysis

For each experimental curve were collected 150-200 flies (30 flies per vial). Every 2–3 days, flies were passed into new vials and dead flies were counted. The survival rate was calculated by the percentage of total flies surviving. Survival curves were analysed by the log-rank method using the web tool OASIS (Online Application for the Survival Analysis) (Yang et al., 2011).

Semi-quantitative PCR

100ng of cDNA reverse transcribed from 4µg of total RNA purified from fly heads, was added to the following reaction mix: 1X PCR Buffer, primers (0.2 µM), $MgCl_2$ (1.5 mM), dNTPs (0.2 mM each), and 2U/rxn of Platinum™ Taq DNA Polymerase (Invitrogen). The thermal profile was: 94 °C for 3 minutes for the initial denaturation, followed by 30 cycles at 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds and a final extension at 72 °C for 7 minutes. PCR products were analysed by agarose gel electrophoresis.

Western blot analysis

To obtain a total protein extract, *Drosophila* brain or head samples were homogenized in Sample Buffer 1X (50 mM Tris-HCl pH 6.8,

100 mM dithiothreitol, 2% sodium dodecyl sulphate, 2.5% glycerol, 0.1% bromophenol blue) and heated at 85 °C for 8 minutes to complete the protein denaturation.
Protein extracts were separated by a 10% SDS-PAGE electrophoresis and electroblotted onto methanol-activated PVDF membranes (Biorad) in CAPS transfer buffer (10 mM CAPS pH 11, 10% methanol) in a semi-dry transfer apparatus (Amersham Biosciences). The membranes were blocked in 5% non-fat dry milk in 1X TBS (Tris Buffered Saline) 0.1% Tween 20 and incubated with the following antibodies diluted in TBST: mouse anti-env 8E7 (1:500, kindly provided by J. Gall), rabbit anti-giotto (1:10000, kindly provided by G. Cestra) and mouse anti-elav (1:500, 9F8A9 DSHB). Proteins of interest were detected with HRP-conjugated goat anti-mouse or anti-rabbit IgG antibody and visualized with the ECL western blotting substrate (GE Healthcare), according to the provided protocol. The chemiluminescence detection was performed on the ChemiDoc XRS+ System (Biorad) and analysed using the included ImageLab software.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed according to Menet (Menet et al., 2010) with few modifications. Approximately 300 µL of fly heads were homogenized in 3 mL of 1X NEB (10 mM HEPES-Na pH 8, 10 mM NaCl, 0.1 mM EGTA-Na pH 8, 0.5 mM EDTA-Na pH 8, 1 mM DTT, 0.5% NP-40, 0.5 mM Spermidine, 0.15 mM Spermine, 1X EDTA-free Complete Protease Inhibitors) with a Polytron homogenizer (Kinematica Swizerland) with a PT300 tip five times (2 min and 1 min on ice) at 3500 rpm and five times at 4000 rpm. The homogenate was

transferred to a pre-chilled glass dounce (Wheaton) and 20 full strokes were applied with a tight pestle. Free nuclei were filtered on a 70 μM strainer and then centrifuged at 6000xg for 10 minutes at 4 °C. The nuclei-containing pellets were suspended in 1 mL of 1X NEB 0.25 M sucrose and centrifuged at 20000xg for 20 minutes on sucrose gradient (0.65 mL of 1.6 M sucrose in NEB, 0.35 mL of 0.8 M sucrose in NEB). The pellet was dissolved in 1 mL of 1X NEB 1% formaldehyde and placed onto a nutator 10 minutes to cross-link nuclei; quenching was done by adding 1/10 volume of 1.375 M glycine. The nuclei were collected by centrifugation at 6000xg for 5 minutes. Nuclei were washed in 1 mL of 1X NEB and then resuspended in 1.5 mL of Lysis Buffer (15 mM HEPES-Na pH 7.6, 140 mM NaCl, 0.5 mM EGTA, 1 mM EDTA pH 8, 1% Triton X-100, 0.5 mM DTT, 0.1% Na-Deoxycholate, 0.1% SDS, 0.5% N-lauroylsarcosine and 1X EDTA-free Complete Protease Inhibitors). Chromatin sonication was performed using a Hielscher Ultrasonic Processor UP100H (100 W, 30 kHz) thirty times for 30s and 30s on ice. Chromatin fragments (300-500 bp) were centrifuged at 15000xg for 10 minutes at 4 °C. To allow the immunoprecipitation, 3μg of H3K9me3 monoclonal antibody (Active Motif) were incubated in the presence of Dynabeads protein G (Invitrogen) for 3 hours at room temperature on a rotating wheel, then chromatin extract was added and placed on a rotating wheel for an overnight incubation at 4 °C. Supernatants were discarded and samples were washed twice in Lysis Buffer (each wash 15 minutes at 4 °C) and twice in TE Buffer (1 mM EDTA, 10 mM Tris HCl pH 8). Chromatin was eluted from beads in two steps: firstly at 65 °C for 15 minutes in 100 μl of Elution Buffer 1 (10 mM EDTA, 1% SDS, 50mM Tris HCl pH 8) and secondly in 100 μl of Elution Buffer 2 (TE 0.67% SDS) for 15 minutes at 65 °C. The combined elute (200 μl) was

incubated overnight at 65 °C to revert the cross-link and treated by 50 µg/ml RNaseA for 30 min at 37 °C and by 500 µg/ml Proteinase K (Invitrogen) for 3 hours at 65 °C. Samples were phenol–chloroform extracted and ethanol precipitated. DNA was resuspended in 15 µl of water and target genes were amplified through qPCR. The heterochromatic control region F22 (Lu et al., 2018) was used as a positive control for H3K9me3 enrichment. Oligonucleotides used as primers for qChIP-PCR analysis are listed below:

412 promoter	**F:** GCCCAGCTGACACCTGATG
	R: TTGGGTTAACAGGGCATAATAGTATATG
412	**F:** AGTTTTTGGTAAATGCAGGGAATACAA
	R: TGGAACTGGGTAGTTCTGAATGACA
1360 promoter	**F:** AAGGAAAAAAGGGCACAAATACAA
	R: TTATGCTGTTCCACTTTTGCAAA
1360	**F:** TCGTGCAAGACAATGAGAGG
	R: GCAACTGGATCCCTTAGCAA
blood promoter	**F:** ATGGGCAACGGCACAGAA
	R: CTTTTCGGCTTGGGTGATGT
blood	**F:** TGCCACAGTACCTGATTTCG
	R: GATTCGCCTTTTACGTTTGC
copia promoter	**F:** CAACACTACTTTATATTTGATATGAATGGC
	R: GTTCTCGCCATCAAACGCGTAC
copia	**F:** GCATGAGAGGTTTGGCCATATAAGC
	R: GGCCCACAGACATCTGAGTGTACTACA
Het-A promoter	**F:** ACCACGCCCAACCCCCAA
	R: GCTGGTGGAGGTACGGAGACAG
Het-A	**F:** CGCAAAGACATCTGGAGGACTACC
	R: TGCCGACCTGCTTGGTATTG
I element promoter	**F:** CGTGCCTCTCAGTCTAAAGCCTC
	R: CCCGGATTAGCGGTATTGTTGTT
I element	**F:** TGAAATACGGCATACTGCCCCCA
	R: GCTGATAGGGAGTCGGAGCAGATA
roo promoter	**F:** CCTCTGCGTAGGCCATTTAC

	R: AAGGCTCGATTGACCAAATG
roo	**F:** CGTCTGCAATGTACTGGCTCT
	R: CGGCACTCCACTAACTTCTCC
F22	**F:** CAGTTGATGGGATGAATTTGG
	R: TGCCTGTGGTTCTATCCAAAC

Histochemical localization of β-galactosidase in PEV analysis

To induce β-galactosidase expression, larvae were heat-shocked at 37 °C for 45 minutes followed by 1 hour of recovery at room temperature. Salivary glands were dissected in 1X PBS, fixed in 4% formaldehyde for 15 minutes, washed in 1X PBS and incubated in 0.2% X-gal (5-bromo-4-chloro-3-indolyl-p-D-galactopyranoside) assay buffer (3.1 mM potassium ferricyanide, 3.1 mM potassium ferrocyanide, 10 mM PB (pH 7.2), 0.15 M NaCl, 1 mM $MgCl_2$) for 3 hours (Piacentini et al., 2009). Salivary glands were immersed in 90% glycerol – 10% 1X PBS and sealed with nail polish. All images were acquired through a stereomicroscope equipped with a Nikon D5000 camera, analysed and further processed using Adobe Photoshop CS6.

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