Cláudia Garcia Belém

Licenciada em Biologia

Retrotransposition and Ageing-associated Neuronal Function Decline

Dissertação para obtenção do Grau de Mestre em

Genética Molecular e Biomedicina

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They thought I was crazy, absolutely mad.

Barbara McClintock (1983)

Abstract

The world population is progressively ageing. It is estimated that by 2050, almost one-fifth of the world population will be aged 65 years or more. Despite the significant increases in life expectancy observed in the last century, health span remained unchanged. Therefore, people live longer but in suboptimal conditions, which frequently lead to the development of age-related diseases, like neurodegenerative diseases. Understanding the molecular and cellular mechanisms underlying ageing and neurodegeneration is crucial and could provide the means to delay, mitigate or even revert the deteriorating effects associated with age-related neurodegeneration. Recent studies have correlated increased expression of retrotransposable elements (REs) with age, which is likely due to the tendency of RE silencing mechanisms to fail with age. Furthermore, it was reported in flies that young individuals with a neurodegenerative decline had premature expression of REs in their brain. However, it remains unclear whether RE expression and mobilization are the cause or a consequence of the age-associated neuron functional decline.

The aim of this dissertation is to determine if RE expression in the central nervous system causes an age-associated neuronal function decline. To answer this, we developed a heterologous and *naïve* inducible RE system that allows specific expression of a human *long interspersed nuclear element 1 (LINE-1 or L1)* in *Drosophila melanogaster* neurons. Negative geotaxis assays were performed in flies aged 2, 20, and 40 days after eclosion to assess the age-associated neurofunctional decline.

Results revealed that the forced expression of *L1* in neurons throughout lifespan does not affect neuronal function. However, both *in vivo* and *in vitro* experiments failed to demonstrate retrotransposition events in the fly. These findings suggest that additional human factors are required in *L1* retrotransposition. Future studies will focus on determining retrotransposition

capacity of *L1* in the fly genome.

Keywords: Ageing, Neurodegeneration, Genomic instability, Retrotransposons, *L1*, *Drosophila melanogaster*.

Resumo

A população humana está a envelhecer. Estima-se que em 2050, cerca de um quinto da população mundial terá 65 anos de idade ou mais. Apesar do aumento significativo na esperança média de vida observado no século passado, o período de vida saudável permaneceu inalterado. Assim, as pessoas vivem mais tempo, mas em condições subótimas, que frequentemente levam ao desenvolvimento de doenças relacionadas com a idade, como as doenças neurodegenerativas. Estudos recentes correlacionaram o aumento da expressão de Elementos Transponíveis (ETs) com a idade, o que provavelmente se deve à tendência dos mecanismos de repressão dos ETs falharem com a idade. Além disso, foi relatada expressão prematura de ETs no cérebro de moscas jovens com declínio da função neuronal. Contudo, permanece por esclarecer se a expressão dos ETs e a sua mobilização são a causa ou uma consequência do declínio funcional neuronal associado à idade.

O objetivo desta dissertação é determinar se a expressão dos ETs no sistema nervoso central causa o declínio funcional neuronal associado à idade. Para responder a esta pergunta, desenvolvemos um sistema heterólogo de ETs indutível e *naïve* que permite expressar especificamente um ET de humano, o *long interspersed nuclear element 1 (LINE-1 or L1)*, nos neurónios de *D. melanogaster*. Realizaram-se ensaios de geotaxia negativa em moscas com 2, 20, e 40 dias de idade para avaliar o declínio neurofuncional.

Os resultados demonstraram que a expressão induzida de *L1* nos neurónios durante a vida da mosca não afeta a função neuronal. Contudo, os resultados obtidos em experiências *in vivo* e *in vitro* não demonstraram evidências de eventos de retrotransposição em mosca. Estes resultados sugerem que existem fatores humanos adicionais envolvidos na retrotransposição de *L1*. Estudos futuros focar-se-ão na determinação da capacidade de retrotransposição de *L1* no genoma da mosca.

Palavras-chave: Envelhecimento, Neurodegeneração, Instabilidade Genómica, Retrotransposões, *L1*, *Drosophila melanogaster*.

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Acronyms

μL microliter

μM micromolar

μg microgram

μm micrometer

A Adenine

AML Amyotrophic Lateral Sclerosis

bp base pairs

cDNA complementary DNA

cm centimeter

CNS Central Nervous System

DABCO 1, 4-Diazobicyclo-(2,2,2-octane)

DAPI 4,6 diamidino-2-phenylindole

DL2 Drosophila line 2

DNA DeoxyriboNucleic Acid

DSB Double Strand Break

EDTA ethylenediaminetetraacetic acid

esiRNA Endogenous Short Interfering RNA

FTLD FrontoTemporal Lobar Degeneration

Gbp Giga base pairs

gDNA genomic DNA

GFP Green Fluorescent Protein

h hour

HEK Human Embryonic Kidney

HeLa Henrietta Lacks

HERV Human Endogenous RetroVirus

IQR Interquartille Range

kb kilo base pairs

kDa kilodalton

L1 LINE-1

LINE Long Interspersed Nuclear Element

LTR Long Terminal Repeat

mg milligram

Mhc Myosin Heavy Chain

min minute

miRNA MIcroRNA

mL milliliter

mM millimolar

moesin Membrane-Organizing Extension Spike proteIN

mRNA messenger RNA

NCD Non-Communicable Disease

ng nanogram

ON overnight

ORF Open Reading Frame

PBS Phosphate Buffer Saline

PCR Polymerase Chain Reaction

pg picogram

piRNA PIWI-Interacting RNA

PIWI P-element Induced WImpy testis

pmol picomole

RC Retrotransposition Competent

RE Retrotransposable Element

RNA RiboNucleic Acid

RNAi RNA Interference

RNP RiboNucleoprotein Particle

rpm rotations per minute

RT Room Temperature

RT-qPCR Reverse Transcriptase quantitative PCR

s second

S2 Schneider line 2

SD standard deviation

sfGFP superfolder Green Fluorescent Protein

SINE Short Interspersed Nuclear Element

siRNA Small Interference RNA

TE Transposable Element

TPRT Target Primed Reverse Transcription

Tris Tris(hydroxymethyl)aminomethane

UAS Upstream Activating Sequence

UTR UnTranslated Region

WT Wild-Type

*

Chapter 1

Introduction

1.1 Ageing World

During the last century, there has been not only a very significant increase in life expectancy (due to improvements in quality of life), but also a decline in population growth, especially in the developed countries. Together, these two phenomena prompted the accelerated ageing of the population (He *et al.*, 2016). People aged 65 years and over represent around 8.5 % (617.1 million) of the world's total population (7.3 billion) and this number is expected to grow in an average annual rate of 27.1 million people (He *et al.*, 2016). Furthermore, while Europe and Northern America are the regions that account for the highest percentage of older people per region (17.4 % and 15.1 %, respectively), about half (55.3 %) of the total older population of the world lives in the Asian continent (He *et al.*, 2016; Kinsella and He, 2009). However, with the increase of the older population there was also an increase in several ageing-associated diseases, namely non-communicable diseases (NCDs), such as cardiovascular and neurodegenerative disorders, cancer, and diabetes (He *et al.*, 2016). This suggests that ageing is a primary risk factor for the development of NCDs.

Even though life expectancy has been increasing in the last century, the average length of healthy life (i.e., health span) has not. People are living longer but the risk of developing NCDs, namely neurodegenerative diseases, that further enhance deterioration and disability of the ageing individual still remains high.

According to Prince *et al.* (2014), the dementia-associated burdens (i.e., financial cost, mortality, and morbidity) in people aged 60 years or more had the biggest increase (around 113 %) among the NCDs between 1990 and 2010. In fact, it is predicted that there are currently 47 million people living with dementia worldwide and the costs associated have been estimated in US$ 818 billion annually (Prince *et al.*, 2016).

It is imperative to understand the molecular and cellular factors underlying ageing and health deterioration in order to gain insight about possible interventions that may slow down, mitigate or even revert the deteriorating effects associated with ageing-related diseases.

1.2 Molecular mechanisms behind ageing

Ageing is the complex and intraindividual time-dependent functional decline that affects most living organisms (reviewed in López-Otín *et al.*, 2013). Hence, ageing is characterized by an intrinsic and inevitable gradual deterioration of physiological integrity, at the cellular and organismal level, responsible for the impaired function, decreased fertility, and increased susceptibility to death by internal and external threats (Comfort, 1964; Partridge and Mangel, 1999).

Two major groups of theories have emerged to explain the processes or mechanisms behind ageing: the programmed ageing and the damage or error-based theories (reviewed in Mercado-Sáenz *et al.*, 2010). The former suggests the existence of an intrinsic biological program that controls and regulates the deterioration of the structural and functional capacity of the organism, whereas the latter defends that the observed deterioration would develop upon a continuous accumulation of molecular and cellular damage (Magalhães, 2011; Mercado-Sáenz *et al.*, 2010). Currently, a combination of these two currents is preferred. In 2013, López-Otín *et al.* outlined and described nine cellular and molecular mechanisms involved in ageing: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intracellular communication.

1.3 Genomic instability

Both endogenous and exogenous sources are continuously imposing deoxyribonucleic acid (DNA) damage, and, consequently, challenging the integrity of the genome. These agents can be labeled as external (or extrinsic), if they originate in the surrounding environment and endogenous (or intrinsic), if they reside within the cell itself. Examples of external agents are

ultra-violet and ionizing radiation exposure and cigarette smoke, whereas internal mutagens encompass DNA replication errors, the formation of free radicals, and the mobilization of transposable elements (TEs) (reviewed in Aunan *et al.*, 2016; López-Otín *et al.*, 2013; Vijg and Montagna, 2017).

Genomic instability refers to a variety of events capable of causing unscheduled alterations within the genome (reviewed in Bapat and Perera, 2007; Pikor *et al.*, 2013). Genomic instability is caused by damage accumulation, which encompasses DNA damage, mutations, and chromosomal alterations (Vijg and Montagna, 2017). DNA damage is related with the DNA structure physical alteration, which includes single and double strand breaks (DSBs) of the DNA backbone, cross-links between bases, and base modifications. Mutations involve insertions, deletions, or substitutions of one or thousands of nucleotides (Vijg and Montagna, 2017). The chromosomal alterations involve gains or loss of either parts or whole chromosomes, translocations, and inversions (Pikor *et al.*, 2013). Mutations and chromosomal alterations are irreversible whereas DNA damage is reversible (*e.g.*, repairable). In order to prevent the accumulation of DNA damage and to keep the viability of the cells, organisms evolved complex and interconnected DNA repair mechanisms (reviewed in Hoeijmakers, 2001; Vijg and Montagna, 2017). Together, these highly conserved mechanisms recognize and fix almost every type of damage inflicted to the DNA, therefore contributing for the maintenance of the genome (Lord and Ashworth, 2012; reviewed in Hoeijmakers, 2001; López-Otín *et al.*, 2013; Milholland *et al.*, 2017; Vijg and Montagna, 2017). Nevertheless, some DNA lesions unavoidably escape these repair mechanisms and tend to accumulate in the genome. Additional genomic instability comes from the generation of errors during DNA damage repair, DNA replication, or chromosomal segregation during cell division (reviewed in Hoeijmakers, 2001; López-Otín *et al.*, 2013; Milholland *et al.*, 2017; Vijg and Montagna, 2017). The occurrence of mutations in any of the genes involved in the genome maintenance mechanism would further lead to a loop of increasing instability. In addition, DNA repair mechanisms tend to fail with age, further promoting the accumulation of damage in cells. The accumulation of genetic damage in somatic cells of an organism plays a determinant role in ageing (Moskalev *et al.*, 2013). In fact it has been reported that mutations affecting DNA-repair system genes are frequently associated with increased frequency of somatic mutations and decreased lifespan in flies (Greer *et al.*, 2013). The same was observed for mice, which displayed a premature ageing (Dollé *et al.*, 2006). In humans, mutations in DNA-repair system genes were found to be correlated with human progeria syndrome patients (reviewed in Vijg and Montagna, 2017). The accumulation of DNA damage and mutations has been directly implicated in ageing and in the development of neurodegenerative

diseases (reviewed in Vijg and Montagna, 2017).

As mentioned above, repetitive elements, such as TEs, are another source of genome instability. In the following section, we will provide further background on TEs (subsections 1.4.1 and 1.4.2), describe how these elements can instigate genomic instability (subsection 1.4.3), and how do the host organisms regulate TEs (subsection 1.4.4).

1.4 Transposable Elements (TEs)

1.4.1 What are TEs?

TEs were initially discovered by Barbara McClintock during her studies of *Zea mays* (maize) in the mid 1940s, work that would award her a Nobel prize in 1983 (McClintock, 1950; reviewed in Biémont and Vieira, 2006; Erwin *et al.*, 2014). While studying the inheritance of pigmentation in maize kernels, McClintock noticed that the color patterns of kernels changed. According to McClintock (1950), this variation was due to the mobilization of genetic units, which she named "controlling elements", from one locus to another. Thirty years later, Shapiro (1969) reported that the insertion of specific DNA fragments, called insertion sequences or IS elements, into the bacterial genome were disrupting a gene (Shapiro, 1969). It was only in 1974, that Hedges and Jacob coined those elements as TEs or transposons after proving that DNA segments carrying antibiotic-resistance genes are able to transpose from one DNA molecule (plasmid) to another (chromosome).

Today, we know that TEs, also known as jumping genes, are discrete and moderately repetitive interspersed DNA sequences capable of moving within a host genome (reviewed in Biémont and Vieira, 2006). TEs can be found in the genome of organisms from all three domains of life (Figure 1.1) (reviewed in Elbarbary *et al.*, 2016; Haren *et al.*, 1999; Huang *et al.*, 2012; Piégu *et al.*, 2015). The percentage of TEs present in the genome of an organism seems to be positively correlated with genome size. For instance, the genomes of *Escherichia coli* (0.0046 pg; 1 pg = ~1 Gbp of DNA), *D. melanogaster* (0.18 pg), *Homo sapiens* (3.5 pg), and *Z. mays* (5.0 pg) are composed by 0.3, 22, 45, and 60 % of TEs, respectively (Alzohairy *et al.*, 2013; Biémont and Vieira, 2006). There are even some cases in plants where TEs make up to 90 % of its genome (reviewed in Ayarpadikannan and Kim, 2014).

1.4.2 TE Classification

TEs can be classified as type I or type II according to their capacity to mobilize via ribonucleic acid (RNA) or DNA intermediates, respectively (Figure 1.2) (reviewed in Ayarpadikannan

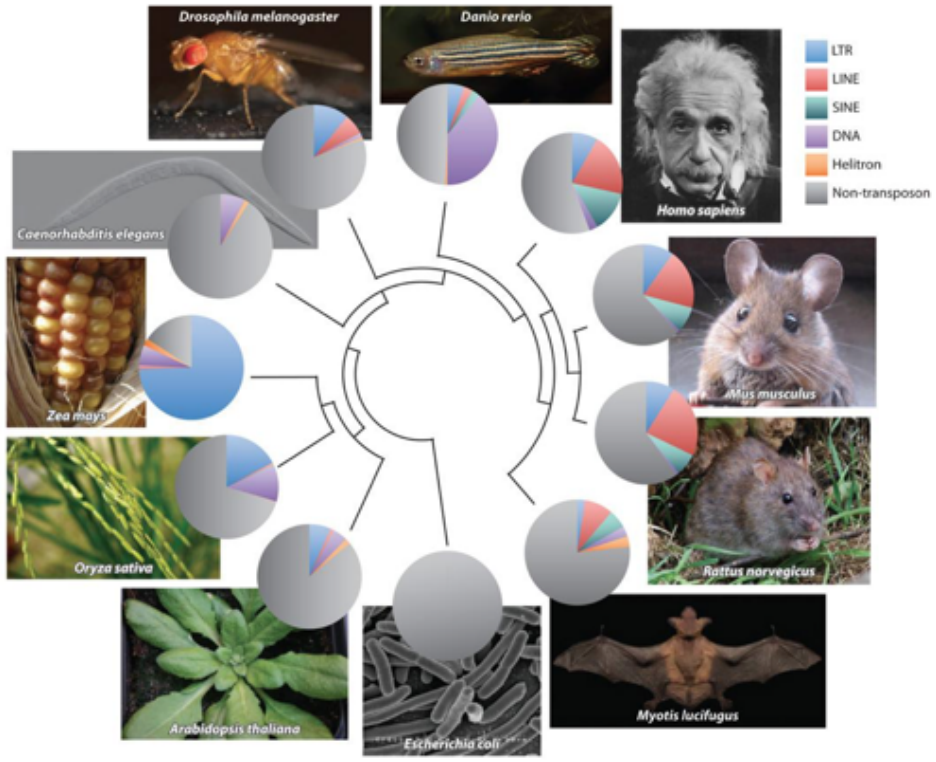

Figure 1.1: The composition of TE differs between different taxa. The circle charts portray the relative composition of different TE classes in each species genome. The evolutionary relationship between species is represented by the phylogenetic tree in the center. *LTR - Long Terminal Repeat*, *LINE - Long Interspersed Nuclear Element*, *SINE - Short Interspersed Nuclear Element* (reproduced from Huang *et al.*, 2012).

and Kim, 2014; Huang *et al.*, 2012; Mita and Boeke, 2016). Type I TEs mobilize using an RNA intermediate, generating new copies in the genome through a process termed retrotransposition (copy-and-paste mechanism). They are called retrotransposable elements (REs), retrotransposons, or retroelements. On the other hand, type II TEs mobilize via DNA intermediates, excising themselves and re-integrating elsewhere in the genome (cut-and-paste mechanism). Type II TEs are termed DNA transposons (reviewed in Huang *et al.*, 2012; Mita and Boeke, 2016).

REs can be separated into two different subtypes according to their structural features. REs flanked by long terminal repeat (LTR) (i.e., repeated sequences of 300 to 1000 bp) are designated LTR retrotransposons, whereas REs without LTRs are entitled non-LTRs retrotransposons (reviewed in Huang *et al.*, 2012; Mita and Boeke, 2016). Non-LTRs retrotransposons are separated into two classes: long interspersed nuclear element (LINE)s and short interspersed nuclear element (SINE). LINEs and SINEs differ in size and structure. LINEs are about 6 kb long, encode the proteins required for the retrotransposition, and are transcribed by a RNA polymerase II,

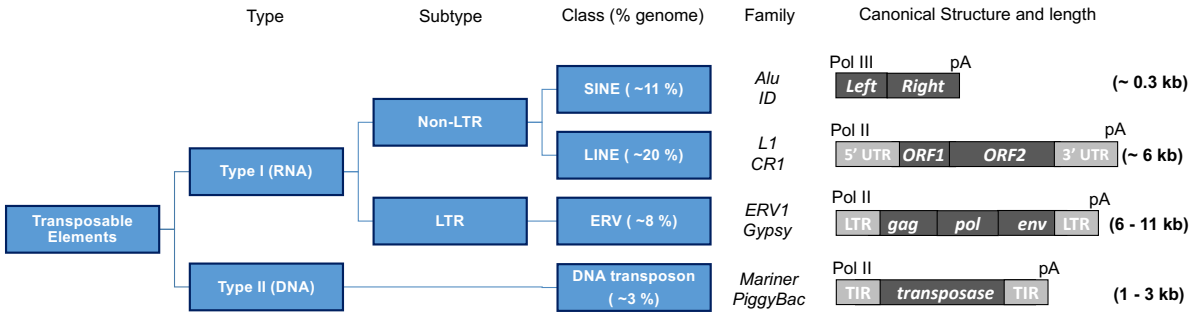

Figure 1.2: Classification and organization of TEs in the human genome. Transposable Elements (TEs) can be divided into type I or type II according to whether they propagate via RNA or DNA intermediates, respectively. Type I TEs are subdivided into Non-LTR and LTR transposons according to structural features. Non-LTR TEs are divided into different classes according to their functional domains, and consequently into different families (2 examples of each were given). Genome percentage is relative to the human genome. Full-length canonical structures are shown with corresponding polymerases and polyadenylation signals. *LTR- Long Terminal Repeat*, *SINE - Short Interspersed Nuclear Element*, *LINE - Long Interspersed Nuclear Element*, *ERV - Endogenous Retrovirus* (adapted from Ishak *et al.*, 2018).

whereas SINEs are ~300 bp long, do not encode proteins, and are transcribed by a RNA polymerase III. Examples of LINEs and SINEs are the *long interspersed nuclear element 1 (LINE-1 or L1)*, a family of LINEs widely present in mammals, and *Alu*, a primate-specific family of SINEs, respectively.

Today, approximately 3, 8, 11, and 20 % of the human genome correspond to DNA transposons, LTR retrotransposons, SINEs, and LINEs, respectively (Lander *et al.*, 2001).

1.4.3 Impact of TEs on genome

1.4.3.1 TEs as mutagens

Though McClintock had previously described TEs as "controlling elements", this idea disappeared by the 60's. At that time, TEs were seen as functionless genomic parasites given their capacity to propagate throughout the genome at the cost of the host and no known selected function. This was also the reason why they were considered to be "selfish DNA" and "junk DNA" (reviewed in Sotero-Caio *et al.*, 2017). The ability to disseminate across the genome provides TEs with an intrinsic propensity to impact the genome at both structural and functional level (reviewed in Seidl and Thomma, 2017). This influence on the genome can range from local instability to large-scale structural variations. Hence, TEs constitute a significant source of mutations in the human genome that can potentially cause diseases. Since REs comprise the majority of TEs in the human genome, we will concentrate on the mutagenic ability of REs.

The mobilization of REs represent a threat to the hosts because it can impact the genome

and the genome structure. Insertional mutagenesis, transduction of flanking sequences, ectopic recombination, modulation of gene expression, and DNA DSBs formation are some of the examples of how REs can reshuffle the genome and alter gene expression (Figure 1.3). Below, we describe in more detail some of these mechanisms, focusing mostly on L1 and Alu effects, which are the most active TEs in the human genome.

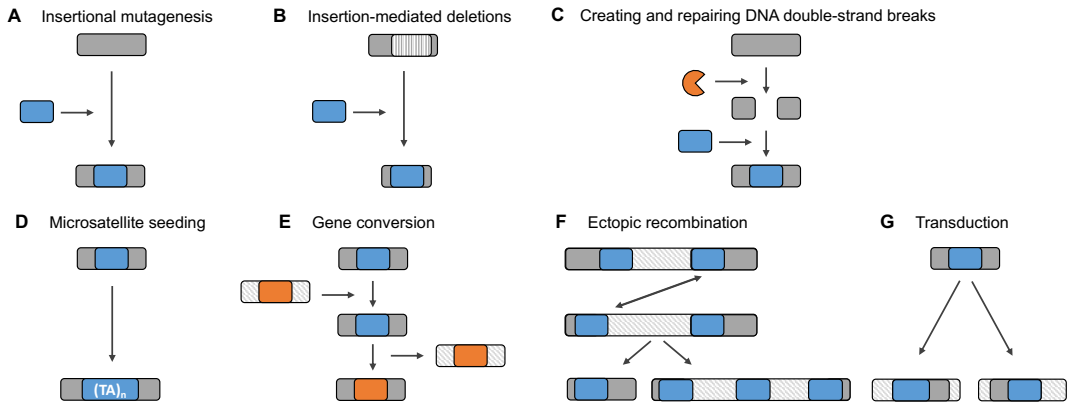

Figure 1.3: Mechanisms through which REs can affect the host genome. Insertion of a TE (blue) into a new location (grey) may cause gene (A) disruption or (B) deletion (light grey) of the site. (C) TE endonuclease (orange) may create and L1 may repair DSBs. (D) Homopolymeric tracts endogenous to retrotransposons may generate microsatellites. (E) Non-reciprocal recombination between homologous transposons (orange and blue), that can lead to TE activation or deactivation.(F) Non allelic recombination may lead to deletions or duplications. (G) During retrotransposition, the flanking regions may be co-retrotransposed with the TE. (reproduced from Cordaux and Batzer, 2009).

REs cause mutations by inserting themselves into functional sequences such as promoters, exons, and enhancers in a process termed insertional mutagenesis (Figure 1.3.A). Such insertions are expected to severely compromise gene function whether it is by interfering with gene transcription, or by interfering with the functionality of a protein. Moreover, the insertion of these elements into the intronic region of a gene can culminate in exon skipping or alternative splicing of that transcript. Besides causing local genomic instability, the insertion of REs into new sites in the genome may produce target site deletions that can range from 1 bp to 130 kb or more (Figure 1.3.B). Even though it happens more rarely than insertional mutagenesis, insertion-mediated deletions do occur and have been reported to occur naturally in the human genome. For example, *L1* integration into the pyruvate dehydrogenase complex – component X - gene led to a 46 kb deletion that culminated in pyruvate dehydrogenase complex deficiency in a human patient (reviewed in Goodier and Kazazian, 2008; Han and Boeke, 2005).

DSBs are among the most deleterious types of genomic damage that can occur in the genome because the DSB-repair mechanisms have a large rate of error. This introduction of errors upon

repair, frequently leads to loss of genetic information and, in some cases, instigates chromosomal rearrangements (Figure 1.3.C) (Longhese *et al.*, 2006). L1 has been linked with the formation of DSBs (Gasior *et al.*, 2006; reviewed in Cordaux and Batzer, 2009; Goodier and Kazazian, 2008). While overexpressing *L1* in mammalian cells, Gasior *et al.* (2006) observed an unexpected increased number of DSBs that did not match the number of real *L1* insertions. This observation suggested that L1 was able to cleave DNA independently of retrotransposition. Furthermore, these results were later shown to be consistent with the findings that *L1* overexpression led to apoptosis and senescence (a state of permanent arrest of the cell cycle) (Belgnaoui *et al.*, 2006; Gasior *et al.*, 2006; Haoudi *et al.*, 2004; Wallace *et al.*, 2008b; reviewed in Goodier and Kazazian, 2008; Richardson *et al.*, 2015). Interestingly, Wallace *et al.* (2008b) showed that open reading frame (ORF) 2 protein (ORF2p), one of the *L1* proteins that has endonuclease activity, alone was sufficient to prevent between 50-60 % of cellular proliferation in human cells. The authors further suggested that the ORF2p-mediated toxicity would be independent of retrotransposition, and that mutation of either the endonuclease or reverse transcriptase domain would lead to a decreased toxicity (Wallace *et al.*, 2008b). Thus, L1 is considered to be one of the sources of DNA DSB formation and, consequently, of genomic instability (reviewed in Cordaux and Batzer, 2009; Goodier and Kazazian, 2008; Richardson *et al.*, 2015).

Post-integration recombination events between genomic retrotransposons are behind the large-scale structural variation in the genome (Figure 1.3.F). The vast copy number of *L1* and *Alu* elements instigate the recombination between non-allelic homologous elements (i.e., ectopic recombination), which can lead to genomic deletions, duplications, inversions or even translocations (reviewed in Cordaux and Batzer, 2009; Goodier *et al.*, 2000; Richardson *et al.*, 2015). Indeed, several studies have identified the occurrence of deletions associated with *Alu*-mediated recombination as the cause of certain cancers and genetic disorders (Callinan and Batzer, 2006). Albeit at a low frequency, deletions associated with L1-mediated recombination have also been reported to cause diseases (reviewed in Cordaux and Batzer, 2009).

Sometimes, the genomic regions that flank REs can be co-retrotransposed with them in a process termed (5' or 3') transduction (Figure 1.3.G). 5' transduction events originate when transcription is initiated from a different promoter which resides upstream of an active non-LTR retrotransposons. Chen *et al.* (2006) proved that 5' transduction events do happen and that it can lead to deleterious effects. Contrarily, due to the presence of inherently weak polyadenylation signals in the 3' untranslated region (UTR) of non-LTR retrotransposons, 3' end processing machinery often bypasses this signal, resulting in the co-transposition of downstream sequences

(3' transduction). Thus, up until 20 % of the time, a second downstream poly(adenine (A)) signal is utilized, leading to the insertion of that 3' flanking genomic DNA into a new chromosomal location (Holmes *et al.*, 1994). Hence, transduction events can lead to exon shuffling and creation of new genes (by mobilizing exons and promoters) (reviewed in Goodier and Kazazian, 2008; Richardson *et al.*, 2015).

1.4.3.2 TEs as an evolutionary driving force

The idea that TEs are important contributors to genetic innovation, and possible drivers of genome evolution was first proposed by Barbara McClintock (Biémont, 2010; McClintock, 1984), but started gaining more widespread acceptance when the human genome reference sequence revealed that half of the human genome was comprised of TEs (Lander *et al.*, 2001). TEs were then considered double-edged swords that despite having a detrimental effect at the individual level by promoting genomic instability, have been a massive driving force in evolution and biodiversity (reviewed in Jurka *et al.*, 2007). For millions of years, TEs have continuously propagated and accumulated in the genome. The presence of stressors and other environmental stimuli or conflicts triggered several outbursts of activity of certain TEs throughout evolution. Those TE outbursts were crucial for their amplification in the genome, and led to the increase in the genome size (reviewed in Horváth *et al.*, 2017). Many of these new insertions were not complete, thus yielding remnants of TEs unable to mobilize (molecular fossils). Several of these fossils are currently being used to determine inter-genetic variation in forensic sciences while others were co-opted by the host to serve their cellular functions (reviewed in Jurka *et al.*, 2007). Below, we describe in more detail some mechanisms through which the mobilization of TEs can be constructive and advantageous to the host cell, focusing mostly on REs.

In some cases, the accumulation of mutations led to the neofunctionalization (*i.e.*, domestication or co-option) of the inserted elements. Certain TEs acquired different functions that often brought phenotypic benefits to the host (Gould and Vrba, 1982; reviewed in Casola *et al.*, 2007; Joly-Lopez and Bureau, 2018). Consequently, the domesticated TEs started evolving under phenotypic rather than self-replicative selection. There are several studies reporting that some TEs were actually co-opted by the host. A clear example of TE neofunctionalization is the mechanism of telomere maintenance in *D. melanogaster*. *Drosophila* does not have telomerase, the enzyme responsible for maintaining telomere length in other eukaryotes, like mammals. Instead, repeated transpositions of *Drosophila* non-LTR REs maintain the chromosome ends, preventing their shortening. Despite being able to mobilize to other places, these REs show a preference for the end of the chromosomes and are rarely found in other genomic regions

(reviewed in Jangam *et al.*, 2017; Pardue and DeBaryshe, 2011a; Pardue and DeBaryshe, 2011b).

Another example whereby TEs have contributed to genome evolution is at the base of the vertebrate adaptive immune system: V(D)J recombination (Jangam *et al.*, 2017; Thompson, 1995). Briefly, V(D)J recombination is the process of somatic recombination that occurs during T and B cell development, where several segments of (Variable, Diversity and Joining) genes are assembled arbitrarily to produce countless unique receptors, capable of recognizing different molecules (reviewed in Lewis, 1994). Recombination activating gene 1 and 2 (RAG1 and RAG2) are the enzymes involved in the segment rearrangement and recombination. The recombination signal sequences that flank the segments are crucial to determine which sequences will be cleaved and joined (reviewed in Lewis, 1994). Both RAGs and the recombination signal sequences are thought to be derived from the transposase and terminal inverted repeats sequences of DNA transposons (Kapitonov and Jurka, 2005; Kapitonov and Koonin, 2015; reviewed in Carmona and Schatz, 2017). Curiously, the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated system (Cas) system, the adaptive immune system of prokaryotes, also seems to result from TE co-option (Jangam *et al.*, 2017).

1.4.4 Regulation of TEs

Given the mutagenic potential and the associated deleterious consequences of a retrotransposition event on the genome, hosts evolved transcriptional and post-transcriptional mechanisms to regulate or even suppress RE activity in both somatic and germ cells (reviewed in Ishak *et al.*, 2018). Although some TE silencing mechanisms in mammals, like the P-element induced wimpy testis (PIWI) pathway, are more specific to the germline, there are others, like DNA and histone methylation, that are also active in somatic cells. The outcome of the cooperation between the different repressing mechanisms is the continuous and dynamic establishment of silencing marks, which are maintained and require the cooperative action of many different proteins (Castro-Diaz *et al.*, 2014; Rowe *et al.*, 2013; reviewed in Slotkin and Martienssen, 2007).

1.4.4.1 Transcriptional REs silencing

Transcriptional strategies of RE silencing include chromatin modifications through DNA methylation and histone modification (reviewed in Cardelli, 2018) and KRAB Zinc-Finger proteins (reviewed in Richardson *et al.*, 2015; Yang and Wang, 2016). We refer to DNA methylation here as the process in which the fifth carbon of a cytosine residue receives a methyl group to form a 5-methylcytosine. In vertebrates, DNA methylation seems to be more restricted to CpG sites, which are repetitive sequences made of dinucleotides of cytosine preceding guanines,

whereas in other eukaryotes, like plants, non-CpG cytosine are also targeted (Holliday and Pugh, 1975; reviewed in Cardelli, 2018). Additionally, DNA methylation is a defense mechanism that promotes the inheritance of TE silencing since the methylation pattern is copied to the new strand upon DNA replication (reviewed in Long *et al.*, 2017). The use of DNA methylation as a TE activity repressing mechanism is conserved in vertebrates, fungi, and some plants (reviewed in Cardelli, 2018). Though invertebrates do not use DNA methylation as a defense mechanism against TEs, they possess other mechanisms that effectively silence these elements (Siomi *et al.*, 2008; Yang and Wang, 2016). The *L1* internal promoter comprises a CpG island which is typically highly methylated (Hata and Sakaki, 1997). In humans, DNA methylation is initially established in primordial germ cells and maintained throughout the life of an organism. The maintenance of this epigenetic repression is thought to control the expression of REs, namely *L1* (and LTR retrotransposons in mice), in somatic tissues and germ cells (reviewed in Richardson *et al.*, 2015; Yang and Wang, 2016).

Just like DNA methylation, some types of histone modifications can promote repressive chromatin states that suppress TE activity (Rowe and Trono, 2011; reviewed in Castro-Diaz *et al.*, 2015). Histone methylation and deacetylation are especially important in tissues where the DNA is hypomethylated, such as in embryonic stem cells (Garcia-Pérez *et al.*, 2010; reviewed in Garcia-Pérez, 2016; Goodier and Kazazian, 2008). Methylation of histone H3 at lysine 9 (H3K9) signals for transcriptionally repressive and inactive chromatin. H3K9 is typically associated with nucleosomes containing TEs. Hence, the occurrence of deleterious mutations in the histone modifying genes result in TE reactivation. An example would be the histone H3K9 methyltransferase gene, *Suv39*, which when mutated, induces TE overexpression in mouse embryonic stem cells (Martens *et al.*, 2005; reviewed in Slotkin and Martienssen, 2007).

Interestingly, the involvement of PIWI proteins and PIWI-interacting RNAs (piRNAs) in the regulation of *de novo* methylation of REs in the animals germline has been suggested (reviewed in Yang and Wang, 2016). Briefly, PIWI proteins are small RNA binding proteins, belonging to the Argonaute family of proteins (reviewed in Castel and Martienssen, 2013; Richardson *et al.*, 2015; Yang and Wang, 2016). PIWI proteins are typically gonad-specific but a somatic role has also been described (reviewed in Castel and Martienssen, 2013; Siomi *et al.*, 2008; Zuo *et al.*, 2016). The RNAs these proteins interact with are typically single-stranded and have between 23 and 31 nucleotides (reviewed in Castel and Martienssen, 2013; Slotkin and Martienssen, 2007; Zuo *et al.*, 2016). Briefly, piRNAs are transcribed from genomic piRNA clusters, exported to the cytoplasm and loaded into PIWI-containing complexes to be used as guides to degrade the complementary TE messenger RNAs (mRNAs) by endonucleolytic processing (reviewed in

Garcia-Pérez, 2016; Goodier and Kazazian, 2008; Richardson *et al.*, 2015; Yang and Wang, 2016). The cleavage of TEs often leads to the formation of additional piRNAs that target the same element (Slotkin and Martienssen, 2007; Yang and Wang, 2016). Although the piRNA pathway is conserved in animals, it operates differently in mice (mammals) and in the fruit fly (reviewed in Castel and Martienssen, 2013). During early development, there is a global derepression of transposons in the mouse male germ line due to DNA demethylation. The DNA methylation pattern is then re-established via the PIWI pathway, in which the piRNAs direct PIWI proteins to transposon enriched regions (reviewed in Castel and Martienssen, 2013). For instance, the mammalian *Rasgfr1* locus, which is usually (DNA) methylated, is located downstream of an LTR retrotransposon whose transcript is targeted by piRNAs (that originate from a genomic piRNA cluster that has a copy of an LTR) during *de novo* methylation in the early development. The LTR is then methylated and thought to facilitate the spreading of methylation to the *Rasgfr1* locus (reviewed in Castel and Martienssen, 2013). Other studies have shown that mice with PIWI proteins mutated exhibit a similar phenotype to mouse lacking a DNA methyltransferase (that promotes the establishment of DNA methylation patterns) (Bourc'his and Bestor, 2004). Besides being unable to undergo meiosis properly, these mice are characterized by DNA hypomethylation of the TE-enriched genomic regions, uncontrolled expression, and propagation of (*L1* and *LTR*) REs in the mouse male germline (Aravin *et al.*, 2008; reviewed in Richardson *et al.*, 2015). Recent studies have shown that in the spermatogonia of adult mice with defects in PIWI proteins, *L1* remain repressed via H3K9 methylation (Di Giacomo *et al.*, 2013; Di Giacomo *et al.*, 2014). In *Drosophila* ovaries, piRNA-mediated silencing of transposons occurs only via H3K9 methylation (Sienski *et al.*, 2012). In the follicle cells, one piRNA cluster is expressed and piRNAs against TEs from the *gypsy* family (LTR), which is very active in *Drosophila* genome, are made. Once the piRNAs interact with the PIWI proteins, there is transcriptional silencing of *gypsy* REs in the nucleus. In the oocyte, on the other hand, there is expression of several piRNA clusters, resulting in the silencing of several active transposons through H3K9 methylation. The presence of PIWI proteins in the nucleus and its interacting RNAs is crucial to the heterochromatinization of these transposons (Klenov *et al.*, 2011, reviewed in Castel and Martienssen, 2013; Yang and Wang, 2016).

1.4.4.2 Post-transcriptional RE silencing

Post-transcriptional mechanisms include the piRNA pathway, small interference RNA (siRNA), microRNA (miRNA), alternative RNA splicing of REs, and premature polyadenylation of REs.

Both RE RNA splicing and premature polyadenylation are considered to be secondary mechanisms of REs silencing and will not be the focus of this section.

Besides targeting TE-enriched DNA regions for DNA and histone methylation, the piRNA pathway also mediates the cleavage of active transposon transcripts, namely in flies (Zhang *et al.*, 2015; reviewed in Castel and Martienssen, 2013; Levin and Moran, 2011; Slotkin and Martienssen, 2007; Yang and Wang, 2016). As mentioned in the previous section (subsubsection 1.4.4.1), the degradation process allows the formation of additional piRNAs, which in turn enables the perpetuation of this effective transposon defense cycle. This cycle is termed "ping-pong" amplification cycle (reviewed in Richardson *et al.*, 2015). In *Drosophila*, the disruption of piRNA pathways leads to derepression of retrotransposons in the male germline (reviewed in Goodier and Kazazian, 2008). Moreover, the deletion of RNAs in a piRNA cluster in mice resulted in the overexpression of *L1* in the spermatocytes (Xu *et al.*, 2008; reviewed in Goodier and Kazazian, 2008).

Another class of small RNAs involved in the silencing of TEs is the siRNA. In *Drosophila*, siRNAs are called endogenous short interfering RNAs (esiRNAs) and are involved in somatic tissue silencing of TEs (Li *et al.*, 2013; Siomi *et al.*, 2008). Unlike piRNAs, esiRNAs are 21 nucleotides long and are ubiquitously expressed. The bidirectional transcription of retrotransposons (and other repetitive sequences) leads to the formation of double-stranded RNAs whose sense and antisense strands originate esiRNAs in a manner dependent on Dicer2, a double stranded RNA ribonuclease (Ghildiyal *et al.*, 2008; Siomi *et al.*, 2008). Once assembled to the specific PIWI protein, esiRNAs target the transcript of the target transposon for cleavage and degradation (Siomi *et al.*, 2008). Moreover, endogenous siRNAs also silence transposons in mice and humans, since the knockout of the homolog *Dicer* in both of them leads to the overexpression of certain REs (like *L1*) in the mouse ovaries, HeLa cells, and HEK cells (Watanabe *et al.*, 2008; Yang and Kazazian, 2006).

An additional mechanism of TE regulation through small RNA is carried out by miRNAs. The *microprocessor* is a nuclear complex, that recognizes the primary miRNA (pri-miRNA) with RNA hairpin structures, cleaves, and processes the pri-miRNA to pre-miRNA (reviewed in Garcia-Pérez, 2016). After being exported to the cytoplasm, the pre-miRNA is further cleaved into mature miRNAs which will direct argonaute proteins - and the associated complex - to the target sequence in cellular RNAs. Consequently, these RNAs are either degraded or untranslatable. *In vitro* studies have shown that the *microprocessor* possesses the ability to recognize structural RNA domains in and bind specifically to *L1* and *Alu* transcripts and cleave them (reviewed in Garcia-Pérez, 2016; Goodier and Kazazian, 2008; Richardson *et al.*, 2015).

Together, all these mechanisms constitute a complex yet very successful network of repressors that control the mobilization of TEs in both germ and somatic cells.

1.4.5 Active TEs in the genome

1.4.5.1 Active TEs in *Drosophila melanogaster* (fruit fly)

TEs constitute around 23 % of the *D. melanogaster* genome. This represents more than 36,810 copies of approximately 100 different TEs (Mackay *et al.*, 2012; reviewed in Barrón *et al.*, 2014; McCullers and Steiniger, 2017; Petrov *et al.*, 2003). However, only around one-third of these are thought to be full length and active due to the accumulation of errors introduced during replication and integration of truncated elements (Hoskins *et al.*, 2002; Kaminker *et al.*, 2002). The active elements in the *Drosophila* genome encompass DNA transposons (16 %), LTR retrotransposons (45 %), and non-LTR retrotransposons (21 %) (Kaminker *et al.*, 2002; reviewed in McCullers and Steiniger, 2017). Elements from all these classes have been shown to be active and mobile not only in the fly germline, but also in somatic tissues like the brain (Li *et al.*, 2013; Perrat *et al.*, 2013). Among the TEs that are expressed and mobile in the brain are the DNA transposons *P-element* and *transib*, the LTR retrotransposons *gypsy*, *copia*, and *roo* (being *roo* the most abundant TE in the fly genome), and the LINE-like elements (non-LTR retrotransposons) *I-element* and *R2* (Hoskins *et al.*, 2002; Kaminker *et al.*, 2002; Li *et al.*, 2013; Perrat *et al.*, 2013; reviewed in Waddell *et al.*, 2014). Cell type-specific gene expression profiling data showed that even though REs are expressed all over the brain, their expression is substantially higher in a particular subset of neurons in the mushroom body (Perrat *et al.*, 2013). Deep sequencing experiments further revealed that the expressed REs are mobile and result in *de novo* insertions all over the brain. However, there seems to be a higher frequency of the mobilization of these elements in the mushroom body-specific neurons and about half of those insertions were near identified genes (Perrat *et al.*, 2013). The authors further showed that the piRNA-mediated RE silencing pathway, that represses REs in the fly germline, was also active in the brain, albeit at reduced levels in the mushroom body-specific neurons, thus explaining the increased expression of REs in those neurons. Some non-LTR elements are also known to be ubiquitously expressed and mobile given their involvement in *Drosophila* chromosomes telomere maintenance (reviewed in Pardue and DeBaryshe, 2011b).

1.4.5.2 Active TEs in *Mus musculus* (mouse)

Unlike what happens in *D. melanogaster*, active TEs in mammals are restricted to specific elements of certain classes. TEs constitute 40 % of the mouse genome (Waterston *et al.*, 2002).

REs are the only known active TEs in the mouse genome. The active REs includes elements from both LTR and non-LTR retrotransposons. Among the LTR retrotransposons, the most successful LTR element is the still active MaLR (with 388,000 copies). Other abundant and very active LTR retrotransposons are the intracisternal-A particles (abbreviated as IAP) and the early-transposons (ETn). In fact, almost 15 % of spontaneous mouse mutants have one of these elements inserted in an allele (Waterston *et al.*, 2002). The active non-LTR retrotransposons are the *L1* (599,000 *L1* derived sequences, comprising ~19 % of the mouse genome) and four different family of SINEs (in a total of 1,498,000 copies corresponding to ~8 % of the mouse genome) (Waterston *et al.*, 2002). It is estimated that of the more than 599,000 *L1s*, only around 3,000 are full-length and competent for retrotransposition (Goodier *et al.*, 2001; Waterston *et al.*, 2002).

1.4.5.3 Active TEs in *Homo sapiens sapiens* (human)

Although approximately 45 % of the human genome is occupied by TEs or TE-derived sequences, only a few elements remain active (Lander *et al.*, 2001; Mills *et al.*, 2006; Waterston *et al.*, 2005). Similarly to the mouse genome, almost every TEs in the human genome are either DNA transposons (~3 %), LTR retrotransposons (~8 %), and non-LTR retrotransposons (~35 %) (Lander *et al.*, 2001). DNA transposons are currently thought to be inactive and incapable of mobilizing. Albeit recent studies reported transposition events of a specific subfamily of LTR retrotransposons, it is believed that LTR elements in general are on a path to extinction. The most active elements in the human genome are *L1* (LINE, ~17 %) and *Alu* (SINE, ~11 %) (Lander *et al.*, 2001; Mills *et al.*, 2007). Several studies have shown that REs are active in the human genome in somatic and germline cells (Akagi *et al.*, 2008; Belancio *et al.*, 2010; Coufal *et al.*, 2009; Dewannieux *et al.*, 2003; Evrony *et al.*, 2012; Halling *et al.*, 1999; Kazazian *et al.*, 1988; Moran *et al.*, 1996; Muotri *et al.*, 2005; Upton *et al.*, 2015; reviewed in Mills *et al.*, 2007). Below we describe *L1* and *Alu* elements in more detail.

1.4.5.3.1 *LINE-1 (L1)* retrotransposons

L1s have been present in mammalian genomes for more than 160 million years and it is speculated that L1 had a key role in the diversification and evolution of this group of animals (reviewed in Richardson *et al.*, 2015). Indeed, there was a burst of L1 activity in the human genome after the divergence of the ancestral mouse and human lineages (around 65-75 million years ago) (Lander *et al.*, 2001). This burst led to a substantial increase in the number of *L1*-derived sequences present in the human genome. Today, more than 500,000 copies of *L1* account for 17 % of the human genome (Lander *et al.*, 2001, reviewed in Faulkner and Garcia-Perez,

2017). Of the more than 500,000 *L1*-derived sequences, only 80 to 100 (~0.02 %) are functional and capable of accomplishing retrotransposition (retrotransposition competent (RC)). The remaining copies (~99.8 %) either accumulated mutations or are truncated, and therefore are incapable of undergoing retrotransposition events (Brouha *et al.*, 2003; Hardies *et al.*, 1986; Voliva *et al.*, 1983). For this reason, they are considered molecular fossils. Since these estimates are based solely on the euchromatic portion of the genome, the number of *RC-L1s* is likely underestimated (Brouha *et al.*, 2003; Lander *et al.*, 2001).

Every known *RC-L1* belongs to one out of the sixteen *L1* subfamilies, the subfamily PA1, and because they are human-specific *L1s*, they have been termed *L1Hs*. The majority of the currently active *L1Hs* belongs to a specific small subset of *L1s*, denominated Transcribed group a subset (Ta-subset) (Beck *et al.*, 2010; Boissinot *et al.*, 2000; Huang *et al.*, 2010; Iskow *et al.*, 2010; Skowronski *et al.*, 1988). In fact, the bulk of retrotransposition in the human population is carried out by only 8 to 10 % of the RC-*L1s* and practically all of them belong to the Ta-subset (Brouha *et al.*, 2003; Sassaman *et al.*, 1997; Skowronski *et al.*, 1988). These highly active elements are referred to as "hot" *L1s* (Brouha *et al.*, 2003). Functional studies performed by Beck *et al.* (2010), Iskow *et al.* (2010), and Huang *et al.* (2010) in which they used different approaches to map the human *L1s* further corroborated the Ta-subset *L1s* as the main responsible for the bulk of retrotransposition. A typical *RC-L1s* is 6 kb long and encompasses a 5'UTR, in which resides an internal promoter with a CpG island, two non-overlapping ORFs, and a 3'UTR (Figure 1.2). The two ORFs, *ORF1* and *ORF2*, are 63 bp apart from one another.

L1 ORF1 and *ORF2* encode different proteins, ORF1p and ORF2p, which are crucial for the retrotransposition process of *L1*. ORF1p is a 40-kDa RNA-binding protein that binds to *L1* transcripts, stabilizing them, whereas ORF2p is a 150-kDa multifunctional protein with endonuclease and reverse transcriptase activities. Together, these two proteins promote the retrotransposition of *L1 in cis* through a process designated target primed reverse transcription (TPRT) (reviewed in Goodier and Kazazian, 2008; Thomas *et al.*, 2012).

L1 retrotransposition initiates with transcription of *L1* and transportation of the mRNA to the cytoplasm where translation of ORF1p and ORF2p occurs. Once translated, ORF1p and ORF2p bind to the *L1* mRNA molecule from which they were coded, *in cis*, to compose a ribonucleoprotein particle (RNP) complex. Then the RNPs enter the cell nucleus and reach the gDNA, where the TPRT integration process begins. In TPRT, the endonuclease domain of the ORF2p recognizes and nicks the target DNA, generating a free 3'-hydroxyl end that is used by the reverse transcriptase domain to prime reverse transcription of *L1* mRNA, and, consequently, integrate *L1*. This mechanism of retrotransposition generates a small target-site duplication of

7-20 bp that flanks the new insertions. Reverse transcription frequently terminates prematurely through a pathway termed abortive retrotransposition and results in the formation of many (5'-) truncated and nonfunctional insertions (Gilbert *et al.*, 2005). According to Lander *et al.* (2001), the majority of LINE-derived repeats are shortened, being on average only 1 kb long. Even though this is one of the most well studied mechanisms of retrotransposition, there are still details that remain unclear, such as the mechanism through which the RNP complexes enter the cell nucleus and the exact function of ORF1p in retrotransposition (reviewed in Thomas *et al.*, 2012).

L1 was first shown to be active and able to retrotranspose in neurons in a pioneer work by Muotri *et al.*, 2005, in which rat hippocampus neural stem cells were transfected with an engineered human *L1* carrying a retrotransposition reporter cassette and then differentiated into neural progenitor cells. Additionally, the ability of L1 to retrotranspose in neurons was further corroborated by *in vivo* studies performed in mice. This work demonstrated not only that L1 is capable of inserting *de novo* copies in the genome of hippocampal neurons during development, but also that the new insertions can affect the neuronal expression of neuronal genes, and therefore influentiate the neuronal cell fate (Muotri *et al.*, 2005). Interestingly, Muotri *et al.* (2005) also revealed that *L1* transcription seems to inversely correlate with the presence of an *L1* repressor, Sox2. During a certain period in the course of neuronal differentiation, *Sox2* expression is decreased, chromatin undergoes remodelling, and *L1* is expressed. Since then, several studies have corroborated not only that *L1* retrotransposes in the central nervous system (CNS), but also in other somatic tissues, albeit at a lower frequency (Baillie *et al.*, 2011; Belancio *et al.*, 2010; Coufal *et al.*, 2009; Evrony *et al.*, 2012; Evrony *et al.*, 2015; Hazen *et al.*, 2016; Macia *et al.*, 2017; Muotri *et al.*, 2009; Muotri *et al.*, 2010; Sur *et al.*, 2017; Upton *et al.*, 2015).

1.4.5.3.2 *Alu* retrotransposons

There are three different families of SINEs in the human genome, albeit only one, the *Alu* family, is currently active (Lander *et al.*, 2001). *Alu* elements, named after an internal *AluI* restriction enzyme recognition site, are primate-specific SINEs that have been present in the hominid lineage for the past 65 million years (reviewed in Batzer and Deininger, 2002). A burst in the retrotransposition of *Alu* elements occurred 40 million years ago and contributed for approximately 80 % of the *Alu* copy number currently present in the human genome. With more than 1,100,000 copies in the human genome (corresponding to ~10 % of the human genome), *Alu* elements are the most successful family of TEs in the human genome (reviewed in Ade *et al.*, 2013; Batzer and Deininger, 2002; Cordaux and Batzer, 2009; Lander *et al.*, 2001). However, not every *Alu* is capable of undergoing retrotransposition mainly due to the accumulation of

mutations that influence its RNA structure and protein binding capacity (Bennett *et al.*, 2008; Deininger, 2011).

Alu elements can be classified into three major subfamilies, J, S, and Y, according to their evolutionary age. *Alu Y* is the youngest and the only active subfamily in the human genome, accounting with being responsible for the bulk of *Alu* retrotransposition in the human genome (Ade *et al.*, 2013; Bennett *et al.*, 2008). Besides being the most dominant *Alu* elements, *Alu Ya5* and *Alu Yb8* are highly polymorphic and therefore contribute for the diversification of human population (Ade *et al.*, 2013; Bennett *et al.*, 2008; Burns and Boeke, 2012).

A typical human *Alu* is a 280 bp dimer, which is ancestrally derived from *7SL RNA* (Ullu and Tschudi, 1984; reviewed in Deininger, 2011). *7SL RNA* is part of the signal recognition particle, a universally conserved ribosome-protein-RNA complex involved in protein sorting (Walter and Blobel, 1982). Human *Alu* elements are composed by a left and a right monomer divided by a short centrally located A-rich sequence (reviewed in Ade *et al.*, 2013; Burns and Boeke, 2012; Cardelli, 2018; Deininger, 2011; Richardson *et al.*, 2015). *Alu* elements also have a poly(A) region in their 3' terminal sequence, which can influence not only their expression, but also their ability to be retrotransposed (Roy-Engel *et al.*, 2002). The left monomer comprises two conserved sequences named box A and box B that correspond to internal RNA polymerase III binding sites, constituting an internal RNA polymerase III promoter. (reviewed in Ade *et al.*, 2013; Burns and Boeke, 2012; Cardelli, 2018; Richardson *et al.*, 2015).

Alu elements, and SINEs in general, are non-autonomous retrotransposons because they do not encode the proteins necessary to mediate their retrotransposition. Therefore, *Alu* elements depend on other retrotransposons proteins, *L1* proteins in particular, to be retrotransposed. Although *L1* ORF1p enhances the retrotransposition efficiency of *Alu*, ORF2p is sufficient to promote new insertions of *Alu* in the genome (Dewannieux *et al.*, 2003; Wallace *et al.*, 2008a). Once transcribed, *Alu* mRNAs assemble into ribonucleoprotein complexes that are thought to help direct and promote the association between *Alu* mRNAs and ribosomes (Ade *et al.*, 2013; Deininger, 2011). The *Alu* complexes encompass the binding of the protein heterodimer SRP9/14 and polyA-binding proteins (PABPs) (reviewed in Deininger, 2011). If any of the *Alu* mRNA associated ribosomes translates an *L1* mRNA, *Alu* mRNA would have privileged access to ORF2p as soon as it finishes being translated. Hence, upon *ORF2* translation from *L1* mRNAs, *Alu* mRNAs hijack the ORF2p to mediate their retrotransposition *in trans*. Like *L1*, *Alu* elements integrate the genome via TPRT process and are also flanked by target-site duplications (reviewed in Ade *et al.*, 2013; Deininger, 2011).

Similarly to *L1*, *Alu* elements have also been shown to be active and able to be retrotransposed (Wallace *et al.*, 1991; reviewed in Mills *et al.*, 2007). Indeed, *Alu* has been established as the cause of many diseases in human patients, as reviewed in Belancio *et al.*, 2008.

In the next section we will focus on recent studies reporting how REs relate with ageing and with neurodegenerative diseases. We will focus mostly on REs given their higher abundance in the human genome.

1.5 Active REs and Disease

1.5.1 REs and ageing

Despite the tight repression that REs are under, there has been accumulating evidence that REs are capable of mobilizing (i.e., generating *de novo* insertions) not only in germline cells, but also in somatic tissues that include brain, liver, and fat (Barbot *et al.*, 2002; Krug *et al.*, 2017; Li *et al.*, 2013; Muotri *et al.*, 2005; Wood *et al.*, 2016; reviewed in Cardelli, 2018; Dubnau, 2018). In fact, several studies have reported that this ability to transpose increases with age (Barbot *et al.*, 2002; Chen *et al.*, 2016; De Cecco *et al.*, 2013a; De Cecco *et al.*, 2013b; Dennis *et al.*, 2012; Hu *et al.*, 2014; Lee *et al.*, 2012; Li *et al.*, 2013; Maxwell *et al.*, 2011; Patterson *et al.*, 2015; Van Meter *et al.*, 2014), and that it mainly results from the progressive weakening of the REs repression mechanisms (Chen *et al.*, 2016; Wood *et al.*, 2016; reviewed in Dubnau, 2018).

The association between ageing and REs derepression (or increased expression) has been verified in various organisms like budding yeasts (Hu *et al.*, 2014; Maxwell *et al.*, 2011; Patterson *et al.*, 2015), worms (Dennis *et al.*, 2012), flies (Chen *et al.*, 2016; Li *et al.*, 2013; Wood *et al.*, 2016), mice (Barbot *et al.*, 2002; De Cecco *et al.*, 2013b; Dupressoir *et al.*, 1995; Lee *et al.*, 2012; Van Meter *et al.*, 2014), and humans (De Cecco *et al.*, 2013a).

Studies in the chronological ageing model *Saccharomyces cerevisiae*, revealed not only that the expression of the yeast RE, *Ty1*, was increased in older populations of yeast, but also that reduction of *Ty1* expression or repression of its activity would lead to a decrease in age-related accumulation of genetic damage (or instability) (Maxwell *et al.*, 2011). This association between ageing and increase in the RE expression was further verified in another yeast model of ageing (Hu *et al.*, 2014; Patterson *et al.*, 2015).

Similar associations between increased RE expression (or activity) and age have been reported in invertebrates. For instance, while looking for virus-like particles through electron microscopy in *C. elegans*, Dennis et al. (2012) showed that the RE, *Cer1*, is activated with age. In *Drosophila*, an RNA deep sequencing approach validated the same trend of increased RE

expression with age in the fat body (a tissue similar to the mammalian liver and adipose tissue) and correlated this increase with a concomitant reduction in heterochromatin in old *Drosophila* individuals (Chen *et al.*, 2016; Wood *et al.*, 2016). It was also documented that RE expression correlated with increased DNA damage and decreased lamin-B expression, which was shown to induce heterochromatin loss and a consequent increase in both RE expression and DNA damage in young individuals (Chen *et al.*, 2016). Additionally, Wood et al. (2016) were able to show direct evidence of the increased RE activity, namely, increased *de novo* insertions with ageing by using a *Gypsy*-trap reporter.

In mice, Barbot *et al.* (2002) and Dupressoir *et al.* (1995) demonstrated, using northern blot and reverse transcriptase quantitative PCR (qPCR) approaches, that liver-specific *IAP* levels were substantially increased in aged mice. Barbot *et al.* (2002) further showed that the specific activation of *IAP* followed a circadian pattern that was dependent on the activation - through DNA demethylation - of the gene in which *IAP* was inserted. The repeated activation of *IAP* throughout mice lifespan would lead to a progressive demethylation and increased expression of *IAP* in aged mice.

Genome-wide chromatin conformation studies performed in human cells have revealed that older cells display characteristic heterochromatin alterations when compared to younger cells: the DNA regions that are normally under a transcriptionally repressive state (gene-poor regions like intergenic regions, where many REs reside, and centromeres), become less repressed (De Cecco *et al.*, 2013a). This loosening of heterochromatin in REs (*L1* and *Alu*) sequences results in the increased expression of those elements, as assayed by RT-qPCR (De Cecco *et al.*, 2013a). The same authors further corroborated the obtained results by demonstrating that aged mice present higher levels of both *L1* (and other REs) mRNA and genomic copy number in their liver (De Cecco *et al.*, 2013b).

Since the increase in RE expression with age is widely conserved among such distant taxa, it has been suggested that RE expression, and RE derepression, is a conserved feature of ageing (reviewed in Cardelli, 2018; Dubnau, 2018; Maxwell, 2015). However, whether RE derepression is a cause (contributes for) or a consequence (a by-product) of the age-dependent loss of cellular function remains unclear.

On the one hand, it is known that the ageing process at the cellular level is accompanied by an extensive chromatin structure remodeling (reviewed in Cardelli, 2018; Han and Brunet, 2012; López-Otín *et al.*, 2013; Maxwell, 2015; Wood and Helfand, 2013). There is also a decrease in the activity and efficacy of the posttranscriptional gene regulators, which includes transcriptional factors and other RNA-based pathways (reviewed in Maxwell, 2015; Wood

and Helfand, 2013). As mentioned before, RE silencing mechanisms encompass DNA and histone methylation, which involve chromatin remodeling, and small RNA-based mechanisms. Furthermore, it is established that environmental stimuli and stress conditions can influence and trigger RE activity and the respective silencing mechanisms (Cardelli and Marchegiani, 2013; Horváth *et al.*, 2017; Mori *et al.*, 2012). Mori *et al.* (2012) demonstrated an age-dependent decrease of Dicer (a protein involved in the small RNA-pathway) expression and miRNAs production in mice. Interestingly, in vitro experiments also revealed downregulation of *Dicer* upon stress conditions (DNA damage and reactive oxidative species). Therefore, it has been hypothesized that increased RE expression with ageing could be the outcome of the gradual relaxation and corruption of the RE silencing mechanisms as a result of the age-associated functional and cellular decline (reviewed in Cardelli, 2018; Maxwell, 2015).

Research involving the longevity regulating protein, SIRT6, *L1*, and mice revealed not only an additional role for SIRT6 in silencing *L1* activity through the formation of highly transcriptionally repressive heterochromatin in the *L1* 5'UTR, but also the stress- and age-related gradual failure of the SIRT6-mediated repression of *L1* (Van Meter *et al.*, 2014). SIRT6 is an enzyme known to promote DNA damage resistance and genomic instability suppression (Mostoslavsky *et al.*, 2006). Van Meter *et al.* (2014) observed that SIRT6 was redistributed in response to either genotoxic stress (which caused DNA damage and genomic instability) and ageing. The redistribution of SIRT6 to other loci would lead to upregulation of *L1* activity (Van Meter *et al.*, 2014). The unconstrained expression of *L1* could further compromise the healthy ageing process by exacerbating the genomic instability and damage present in the ageing cells (Van Meter *et al.*, 2014; reviewed in Maxwell, 2015).

REs are a known source of DNA damage (Belgnaoui *et al.*, 2006) and, consequently of genomic instability, inducing toxic effects at a cellular level (Tan *et al.*, 2011; Tarallo *et al.*, 2012; reviewed in Maxwell, 2015). There are several studies that suggest that REs may contribute to the ageing process rather than being a consequence of it. Dietary restriction in yeast, worms, and flies has been reported to significantly increase the healthy lifespan of those animals (reviewed in Fontana *et al.*, 2010). However, the mechanisms behind this process are unknown. Recently, Wood *et al.* (2016) reported that besides extending the healthy lifespan of flies, dietary restriction also suspends the age-associated expression of REs and the rate at which these elements mobilize. These results suggest that RE expression and ageing are strongly related and maybe intertwined. Moreover, interfering with the production of proteins involved in the small RNA-based pathway of RE silencing (in somatic tissues), like the PIWI protein AGO2 in flies, leads to an altered pattern of RE expression and lifespan (Li *et al.*, 2013). Namely, *AGO2* mutant

flies revealed a premature desilencing of REs, memory impairment (which was rescued with the ectopic expression of an *AGO2* transgene), and a shortened lifespan (Li *et al.*, 2013). Taken together the evidence described above is consistent with the hypothesis that RE activation leads to the age-dependent decline in health (reviewed in Cardelli, 2018; Dubnau, 2018; Fontana *et al.*, 2010; Maxwell, 2015).

Interestingly, it has been established that in what concerns retrotransposition events in somatic tissues, brain is the tissue where RE mobilization occurs with the highest frequency (Baillie *et al.*, 2011; Coufal *et al.*, 2009; reviewed in Solyom and Kazazian, 2012). Indeed, unconstrained RE activity has been hypothesized to contribute to the development of brain disorders, such as schizophrenia and Rett syndrome (Bundo *et al.*, 2014; Guffanti *et al.*, 2016; Muotri *et al.*, 2010). In the following section, we explore more deeply the link between RE derepression and the development of neurodegenerative diseases.

1.5.2 REs and neurodegeneration

REs and their unrestricted activity have been associated with neuronal decline and many ageing-related diseases, namely neurodegenerative diseases. Indeed, the nervous system seems to be a hotspot for RE derepression, as suggested by several reports associating several neurodegenerative disorders with derepressed REs like LTR, SINEs (*Alu* and *Alu*-like), and LINEs (*L1* and *LINE*-like) (Table 1.1) (Li *et al.*, 2013; reviewed in Dubnau, 2018; Thomas *et al.*, 2012). However, whether or not RE derepression is a cause or consequence of the neuronal dysfunction remains unclear.

Besides the direct mutagenic role associated with TEs mobilization, TEs may also trigger strong neuroinflammatory responses that worsen the pathogenicity of the diseases (reviewed in Kassiotis and Stoye, 2016). Indeed, the accumulation of *Alu* RNAs has been shown to provoke an immune response that leads to the degeneration of the retinal pigmented epithelium in age-related macular degeneration, which consequently leads to blindness (Tarallo *et al.*, 2012). Interestingly, knockdown of *DICER1*, a miRNA-processing enzyme, induces accumulation of *Alu* and *Alu*-like SINEs and degeneration of human and mouse retinal epithelium, respectively (Kaneko *et al.*, 2011). Subsequent studies performed by Tarallo *et al.*, 2012 revealed that in the absence of DICER1, *Alu* transcripts are not cleaved and degraded. Therefore, the *Alu* transcripts accumulate in the cell and activate the NLRP3 inflammasome, thereby inducing a toxic response that culminates with cell apoptosis.

The involvement of REs in neurodegeneration is further corroborated by several evidence

Table 1.1: Evidence of association between neurodegenerative diseases and TEs.

Disease	Transposable elements	Disease genes	Models	References
Age-related Macular Degeneration	Accumulation of *Alu* mRNAs induce an immune response that leads to inflamation and cell apoptosis.	*DICER1*	human, mice	Kaneko *et al.*, 2011; Tarallo *et al.*, 2012
Aicardi-Goutiéres syndrome (AGS)	ADAR1 primarily edits *Alus* in RNA Pol II transcribed mRNAs, ADAR1 knockout neuronal progenitor cells exihibit spontaneous interferon production, inhibition of mRNA translation, and apoptosis; *L1* activity is upregulated in several models with AGS-related mutations.	*ADAR1* (1q), *SAMHD1*, *TREX1*, *IFIH1*	human, mice	Chung *et al.*, 2018; Zhao *et al.*, 2013
Alzheimer's disease	Increased activation of *L1* and human endogenous retroviruses (HERVs)s in 638 human brains from Alzheimer patients; Expression of the human *Tau* gene in the fly brain resulted in the global activation of TEs and its associated genomic instability.	*MAPT*, *APP*, *PSEN1* (14q),	human, flies	Guennec *et al.*, 2017; Guo *et al.*, 2018
	1 patient with *Alu*-mediated *PSEN1* exon 9-10 deletion.	*PSEN2*		
Amyotrophic Lateral Sclerosis - Frontotemporal dementia (ALS-FTD)	Increased expression of HERVs and LINEs resulted in neurotoxic effect and led to motor neurodegeneration in mice.	*C9ORF72* (9p)	human, mice	Li *et al.*, 2012; Prudencio *et al.*, 2017
Sporadic Amyotrophic Lateral Sclerosis (ALS)	Increased expression of HERVs (and other REs) in brain tissues of ALS patients; forced expression of *TDP-43* in the fly brain resulted in activation of *gypsy* (and other REs), thus resulting in the neurodegenerative phenotype in the flies.	*TDP-43*	human, mice, flies	Douville *et al.*, 2011; Krug *et al.*, 2017; Li *et al.*, 2015
Amyotrophic Lateral Sclerosis type 12	5 patients with different types of *Alu*-mediated *OPTN* exon deletions.	*OPTN* (10p)	human	Iida *et al.*, 2012; Maruyama *et al.*, 2010
Ataxia telangiectasia	*Alu*-mediated exonization in *ATM* intron 20; increased genomic *L1* copy number in hippocampi from ataxia telangiectasia patients.	*ATM* (11q)	human, mice	Coufal *et al.*, 2011; Pagani *et al.*, 2002
Epilepsy	5 patients with *Alu*-mediated rearrangement of portions of *ALDH7A1* ; 3 patients with Alu-mediated CDKL5 deletions of various sizes.	*ALDH7A1* (5q), *CDKL5* (Xp)	human	\cite{Mefford2015, Erez2009}
Fragile X-associated tremor ataxia syndrome (FXTAS)	Excess rCGG repeats induce the activation of gypsy in flies. Knockdown of *gypsy* in flies with FXTAS rescued the WT phenotype.	*FMR1*	flies	Tan *et al.*, 2011
Friedreich ataxia	Expanded GAA triplet repeat in central *Alu* linker located in *FXN* intron 1 with allelic suppression.	*FXN* (9q)	human	Clark *et al.*, 2004
Parkinson's disease	1 patient with *Alu*-mediated *PARK2* exon 10 deletion; 1 patient with *Alu*-mediated DJ1 exon 1-5 deletion.	*PARK2* (6q),	human, mice	Bonifati *et al.*, 2003;
	L1 inhibition in mice under oxidative stress partially prevent the neurodegenerative phenotype.	*DJ1* (1p)		Morais *et al.*, 2016; The *et al.*, 2017
	L1 inhibition in mice under oxidative stress partially prevent the neurodegenerative phenotype.			
Rett Syndrome•	Increased rates of *L1* retrotransposition in mouse models and human patients.	*MeCP2*	human, mice	Muotri *et al.*, 2009
Schizophrenia	Increased genomic *L1* copy number in the prefrontal cortex of patients, iPSCs with 22q11 deletion, and mouse and macaque models.		human, mice, macaque	Bundo *et al.*, 2014
Multiple Sclerosis (MS)	Association of HERVs in brain tissue of MS patients.		human	Morandi *et al.*, 2017

• Rett Syndrome is considered a neurodegenerative disorder and not a neurodegenerative disease. This table was adapted from Krestel and Meier, 2018

regarding human LTR retrotransposons, namely HERV (Table 1.1) (Douville *et al.*, 2011; Li *et al.*, 2012; Li *et al.*, 2015; Morandi *et al.*, 2017; Prudencio *et al.*, 2017). HERV expression has been associated with multiple sclerosis, a chronic demyelinating disease of the CNS that ultimately leads do neurodegeneration and other progressive neurodegenerative diseases like amyotrophic lateral sclerosis (AML) and frontotemporal lobar degeneration (FTLD) (Douville *et al.*, 2011; Li *et al.*, 2012; Li *et al.*, 2015; Prudencio *et al.*, 2017; reviewed in Morandi *et al.*, 2017). Recent evidence from meta-analysis and polymerase chain reaction (PCR)-based studies revealed that TDP-43 protein interacts and binds to the LTR sequences of REs (Li *et al.*, 2012; Li *et al.*, 2015). TDP-43 protein is commonly found in cytoplasmic inclusions of individuals with both AML and FTLD (reviewed in Waddell *et al.*, 2014). The involvement of REs was further corroborated by a recent study in which the human *TDP-43* gene was introduced in *Drosophila* genome (Krug *et al.*, 2017). Forced expression of *TDP-43* in *Drosophila* neurons and glia induced RE expression and recapitulated several neurodegenerative characteristics seen in human AML patients (Krug *et al.*, 2017). Genetic repression of *gypsy*, one of the REs induced by forced *TDP-43* expression, resulted in a reduced neurotoxic effect, suggesting that REs contribute to TDP-43-mediated toxicity. The pharmacological inhibition of retrotransposition also led to the same phenotype. In the same study, Krug *et al.* (2017) revealed that *TDP-43* expression in *Drosophila* triggers the expression of *gypsy* by disrupting siRNA-mediated silencing of REs. This derepression ultimately leads to the DNA damage-mediated programmed cell death. Higher expression of REs was also found in *post mortem* brains of FTLD patients (Li *et al.*, 2012; Prudencio *et al.*, 2017).

Studies in mouse models of Parkinson's disease reported that induction of acute oxidative stress in post-mitotic nerve cells instigates *L1* expression, the formation of DNA DSBs, and cell death (The *et al.*, 2017). By using either the reverse transcriptase inhibitor, stavudine, or a siRNA against *ORF2* in animals under oxidative stress, The *et al.* (2017) were able to partially prevent the neurodegenerative phenotype. Together, these findings suggest that *L1* overexpression has a role in the stress-induced neuronal death.

All these studies corroborate a direct and active role of REs in the neuronal function decline seen in several neurodegenerative diseases. However, none of the studies performed until now was able to clearly determine whether RE expression (and activity) is a cause or a consequence of the neurodegenerative toxicity. Nevertheless, research on neurodegenerative diseases related with TDP-43 proteins seems to support the idea that REs are behind the development of sporadic forms of such diseases (Krug *et al.*, 2017).

Furthermore, research performed in mice also suggest that L1-mediated genomic instability

might trigger cell death in age-related diseases, namely diseases involving neurodegeneration, since the reduction of *L1* expression resulted in a neuroprotective effect (The *et al.*, 2017). However, although these two studies show a causal effect of REs on neurodegeneration, the results are based on RNA interference (RNAi) against *gypsy* or anti-reverse transcriptase activity drug experiments. Both these approaches have side-effects which have not been ruled out in such studies. For instance, *gypsy* RNAi might be affecting expression of normal genes that happen to have *gypsy*-related sequences within, whereas the anti-reverse transcriptase drug could be affecting the telomere physiology (which also depends on reverse transcriptase activity), amongst others. Establishing the causal role between REs and neurodegeneration is important because RE derepression could represent a therapeutic target to prevent or mitigate the age-related neuronal decline. The development of drugs or therapeutics that would specifically avoid RE derepression or RE activity would help prolong the productive life of neurons, thereby promoting healthy ageing.

The purpose of this dissertation was to directly test whether RE activity could be a cause of neuronal function decline and degeneration. Based on the literature described above, we assume a model where, as the organism ages, the surveillance mechanisms of REs tend to be weakened, thereby unleashing REs (Figure 1.4). Once expressed, REs are able to undergo *de novo* transposition events, thus generating genomic instability and DNA damage. The accumulation of such insults may trigger either inflammatory processes or programmed cell death, both leading to neurodegeneration. We therefore hypothesize that direct expression of a RE in the neurons of a living animal throughout its lifespan, in conditions where the expression and activity of the RE are naïve to the host RE surveillance system (*i.e.*, the RE is immune to transcriptional and post-transcriptional repressive systems), will lead to progressive loss of neuronal function.

To directly test our hypothesis we have forced the expression of a human RE, *L1*, in the fly (*D. melanogaster*) CNS and assayed for neuronal function in ageing animals by performing a behavioral assay (negative geotaxis assay) 2, 20, and 40 days after eclosion. The results were negative for both of the tested lines, thus suggesting - *a priori* - that RE expression does not influence neurodegeneration (seen with age). We then carried out several *in vitro* and *in vivo* experiments to determine if the human *L1* was able to retrotranpose as expected in *Drosophila*. We conclude that we should take the result with caution that RE expression does not induce an age-dependent loss in neuronal function, as we have not yet been able to provide definitive evidence that human L1 retrotransposes efficiently in the fly nervous system. We speculate that L1 might require additional specific human factors (that are absent in flies) to retrotranspose.

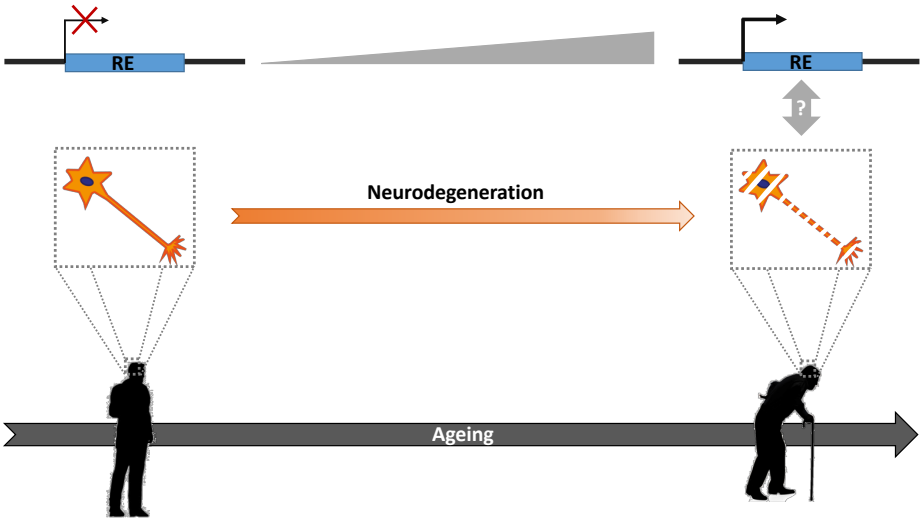

Figure 1.4: Hypothesis: RE expression in neurons contributes to the neuronal function decline and degeneration associated with age. As the organism ages, RE expression, which is normally silenced by the host cells, tends to increase. Once expressed, REs (like the human *L1*), start insulting the stability of the genome by trying to retrotranspose. The accumulation of DNA damage and genome instability over time leads to the gradual functional and cellular deterioration of neurons (neurodegeneration) which can culminate in the development of a neurodegenerative disease.

1.6 Rationale

1.6.1 *D. melanogaster* as the ideal model

The invertebrate model *Drosophila melanogaster* has been used in research for the last hundred years. Since then, a lot has been discovered in *Drosophila* and extrapolated to other model organisms. *Drosophila* and humans share many basic biological, physiological, and neurological properties. The overall homology between fly and mammal nucleotides is 40 %, and about 75 % of the genes related to human diseases are known to have functional orthologs in the fly (Fortini *et al.*, 2000; Pandey and Nichols, 2011; Reiter *et al.*, 2001; Rubin *et al.*, 2000). In fact, several human diseases have been successfully recapitulated in flies through transgenic expression of the human genes associated with those diseases, like the amyotrophic lateral sclerosis- and frontotemporal lobar degeneration-associated gene *TDP-43* (Krug *et al.*, 2017).

Drosophila is a valuable model to study human neurological diseases and neurodegeneration (reviewed in Lloyd and Taylor, 2010). For instance, the *NTE* gene which is nowadays known to be important in neuronal development for the maintenance of stability of the membranes that surround neurons, in particular of the axons, was initially discovered in a screen performed in *Drosophila* (Akassoglou *et al.*, 2004; Heisenberg and Böhl, 1979; Rainier *et al.*, 2008; reviewed in Lessing and Bonini, 2009). Alzheimer's, Parkinson's, and Huntington's disease are some of the

diseases that have been studied in the fly model (Lessing and Bonini, 2009; Lloyd and Taylor, 2010; Pandey and Nichols, 2011).

Large *Drosophila* populations can be rapidly generated, as their maintenance is relatively simple and cheap, and their life cycle is small and fast-paced (~10 days at 25 ºC). The use of insect species, like *D. melanogaster*, is ethically recommended as part of an initiative to reduce, replace, and refine research performed in large, warm-blooded experimental animals, such as mice and chimpanzees. There are several genetic tools available in *Drosophila* that allow to manipulate and edit the fly genome in a temporal and spatial manner (reviewed in Piper and Partridge, 2018).

Flies have a relatively short lifespan of about two-three months (reviewed in Sun *et al.*, 2013). Nevertheless, flies show clear symptoms of ageing as soon as one month after eclosion (Figure 1.5). Symptoms of ageing (otherwise considered as ageing-associated markers of loss of function) in the fly include reduced feeding, decreased reproductive capacity (decreased egg production, laying, and hatching), and decline in neuronal function, which encompasses impaired learning, memory, and debilitated negative geotaxis response (reviewed in He and Jasper, 2014; Piper and Partridge, 2018).

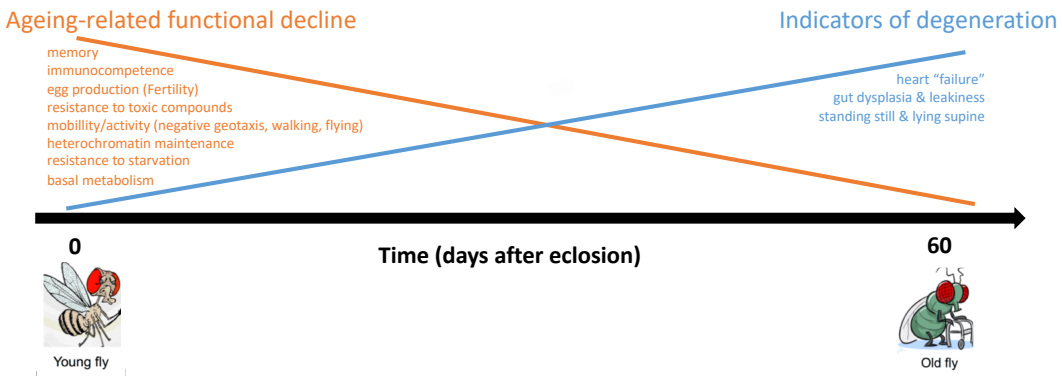

Figure 1.5: Flies show physiological signs of ageing. Adapted from Piper and Partridge, 2018.

Several tools have been developed for and utilized in *Drosophila* to determine the influence of particular genes in a specific subset of cells or tissue in ageing (and lifespan) (reviewed in He and Jasper, 2014). There are diverse genetic approaches to generate mutations and manipulate expression, which account for insertion mutagenesis mediated by P-element, gene expression alterations by *GAL4-upstream activating sequence (UAS)* system (described in more detail below), and RNAi-mediated knockdown of genes (reviewed in Sun *et al.*, 2013).

Physiological, biochemical, and behavioral assays are normally carried out to verify if the mutated or manipulated gene influences ageing (or neurodegeneration) (Sun *et al.*, 2013). In this work we will focus on the assays utilized to study neurodegeneration and ageing in flies.

For instance, Li *et al.* (2013) resorted to a well-established learning and memory assay to study the correlation between age-dependent neurofunctional decline and activation of TEs (Li *et al.*, 2013). One of the most simple assays used to study neurodegeneration (and ageing) in flies is the mean and maximum lifespan measurement, which consists in counting the number of dead flies throughout time (reviewed in He and Jasper, 2014; Lessing and Bonini, 2009; Sun *et al.*, 2013). However, maximum lifespan is a demographic assay and does not provide details on age-related neuronal dysfunctions. Therefore, there are additional strategies to study those changes that range from more classic approaches, like histology, to biochemical and functional methods, like quantification of protein aggregation and characterization of fly locomotor behavior, respectively (reviewed in He and Jasper, 2014; Lessing and Bonini, 2009). Locomotor activity is one of the parameters used to study health span (Sun *et al.*, 2013). The negative geotaxis assay (also known as climbing assay) is a quantitative and non-invasive approach to measure the locomotor decline associated with ageing (due to muscle and respective innervation function alteration) and neurodegeneration. The assay is based on the natural negative geotactic behavior of flies, especially when startled. Once tapped to the bottom of a tube, WT flies tend to climb upwards quickly. This climbing ability is lost progressively as the animals age, and is translated into failed attempts to climb and falling to the bottom of the tube (reviewed in He and Jasper, 2014; Sun *et al.*, 2013). Advantages of the negative geotaxis assay in studying neuronal function decline include the robustness of the behavior, the large amount of individuals that can be tested simultaneously, and the fact that it enables to assess for a common feature inherent to human neurodegenerative disorders, which is ataxia (Lessing and Bonini, 2009). Another major advantage of this assay is the simplicity of the set up, thereby making it very affordable.

The genome of *D. melanogaster* is one of the best studied and annotated genomes among eukaryotes. Although flies have several active LINE-like elements, they lack a true homolog of the mammalian *L1* and SINEs (Kramerov and Vassetzky, 2011; Perrat *et al.*, 2013; reviewed in Kapitonov *et al.*, 2009). Therefore, introducing a human *L1* or *Alu* in the fly genome would permit studying the role of RE derepression in the ageing-associated neurofunctional decline without interference of the repression mechanisms, since the mechanisms would not recognize the extraneous REs. Hence, we would be able to study the *L1* activity and its consequences directly, which is crucial to determine a causal relationship. A previous study, in which both human and mouse *L1* were introduced in chicken cells, demonstrated that *L1* was able to mediate retrotransposition events in a new host (chickens lack an endogenous source of *L1*), suggesting that *L1* does not require additional factors to mobilize in the genome (Suzuki *et al.*,

2009; Wallace *et al.*, 2008a). Having this in mind, we hypothesized that the system would work in the flies as well. The system used is described in the following section (Section 1.6.2).

1.6.2 Strategy

To directly investigate if RE derepression causes the neuronal function decline associated with age, we have established an unique heterologous system that allows tissue specific expression of the human *L1* in flies (Figure 1.6). More specifically, we have resorted to the binary system *GAL4-UAS* to control the expression of *L1* temporally and spatially. The *GAL4-UAS* system is a powerful genetic tool developed in *Drosophila* in 1993 to restrict the expression of a gene of interest to specific tissues (Brand and Perrimon, 1993; Poirier and Seroude, 2005). In flies, the *GAL4-UAS* expression system comprises two principal components, which are usually separated into two different transgenic flies: the yeast GAL4 transcriptional factor and the gene of interest under the regulatory control of a *UAS* promoter. In a transgenic line, a native tissue-specific promoter (also known as driver) drives the expression of *GAL4*, which cannot activate anything because there is no UAS present. On the other hand, the other transgenic line has a *UAS* promoter regulating the expression of an effector gene (the gene of interest), which is silent given the absence of GAL4. Only when these two transgenes are placed in the same fly, there will be expression of the effector gene in a tissue-specific manner. Thus, by crossing these two transgenic lines, the offspring will have both transgenes in every cell. However, only the cells in which the driver is "ON" will have the effector gene being expressed (Brand and Perrimon, 1993). Hence, we can restrict the expression of *L1* to the CNS by crossing a fly line containing GAL4 under the regulatory control of a pan-neuronal driver, like *elav*, *nSyb*, or *GMR57C10* with a transgenic line carrying a *UAS* sequence regulating a human *L1* (Wagstaff *et al.*, 2011).

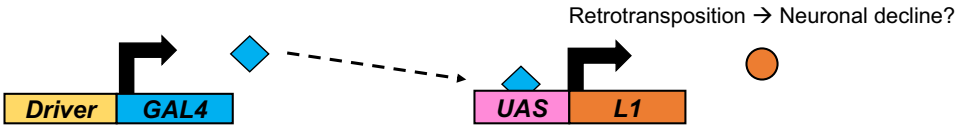

Figure 1.6: Schematic illustration of the human LINE system. A "Driver"(yellow rectangle), for instance, a *Drosophila* pan-neuronal promoter, regulates the expression of the transcriptional activator, *GAL4* (blue rectangle). Once expressed, the GAL4 protein (blue diamond) binds to the *UAS* sequence (pink rectangle) in *trans* and activates the expression of the effector gene, *L1* (orange rectangle). *L1* proteins (ORF1p and ORF2p, orange circle) are produced and mediate retrotransposition, which, according to our hypothesis, would lead to the neuron functional decline associated with age.

To demonstrate that the human *L1* is functionally retrotransposing in *Drosophila*, we have engineered a SINE reporter element (Figure 2.3) using a similar strategy as Belgnaoui *et al.*

(2006). The cellular readout chosen for this system is the production of sfGFP. The *Alu* reporter element comprises the synthetic human *AluYb8* (hereafter, *Alu*) with a GFP reporter cassette placed towards *Alu* 3'UTR in the antisense orientation (*SINE[Act-sfGFP]*) (Dewannieux *et al.*, 2003; Wallace *et al.*, 1991). The GFP cassette consists of a constitutive *Drosophila Actin5c*-promoter (*Act5c*) regulating a *de novo*-synthetized *sfGFP*, an inverted intron (intron 16) of the *Drosophila Mhc* gene, *MhcInt16*, placed in the same orientation as *Alu* (disrupting the *sfGFP* sequence), and a *SV40 PolyA* terminator sequence (Pfeiffer *et al.*, 2010; Qin *et al.*, 2010). To ensure the expression of *Alu* and the splicing of the intron, *Alu* was placed under the regulatory control of a *Drosophila* alpha-Tubulin at 84B (*Tubulin*, *Tub*) promoter, *Tub-SINE[Act-sfGFP]*. In this system, to produce sfGFP, *Alu* must be transcribed, post-transcriptionally processed, and retrotransposed into the genome *in trans* by *L1* proteins. The splicing of the intron is crucial to remove the intron and enable the production of a functional sfGFP after retrotransposition of the spliced transcript into the genome. The amount of retrotransposition can be quantified by direct inspection of the adult CNS following *UAS-L1* expression in the CNS using a neuronal driver such as *elav-Gal4* in the presence of the *SINE[Act-sfGFP]* construct. Although the *Alu* reporter element increases the complexity of our system, it also provides an easier and faster mode of detecting retrotransposition events due to its shorter size and rapid retrotransposition capacity (Kroutter *et al.*, 2009; reviewed in Deininger, 2011).

1.7 Aims

This dissertation proposes to determine if RE expression causes neuronal degeneration and/or neurofunctional decline associated with age. Additionally, we will seek for evidence that human *L1* can retrotranspose in *Drosophila* cells. To achieve these objectives, we will:

- Generate *L1* and *Alu* transgenic lines;
- Analyse the expression of *L1* in the transgenic lines;
- Characterize the locomotor ability of flies expressing *L1* in the nervous system;
- Characterize the production of functional L1 ORF2p in the context of a full-length *L1 in vivo* and *in vitro*;
- Test the *L1*/*Alu* system *in vitro* and *in vivo*;
- Characterize the production of functional L1 ORF2p from a shorter version of *L1 in vitro*;
- Characterize the *L1 ORF2*/*Alu* system *in vitro*.

Chapter 2

Materials and Methods

2.1 *Drosophila melanogaster*: fly lines and crosses

All the stocks and crosses were maintained at 25 ºC under a controlled atmosphere with 70 % humidity on cornmeal-agar medium. Mutant or transgenic fly stocks used in these studies are described on Table 2.1, as well as their source. The *UAS-L1* and the *Tub-Alu[Act-sfGFP]* transgenic lines were generated via P element insertion (Rubin and Spradling, 1982). Briefly, plasmids containing those constructs flanked by the ends of the P element (3' P and 5' P) were co-injected with a plasmid carrying the P element gene into *w[1118]* embryos. Existing fly stock lines were used to generate new stocks through genetic crossing.

2.2 Startle-induced Negative Geotaxis Assay

The startle-induced negative geotaxis assay, also known as climbing assay, is a broadly used assay to study locomotor capacity and its age-dependent decline (Ganetzky and Flanagan, 1978). This assay exploits the negative geotaxis response that flies exhibit after being startled (Figure 2.1). After being tapped to the bottom of the tube, WT flies rapidly climb up the vial. As flies age, this ability is gradually lost and older flies are no longer able to climb to the top of the vial.

The assay was performed as previously described (Barone and Bohmann, 2013). Flies were collected between 0–24 hours (h) after eclosion. The flies were transferred into new food twice a week. Before the assay, flies were submitted to two acclimatization periods. Firstly, groups of

Table 2.1: List of the *D. melanogaster* stocks used in the experiments.

Category	Short name (ID)	Genotype	Origin
Control	*w[1118]*	*w[1118]; ; ;*	Bloomington Stock Center 3605
drivers-GAL4	*57C10>*	*w[1118]; P{y[+t7.7] w[+mC]=GMR57C10-GAL4}attP2*	Bloomington Stock Center 39171
drivers-GAL4	*arm>*	*; armGAL4;*	Pedro Domingos' lab (ITQB)
drivers-GAL4	*pBDP>*	*w[1118]; P{y[+t7.7] w[+mC]=GAL4.1Uw}attP2*	Carlos Ribeiro's lab (CCU)
drivers-GAL4	*elav>*	*elav-GAL4; ;*	Christen Mirth's lab (IGC)
drivers-GAL4	*nSyb>*	*; ; nSyb-GAL4;*	Rita Teodoro's lab (CEDOC)
Tub-Alu[Act-sfGFP]	*Alua>*	*; If/CyO; Alu_M31-F1;*	Alisson Gontijo's lab (CEDOC)
Tub-Alu[Act-sfGFP]	*Alub*	*; If/CyO ; Alu_F25-M1;*	Alisson Gontijo's lab (CEDOC)
Tub-Alu[Act-sfGFP]	*Aluc*	: Alu_F21-M5; MKRS/TM6B	Alisson Gontijo's lab (CEDOC)
Tub-Alu[Act-sfGFP]	*Alud*	; Alu_M51-F1 /CyO; MKRS/TM6B;	Alisson Gontijo's lab (CEDOC)
Tub-Alu[Act-sfGFP]	*Alue*	; Alu_F35-M1 /CyO; MKRS/TM6B;	Alisson Gontijo's lab (CEDOC)
Tub-Alu[Act-sfGFP]	*Aluf*	; Alu_F34-M1 /CyO; MKRS/TM6B;	Alisson Gontijo's lab (CEDOC)
Tub-Alu[Act-sfGFP]	*Alug*	; If/CyO; Alu_M53-M1/TM6B;	Alisson Gontijo's lab (CEDOC)
Tub-Alu[Act-sfGFP]	*Aluh*	Alu_F21-M3-F1; If/CyO ; MKRS/TM6B;	Alisson Gontijo's lab (CEDOC)
Tub-Alu[Act-sfGFP]	*Alui*	Alu_F32_M1/FM7c; +/bal; +/bal;	Alisson Gontijo's lab (CEDOC)
UAS-L1	*L1a*	*; L1_M68-F1; ;*	Alisson Gontijo's lab (CEDOC)
UAS-L1	*L1a_bal*	*; L1_M68-F1 ;MKRS/TM6B;*	Alisson Gontijo's lab (CEDOC)
UAS-L1	*L1b*	*; If/CyO ; MKRS/TM6B; L1_F1_F4*	Alisson Gontijo's lab (CEDOC)
UAS-L1	*L1c*	*; ; L1_F1_M3;*	Alisson Gontijo's lab (CEDOC)
UAS-L1	*L1d*	*; ; L1_F1_F2 ;*	Alisson Gontijo's lab (CEDOC)
UAS-L1	*L1e*	*; ; L1_M16_M2/TM6B*	Alisson Gontijo's lab (CEDOC)
UAS-L1	*L1f*	*; ; MKRS/TM6B; L1_M3_F1*	Alisson Gontijo's lab (CEDOC)
UAS-L1	*L1g*	*; ; L1_M13_F1;*	Alisson Gontijo's lab (CEDOC)
UAS-L1	*L1h*	*; L1_M68_F1; elavG5-G4;*	Alisson Gontijo's lab (CEDOC)
UAS-L1; Tub-Alu[Act-sfGFP]	*L1a+Alub*	*; L1_M68-F1; Alu-F25;*	Alisson Gontijo's lab (CEDOC)

5 to 20 male flies were placed at RT for 5 minutes (min). Secondly, the flies were transferred to empty climbing vials where they were allowed to adapt for 5 min (Figure 2.1, 1-2). Then, the assays started with the tapping of the flies to the bottom of the vial (Figure 2.1, 3). Fly behavior was recorded for 15 seconds (s). Each assay comprised five tappings with 1 min intervals inbetween. The percentage of flies climbing above 2, 4, and 6 cm, 5 and 10 s after initiation of the assay was scored (Figure 2.1., 4-5). As demonstrated in Appendix A Figure A.1, flies locomotor ability tends to decrease with age regardless of the condition scored. Since the decrease in the locomotor ability was best described in the conditions C (above 4 cm, after 5 s) and D (above 6 cm, after 5 s), the percentage of males above 6 cm after 5 s for each assay was plotted using box plots. Between 56 and 254 flies of each genotype were assayed 2, 20, and 40 days after eclosion at the same period of the day, at RT, and under the same light conditions. Each genotype was tested between 5 and 24 times.

Two independent assays were performed with two different *UAS-L1* (hereafter, *>L1*) transgenic lines (*>L1a* and *>L1e*) (Table 2.1). In the first experiment, test animals were obtained from the cross between *>L1a* and the pan-neuronal GAL4 driver lines, GMR57C10, nSyb and elav (hereafter, *57C10>*, *nSyb>*, and *elav>*, respectively). To control for background and transgene insertion effects, animals from several control genotypes were assayed. These included flies resulting from crosses between the GAL4 driver lines and the background control stock, *w[1118]* (*nSyb>*, *elav>* and *57C10>*), between the control driver line, *pBDP-GAL4* (hereafter, *pBDP>*) and the *>L1a* line (*pBDP>L1a*), and between the *>L1a* and *w[1118]* (*>L1a*). *pBDP>* carries an

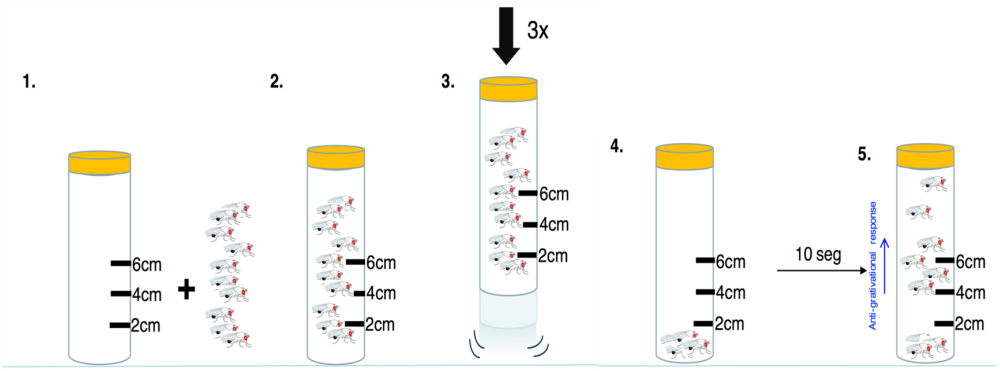

Figure 2.1: Representative scheme of the negative geotaxis assay procedure. Flow diagram illustrating the negative geotaxis assay used to monitor the age-dependent locomotor behaviour in flies. After tapping a test tube containing groups of 5-20 male flies, their anti-gravitational response was recorded and posteriorly analysed (1-5). The anti-gravitational response of flies with increasing ages was scored as the percentage of males that climbed above 6 cm, 5 s after initiating the assay.

"empty" GAL4 driver, identical to the *57C10>* line (*i.e.*, has the same genetic background), but without the neuronal enhancer. In the second experiment, test animals were obtained from the crosses between *>L1e* and *57C10 >*, *w[1118]*, and *pBDP> (57C10>L1e, >L1e, and pBDP>L1e*, respectively). The cross between *57C10>* and *w[1118]* animals was included as a GAL4 driver control.

2.3 Plasmids

To assemble a *pUASTN-LINE1* (hereafter referred to as *pUAS-L1*), the plasmid *pBS-L1PA1-CH-mneo* (Addgene plasmid # 51288; gift from Astrid Roy-Enge) was digested with *NheI* and *EcoRI* enzymes (Wagstaff *et al.*, 2011). The resulting *L1_ORF1_ORF2* fragment was inserted into a *pUASTN* previously co-digested with both *NheI* and *EcoRI* (Figure 2.2). *pUASTN* was made by introducing new restriction sites by annealed oligo cloning in the original *pUAST* (Brand and Perrimon, 1993). Briefly, *pUAST* was opened with *EcoRI* and *BglII*, and posteriorly ligated to overhangs resulting from the annealing of two primers. This cloning resulted in the conversion of the original *EcoRI-BglII* fragment into *NheI-AgeI-EcoRI*, generating the *pUASTN* plasmid (Figure 2.2). This work was done by Fabiana Heredia and André Macedo.

To generate the SINE retrotransposition reporter element (*pTub-Alu[Act-sfGFP]*), a human *AluYb8* sequence containing a docking site with specific restriction enzyme sites, *NcoI* and *AgeI*, towards its 3' end was used (Figure 2.3). To assemble the GFP reporter cassette, a *Drosophila Actin5c*-promoter (*Act5c*) sequence was amplified and new *AgeI* restriction sites were introduced in both extremities of the sequence through PCR. The resulting *Act5c* PCR product

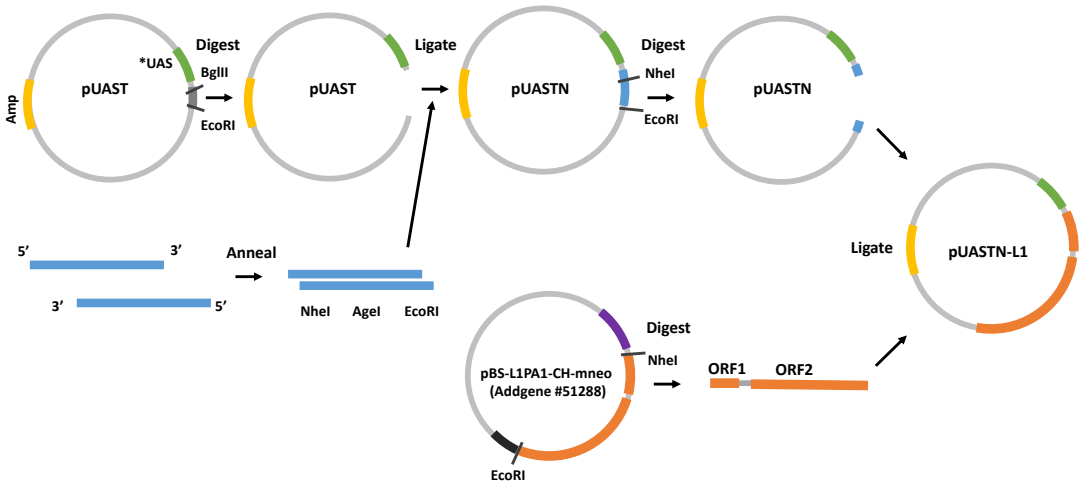

Figure 2.2: Cloning scheme for the construction of *pUASTN-L1*. The *pUAST* plasmid was modified by introducing *NheI*, *AgeI*, *and EcoRI* restriction sites through primer annealing and ligation to a previously *BglII* and *EcoRI* digested *pUAST*. *L1* was obtained from a *NheI* and *EcoRI* digestion of *pBS-L1PA1-CH-mneo* (Addgene # 51288). Only the restriction enzymes used in the procedure are shown. The fragments in the scheme are out of scale.

was digested with *AgeI* and inserted into a plasmid containing a *de novo*-synthetized *sfGFP* sequence disrupted by the *MhcInt16* intron, that had been previously opened with *AgeI*. *XhoI* and *NcoI* restriction sites were introduced into the 5' and 3' extremities of the *SV40 PolyA* terminator sequence via PCR amplification. The resulting PCR product was then co-digested with both *XhoI* and *NcoI*, and ligated to the *Act5c-AgeI-sfGFP* plasmid previously co-digested with the same enzymes (*XhoI* and *NcoI*), thus generating the *Alu[Act-sfGFP]* reporter cassette. To guarantee production of the *Alu[Act-sfGFP]* reporter element and splicing of the *MhcInt16*, a constitutive *Drosophila* alpha-Tubulin at 84B (*Tubulin*, *Tub*) promoter was placed before the element using the *KpnI* and *XhoI* restriction sites, as depicted in Figure 2.3. The *Tub* promoter was obtained from a *pCaSpeR4* backbone (JB25 plasmid, gift from J Bischof, from the Basler lab (UZH, Switzerland)). This work was done by Fabiana Heredia and André Macedo.

The *pUASTN-L1_ORF2* (hereafter referred to as *pUAS-ORF2*) plasmid was generated by cloning an *AgeI-EcoRI* digested *L1_ORF2* (present in *pBS-L1PA1-CH-mneo*) fragment into the previously *AgeI-EcoRI* digested *pUASTN* vector (Figure 2.4).

To confirm the sequence of the final plasmids, a reaction of 100 ng/μL of plasmid DNA with 10 pmol/μL of primer was subjected to Sanger sequencing (STABVIDA Sanger sequencing service). Since Sanger sequencing provides low quality sequences at the beginning and at the end of the sequence, two different primers flanking each side of the inserts were chosen for each plasmid, thus assuring high-quality sequencing for the constructs through comparison. Table A.1 in Appendix A lists the plasmids used in this dissertation.

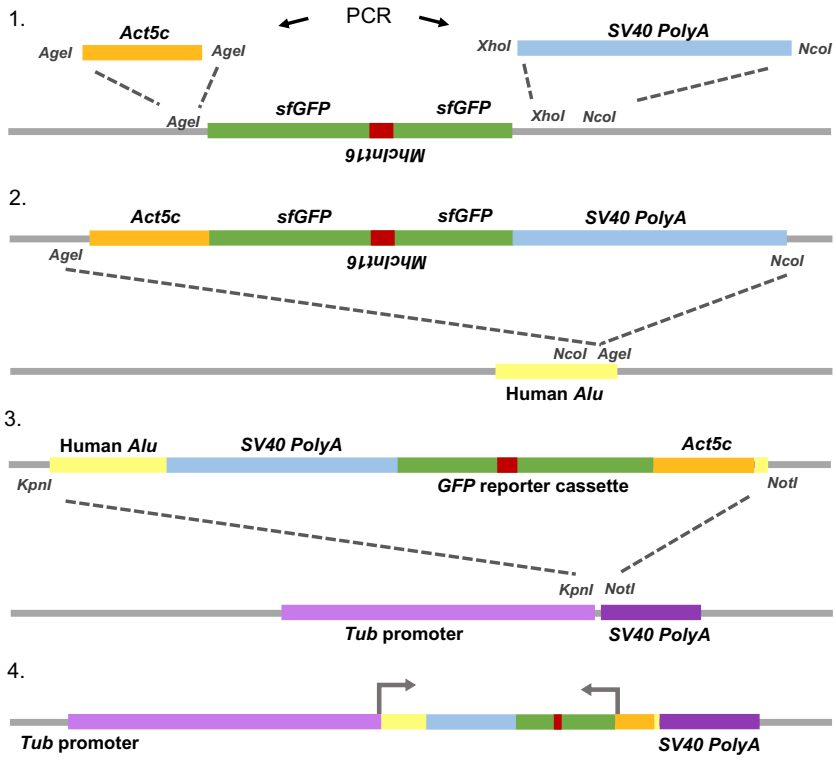

Figure 2.3: Cloning strategy for the construction of the *pTub-SINE[Act-sfGFP]*. 1. A *Drosophila Actin5c* promoter (orange) and a *SV40 polyA* terminator (blue) sequence were amplified through PCR, digested with *AgeI* and co-digested with *XhoI* and *NcoI*, respectively. The resulting fragments were cloned into a plasmid containing a *de novo* synthetized *sfGFP* (green) with a disrupting *MhcInt16* (red), producing a GFP reporter cassette. 2. The cassette was introduced into a plasmid containing the human *Alu* sequence (yellow) in the opposite direction by co-digestion with *AgeI* and *NcoI*, producing the *Alu[Act-sfGFP]*. 3. The *Alu[Act5c-sfGFP]* was cloned into a plasmid containing a *Drosophila* tubulin promoter (*Tub*, light purple) via *KpnI* and *NotI* co-digestion. 4. Representation of the final plasmid, *pTub-Alu[Act-sfGFP]*. Only the restriction enzymes used in the procedure are shown.

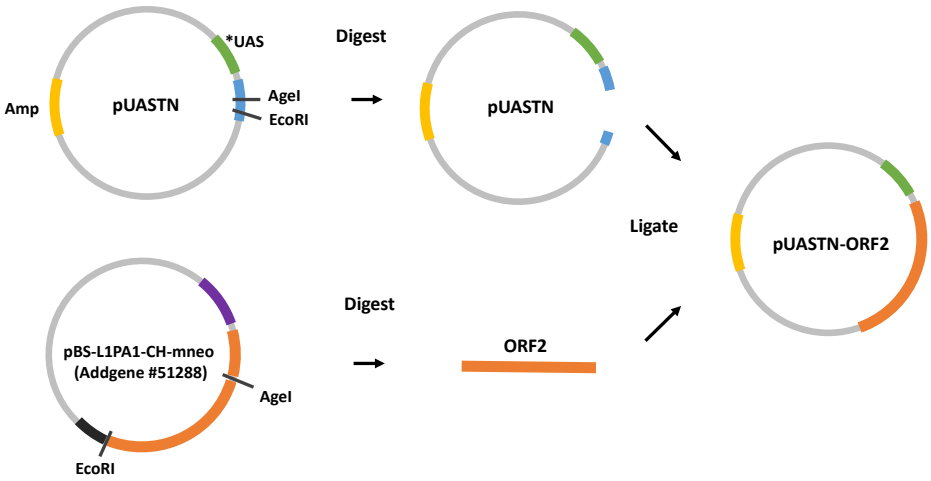

Figure 2.4: Cloning scheme for the construction of *pUAS-ORF2*. *pUASTN* was digested with *AgeI* and *EcoRI* to open and ligate with a previously *AgeI* and *EcoRI* digested ORF2 fragment (from *pBS-L1PA1-CH-mneo* (Addgene # 51288)). Only the restriction enzymes used in the procedure are shown. The fragments in the scheme are out of scale.

2.4 Polymerase Chain Reaction (PCR)

PCR was used together with gel electrophoresis to perform colony, digestions, and transfections screenings.

Bio-Rad's T100^{TM} Thermal Cycler and NZYtech's Supreme NZYTaq 2x Green Master Mix were used for standard PCR reactions. For this purpose, the manufacturer's instructions were followed. The PCR cycle parameters and the primers used are depicted in Table 2.2 and 2.3, respectively.

Table 2.2: List of the PCR and RT-qPCR programs followed in the experiments.

			Cycle			
		Initial denaturation	**Denaturation Step**	**Annealing Step**	**Elongation/Extension Step**	**Final extension**
	Temperature (°C)	95	95	60	72	72
PCR (35 cycles)	Time	60 s	30 s	30 s	60 s / kb	5 min
qPCR (45 cyles)	Time	10 min	10 s	10 s	10 s	

Table 2.3: List of primers used in PCR and RT-qPCR experiments.

Technique	**Target**	**Primer**	**Sequence (5' - 3')**	**Expected Band Size (bp)**
PCR,	*Line-1 ORF2*	*L1orf2-F*	CACTGCTGGTGGGACTGCAA	167
RT-qPCR;		*L1orf2-R*	AACATCCGGGTGCAGGTGTC	
PCR,	*sfGFPcassette with intron*	*SuperfolderGFP_F*	CCTATGGCGTGCAATGCTTC	467
RT-qPCR;		*SuperfolderGFP_R*	CAGCACCATATGATCCCGCT	
PCR;	*sfGFPcassette without intron*	*SuperfolderGFP_F*	CCTATGGCGTGCAATGCTTC	239
		sfGFP_no_MHCintron_1_R	ATTATATTCGAGCTTGTGGC	
RT-qPCR;	*rp49*	*NewRP49_p6_F*	TTGAGAACGCAGGCGACCGT	96
		NewRP49_p6_R	CGTCTCCTCCAAGAAGCGCAAG	

2.5 Gel electrophoresis and purification

Agarose gel electrophoreses were used as a read-out for PCR products, digestion of plasmids (with restriction enzymes) and DNA quality control (*e.g.*, to monitor plasmid integrity). Between 0.7-2.0 % (w/v) agarose powder (NZYtech) was mixed with Tris-acetate-EDTA (TAE) 1X (NZYtech) and further stained with 0.004 % (v/v) GreenSafe Premium (NZYtech) to allow DNA visualization. Since agarose concentration determines the distance between DNA bands of a given length, the choice of which concentration and percentage of agarose to use depended on the expected fragment sizes. Hence, digestion products with smaller sizes (~0.2 kb) were resolved on 2 % agarose gels, whereas 0.7 % agarose gels were used for both separation of 5-10 kb DNA fragments as suggested by Burland *et al.* (1996) and Voytas (2001), and for DNA quality verification. Amplicons were usually resolved in either 1.2 % or 0.7 % agarose gels, according to the expected size. Additionally, the molecular marker NZYDNA Ladder III and 6x NZYDNA loading dye (NZYtech) were used to recognize the approximate molecular weight of

the DNA after migration and to prepare DNA samples for loading. ChemiDoc^{TM} XRS (Bio-Rad Laboratories) and its system were used for gel imaging.

Almost every cloning strategy required DNA purification of either amplicons or digested fragments from agarose gels following electrophoresis. For this purpose, the desired bands were cut and purified with NZYGelpure (NZYtech), following the instructions of the manufacturer. DNA concentrations and quality were measured using Nanodrop^{TM} 2000 Spectrophotometer (Thermo Scientific). The samples would then be either used for cloning or stored at -20 ºC.

2.6 DNA ligation, competent cell transformation and plasmid DNA isolation

DNA insert ligation into vector DNA was performed by following the T4 DNA ligase protocol from ThermoFisher Scientific. Briefly, we joined 50-100 ng of vector and insert in a molar ratio of 1:3 (vector to insert). In order to calculate the exact amount of insert DNA to add, we resorted to Equation 2.1. Both the vector and the insert were heated at 65 ºC for 5 min before being added to the ligation mixture. After mixing, the reaction was incubated at RT for 3-4 h and then shifted into 4 ºC ON. For every ligation, a negative control (without insert) was also made.

$$\frac{\text{Vector amount (ng)} \times \text{Insert size (kb)}}{\text{Vector size (kb)}} \times \text{Molar ratio } \frac{insert}{vector} = \text{Insert amount (ng)} \qquad (2.1)$$

Once the DNA ligation was completed, 5 or 10 μL were used to transform 50 or 100 μL of chemically competent NZY5α cells (NZYtech), according to the instructions of the manufacturer. However, 0.6 milliliters (mL) of warm (37 ºC) liquid SOC Medium (NZYTech) were added and the cells were shaken for more than 1 h (typically, 1.5 h). Then, the cells were centrifuged at 0.5 xG for 5 min and the cell pellet was resuspended in 100 μL of SOC, which were then plated on ampicillin plates (100 $\mu g_{\text{ampicilin}}$ / mL_{LBagar} (w/v)) and incubated for 16 h. The resulting colonies were screened by performing a colony PCR. Briefly, 1-3 positive colonies were picked and cultivated in ampicillin supplemented LB medium for 16 h at 37 ºC with vigorous shaking (225 rpm). To isolate and purify the plasmid from bacteria we resorted to the NZYMiniprep kit from NZYtech and followed the manufacturer's protocol. The only changes to this protocol were the addition of an additional washing step with Buffer A4 and the subjection of the kit's column to the same volume of AE twice, i.e., repetition of the elution step, to increase the elution efficiency. After measuring the concentration of the plasmid DNA, 1 μg of DNA was sent, together with the desired primer(s), to STAB VIDA for sequence verification through

Sanger sequencing. The results were then analyzed with Clustal Omega Multiple Sequence Alignment (Sievers *et al.*, 2011).

2.7 Genomic DNA (gDNA) extraction

To extract gDNA from cells in culture, we used the High pure PCR Template Preparation kit from ROCHE. Briefly, the cells were resuspended in the medium and transferred to a 1.5 mL eppendorf. The cells were then centrifuged at 0.8 xG for 3 min and resuspended in 200 μL of PBS. After that, the same volume of Binding Buffer and 40 μL of 20 mg/mL proteinase K were added before mixing and incubation for 10 min at 70 ºC. Isopropanol was added prior to the inhibition and washing steps. Finally, the gDNA was eluted in 50 μL of pre-warmed elution buffer and stored at -20 ºC for further analysis.

Regarding the extraction of gDNA from flies, the brain of male flies aged 3-7 days or the CNS of wandering 3rd instar larvae were dissected and collected. To maintain consistency among the experiments, only male flies were used. The methods used in the extraction of these 2 different samples were different: we followed High pure PCR Template Preparation kit manual to extract the gDNA from Larvae brains, whereas the gDNA from adult brains was extracted with DNA extraction buffer (10 mM Tris-Chlorine (Cl) at pH 8.2, 1 mM EDTA, and 25 mM Sodium chloride (NaCl), hereafter referred to as buffer A) (Carvalho *et al.*, 2009). The Buffer A extraction protocol consists in the maceration of flies using pellet pestles and 100 μL of Buffer A. Once macerated, the samples are treated with 1 μL of proteinase K (Roche) and incubated at 37 ºC for 1 h. After this period, proteinase K is inactivated by a 5-min incubation at 95 ºC. Lastly, a centrifugation step of 5 min at 5.0 xG allows the separation of tissue and cellular debris, which stay in the bottom, from the gDNA that remains in the supernatant.

2.8 RNA extraction

To characterize and evaluate the relative *L1* and *Alu* mRNA levels in the transgenic fly lines, total RNA was extracted from the heads of 1 to 2 days old male flies (Table 2.4) using both trizol (Tripure Isolation Reagent, Roche) and a spin column-based RNA purification kit (High Pure RNA Tissue kit, Roche) and DNAse-I treated, following instructions of the manufacturers. Briefly, heads were macerated using pellet pestles and homogenized in 800 μL trizol. Posteriorly, the samples were transferred to a purifying column where they were treated with DNAse I, washed and eluted in the minimal volume possible to assure higher concentrations of RNA. A second DNAse treatment, TURBO DNA-free^{TM} Kit enzyme (Invitrogen), was exploited to

prevent gDNA contamination. The RNA concentration of each sample was measured using a Nanodrop^{TM} 2000 spectrophotometer.

Table 2.4: List of the genotypes tested, the number of collected heads and the RNA concentration obtained.

Driver	Gene of interest	# heads	RNA concentration (ng/μL)
BDP >	*L1a*	32	199.1
BDP >	*L1b*	32	182.9
BDP >	*L1c*	51	243.9
BDP >	*L1d*	26	162.8
BDP >	*L1e*	36	185.5
BDP >	*L1f*	40	205.0
BDP >	*L1g*	41	204.7
57C10 >	*L1a*	46	226.8
57C10 >	*L1b*	37	200.9
57C10 >	*L1c*	31	195.4
57C10 >	*L1d*	31	191.1
57C10 >	*L1e*	32	210.7
57C10 >	*L1f*	37	209.7
57C10 >	*L1g*	36	233.7
Tubulin >	*Alua*	73	371,8
Tubulin >	*Alub*	59	254,6
Tubulin >	*Aluc*	78	363,0
Tubulin >	*Alud*	66	328,9
Tubulin >	*Alue*	34	224,2
Tubulin >	*Aluf*	58	314,3
Tubulin >	*Alug*	48	234,2
Tubulin >	*Aluh*	64	330,7
Tubulin >	*Alui*	45	305,2

2.9 Complementary DNA (cDNA) preparation

cDNA was prepared for RT-qPCR analysis from DNAse-TURBO treated RNA samples. For this purpose, Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) was used, according to the manufacturer's protocol, for a total volume of 10 μL *per* reaction. Afterwards, the cDNA samples were diluted at least 3 times (for a final volume of 30 μL or more) to prevent any eventual inhibition of the RT-qPCR reaction caused by reagents used during the cDNA synthesis. To test for gDNA contamination in the RNA samples, all the samples used for the cDNA synthesis were simultaneously used in a parallel reaction in which the reverse transcriptase enzyme was replaced by MilliQ water. These additional reactions are called reverse transcriptase negative controls (RT- controls).

2.10 Reverse Transcriptase quantitative PCR (RT-qPCR)

RT-qPCR was performed using the FastStart Essential DNA Green Master dye and polymerase (Roche) and a Lightcycler®96 (Roche). The final volume for each reaction was 10 μL, consisting of 5 μL of dye and polymerase (master mix), 2 μL of cDNA sample and 3 μL of specific primer pairs (1 μM) (Table 2.3). The relative *L1* and *Alu* mRNA levels were determined according to Equation 2.2. The geometric means ± SD of 3 technical replicates were plotted.

$$\%_{rp49} = 2^{-(\Delta Cq_x - \Delta Cq_{rp49})} \times 100 \quad (2.2)$$

where: $x = L1$ or Alu, $rp49$[1] = control

The RT-qPCR program used is depicted in Table 2.2. The fluorescent intensity of SYBR Green was surveyed at the end of each extension step.

2.11 Cell culture conditions, DNA preparation and transfection

Drosophila line 2 (DL2) cells, also known as Schneider 2 (S2), are spherical semi-adherent cells likely derived from a macrophage-like lineage. DL2 stem from a primary culture of late stage (20 to 24 h old) *D. melanogaster* embryos (Schneider, 1972). DL2 cells were cultured in Schneider's Drosophila Medium (Biowest) supplemented with 10 % (v/v) of heat-inactivated Fetal Bovine Serum (FBS, Life Technologies) and 1 % (v/v) of antibiotic solution (Penicillin-Streptomycin (Pen-strep), Sigma). The cells were maintained in T25-flasks at 25 ºC under normal atmospheric conditions. DL2 cells were subcultured every 3 to 4 days in a cell medium ratio of 1:20, upon reaching 80-90 % cell confluency, to maintain an appropriate mitotic index and to avoid cell death triggered by nutrient absence.

Plasmid DNA was purified using NZYtech miniprep columns, according to the guidelines of the manufacturer. DNA quality was checked by electrophoresis in 0.7 % agarose – GreenSafe gels. Only high quality plasmids were used for transfection.

For transient transfections, 2 x 10^5 DL2 cells were seeded *per* well (in 24-well plates) in medium without antibiotics. Upon reaching 80 % cell confluency, 24 h after seeding, DL2 cells were transfected with a mixture containing 1 μg of total plasmid (each plasmid in equimolar amounts), FuGENE® HD Transfection Reagent (Promega) in a 1:3 DNA (μL)/ FuGENE (μL) ratio, and Schneider's Drosophila Medium (up until 25 μL) *per* well. After a 10 min incubation, the plasmid mixture was added to the cells, which were posteriorly incubated at 25 ºC. 48 or 96 h after incubation, the cells were either fixed and stained or harvested and processed for subsequent analysis. The transfections were made in duplicates.

2.12 Immunostaining

Brains from both wandering 3rd instar larvae and adult flies aged 3-7 d were dissected in Schneider's Drosophila Medium and fixed in 4 % (v/v) Formaldehyde (Sigma) diluted in 1X PBS. Brains were dissected and stained as described by Weitkunat and Schnorrer, 2014.

[1] *rp49* or Ribosomal protein L1 32 gene, *RpL32*, is an endogenous and constitutively expressed gene in *Drosophila.*

Briefly, after having their posterior end cut out, larvae were inverted through their anterior end, leading to the exposure of their brain. For the adult brains, the dissection procedure consisted in decapitation, then making a shallow incision with a forceps in the region between the eye and the proboscis. Next, the eye capsule and the rest of the cuticle are removed while holding the proboscis down. Once the brains were dissected, they were fixed for 20 min in shaking conditions and washed in 0.3 % (v/v) PBS-Triton X-100 (PBT). All tissue samples were blocked in 1 % (w/v) Bovine Serum Albumin (BSA) in PBT blocking solution for at least 1 ON (and up until 48 h) at 4 ºC while shaking. Subsequently, the samples were stained with the primary antibodies at 4 ºC for 48 h while shaking. Then, the tissues were washed 3x (or more) with PBT for 30 min and incubated ON at 4 ºC with the secondary conjugated antibodies in the shaker. The samples were washed 3x in PBT and nuclei were stained by incubating the samples in 0.001 % (w/v) of DAPI (Sigma) for 10-15 min at RT while shaking. After washing 3x with either PBT and PBS, the tissues were mounted by transferring them to a previously prepared slide (that has 2 coverslips flanking a region in the middle in which the tissues are put), by removing all the tissues that are attached to the brain, and by adding mounting medium (16 mL Glycerol, 4 mL PBS 1X and 0.4 g DABCO (Sigma)). Typically, 5–10 brains were mounted for each genotype and 1 representative image per genotype is depicted in the figures.

A similar immunocytochemistry protocol was used with DL2 cells, but the incubation times and the washing solutions were different. 48 h or 96 h after transfection, the cells were fixed in 4 % (v/v) formaldehyde solution for 20 min. Subsequently, the fixed cells were permeabilized with 0.1 % (v/v) PBT for 10 min. Cells were then washed with PBS, incubated with 1 % (w/v) PBS-BSA for 1 h at RT, and posteriorly incubated with the primary antibody either ON at 4 ºC or for 2 h at RT. Cells were subsequently washed and incubated with the secondary antibody, away from light, for a minimum of 2 h at RT. Finally, after a 0.001 % (w/v) DAPI incubation for 10 min (away from light), the coverslips were mounted on the slides. Typically, 5–15 microphotographs were taken for each well and 1 representative image per genotype is depicted in the figures.

Primary antibodies used for immunofluorescence include: rabbit polyclonal anti-GFP (Life Technologies, A11122), mouse monoclonal anti-γH2AV (DSHB, UNC93-5.2.1-S), and mouse monoclonal anti-ORF2p (Kerafast, ETL001). The secondary antibodies used were: goat Alexa fluor 488 anti-rabbit (Invitrogen, A11070), goat Alexa fluor 488 anti-mouse (Life Technologies, A11029), Alexa fluor 594 goat anti-mouse (Invitrogen. A11020), goat FarRed (Cy5) anti-mouse (Jackson Immuno Research, 115-175-166). Additional information on the antibodies and dilutions used is depicted in Table 2.5.

Table 2.5: List of primary and secondary antibodies used for the immunostaining procedures.

Antibodies	Conjugated	Recognize	Host	Concentration	Supplier
Primary		GFP	Rabbit	1:200 (tissue) 1:500 (cells)	Life Technologies
		γH2AV	Mouse	1:500 (tissue) 1:2500 (cells)	DSHB
		L1_ORF2p	Mouse	1:20 (tissue) 1:50 (cells)	Kerafast
Secondary	Alexa Fluor 488	rabbit antibodies	Goat	1:250	Invitrogen
	Alexa Fluor 488	mouse antibodies	Goat	1:250	Life Technologies
	Alexa Fluor 594	mouse antibodies	Goat	1:250	Invitrogen
	Far Red (Cy5)	mouse antibodies	Goat	1:250	Jackson Immuno Research

2.13 Microscopy and image analysis

Images were acquired with a Zeiss Z2 widefield fluorescence microscope equipped with a Plan-Apochromat 20x air, an EC Plan-NeoFluar 40x oil immersion and a Plan-Apochromat 63x oil immersion objective lens (NA 0.80, 0.75 and 1.40, respectively) or with a Zeiss LSM 710 Confocal microscope. To compare the levels of the desired proteins between different conditions, each condition was imaged with the same settings. Exposure time and Laser intensity were set on the brightest condition and reused on the remaining conditions. Images were processed with Fiji (Schindelin *et al.*, 2012).

2.14 Statistics

Fly locomotor ability data were recorded in Microsoft Windows Excel spreadsheet and plotted, with R, in box plots representing median, 25 and 75 % quartiles (Tukey's method). Whiskers extend to 1.5 times the IQR. Values above or below the whiskers are considered outliers. A two-way analysis of variance (ANOVA) was performed to determine significant effects of L1 expression on each day of assay. Alpha was set at 0.05 to consider statistical significance. Data from the relative expression of *UAS-L1* and *Tub-Alu[Act-sfGFP]* transgenic lines were plotted in bar plots with GraphPad.

Chapter 3

Results

RE derepression has been associated with age-dependent neurophysiological decline, which is aggravated in neurodegenerative diseases (Guo *et al.*, 2018; Krug *et al.*, 2017; Li *et al.*, 2013; Tan *et al.*, 2011; reviewed in Dubnau, 2018). However, it is not clear whether RE derepression is a cause or consequence of ageing and related neurodegenerative diseases. To test the hypothesis that RE expression causes the neuronal decline associated with ageing, our strategy was to generate an heterologous system where human *L1* expression is spatially controlled in an animal *naïve* to this RE, the *Drosophila* fly. With this system, we can express human *L1* in fly neurons and quantify neuronal function of young and aged flies using a negative geotaxis assay.

3.1 UAS-L1 transgenic lines respond to neuronally-expressed GAL4

The negative geotaxis assay, also known as climbing assay, is a widely used functional assay to monitor locomotor behavior and its age-dependent decline in *Drosophila* (Figure 2.1). This assay is based on the negative geotactic response that *Drosophila* displays when startled. WT *Drosophila* respond robustly to this assay when young (e.g., 2 day-old flies after eclosion), resulting in the rapid climbing of the flies to the top of the vial. As flies age, this ability is gradually lost and they are no longer able to climb to the top of the vial (A). Hence, the longer the flies take to reach the top of the tube, the worse is their locomotor behavior. Having this and our hypothesis in mind, we expect a significantly decreased percentage of flies in the top of the tube when *L1* is expressed in the fly neurons.

To restrict the expression of *L1* to neurons in the fly, we resorted to the GAL4-UAS system (Figure 1.6) as depicted in Figure 3.1.A. For this purpose, transgenic lines containing the *UAS-L1* construct were successfully generated. In order to see whether these transgenic lines were responding to GAL4, we crossed each *UAS-L1* (hereafter, *>L1*) line with a transgenic stock line containing a pan-neuronal promoter regulating the expression of a *GAL4*, *57C10*, and determined the relative expression of *L1* in the offspring (*57C10>L1*) brain using RT-qPCR (Figure 3.1.B). The relative expression was then compared with a control driver condition in which each *UAS-L1* transgenic line was crossed with a stock line, *pBDP-GAL4*. *pBDP>* carries an "empty" GAL4 driver, identical to the *57C10>* line (*i.e.*, has the same genetic background), but without the neuronal enhancer. Hence, *pBDP>L1* flies are expected to have no (or basal) expression of *L1*. GAL4-dependent induction of *UAS-L1* expression in neurons caused an observable increase of *L1* transcription in 6 out of the 7 tested lines, ranging from 15- to 205-fold compared to the control. The two lines with the largest fold increase in *L1* mRNA levels, L1a and L1e, were used in the following experiments.

3.2 Forced expression of *L1* does not affect neuronal function

To test whether neuronal *L1a* expression has any effect on locomotor activity throughout the ageing process, we collected *57C10>L1a* flies and submitted them to the climbing assay 2, 20, and 40 days after eclosion, as portrayed in Figure 2.1. We included two additional independent neuronal drivers, *nSyb-GAL4* and *elav-GAL4* to ensure robustness of our results. The tested animals were obtained by crossing the *L1a* transgenic line with the respective driver stocks. To control for background and transgene insertion effects, we also assayed animals from several control genotypes. These included animals obtained from crosses between the driver lines and the background control stock, *w[1118]* (*nSyb>*, *elav>* and *57C10>*), between the control driver line *pBDP>* and the *>L1a* line (*pBDP>L1*), and between the >L1a stock and *w[1118]* (*>L1a*).

The forced expression of *L1* in *D. melanogaster* neurons throughout lifespan did not significantly affect the results of the climbing assay (Figure 3.2.A). Though *57C10>L1* flies had a statistically significant decrease in the locomotor ability compared to different controls at age 20 (days), the same was not observed when *L1* expression was driven by the other neuronal drivers, *nSyb>* and *elav>* (Table A.2, Appendix A). Therefore, we conclude that neuronal *L1* expression has no detectable effect on fly locomotor activity as measured in the climbing assay. This suggests that *L1* expression in neurons does not worsen the neurofunctional decline that is associated with ageing in flies.

To confirm the results obtained with the first line, we next tested the *UAS-L1e* transgenic

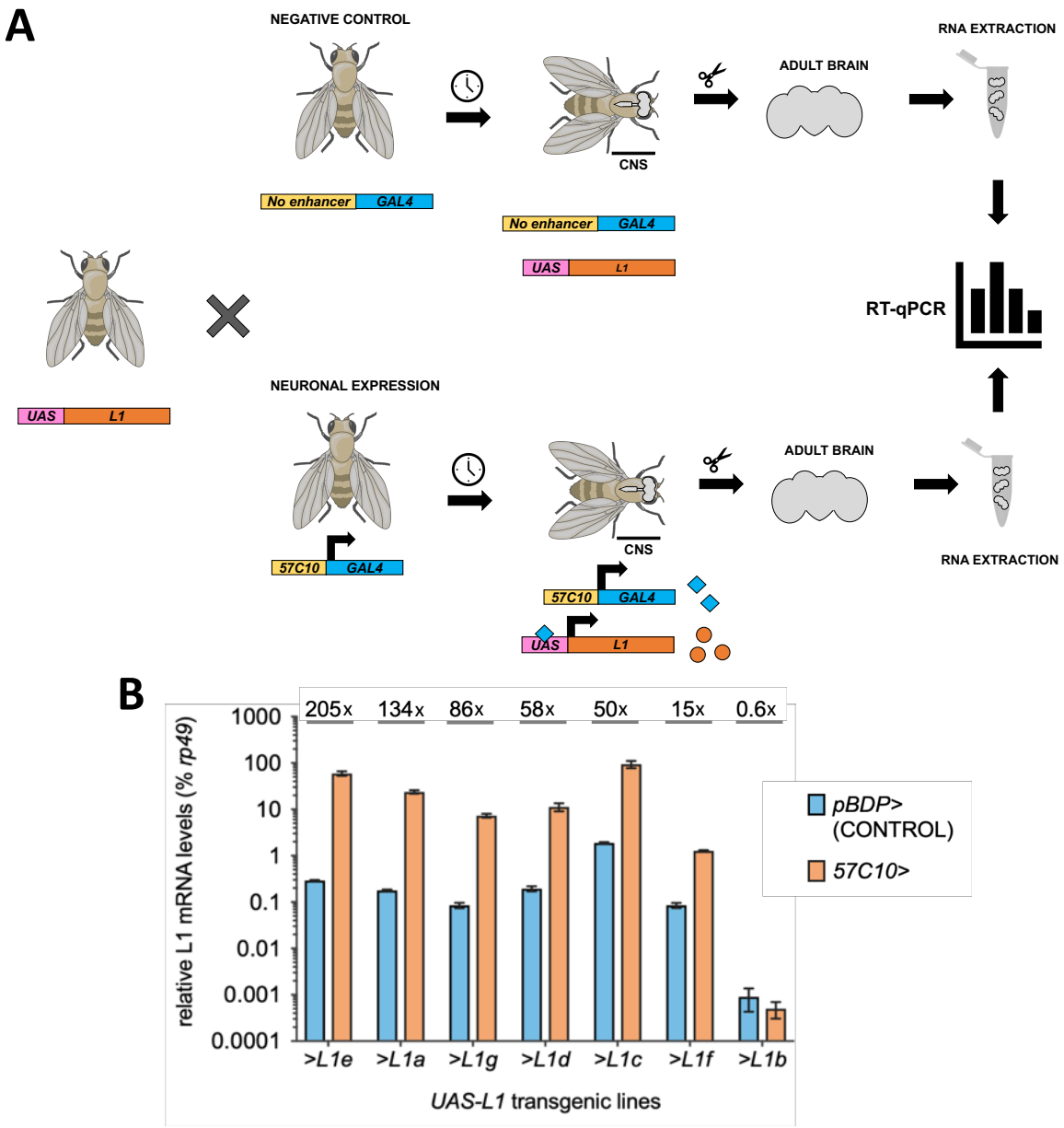

Figure 3.1: *UAS-L1* transgenic lines are responsive to a pan-neuronal GAL4 driver. (A) Schematic representation of the protocol followed to determine the responsiveness of the *UAS-L1* transgenic lines to the neuronally-expressed *GAL4*. Seven *UAS-L1* transgenic lines (*>L1a-L1g*) were crossed to the pan-neuronal GAL4 driver *57C10>* or the negative control driver (*pBDP>*). RNA was then extracted from the heads of the resulting flies and relative *L1* transcript levels were determined by RT-qPCR. (B) Bar plot of the relative abundance of *L1* transcripts, namely *L1 ORF2*, in adult heads of seven transgenic fly lines carrying independent insertions of the *UAS-L1* transgene (*>L1a-g*) crossed to the pan-neuronal GAL4 driver *57C10>* (orange bars) or the negative control driver *pBDP>* (light blue bars). Values represent geometric means ± SD of *L1* mRNA levels relative to *rp49* levels (*rp49* = 100 %). The bars have been ordered according to the largest to smallest fold-induction of *L1* expression.

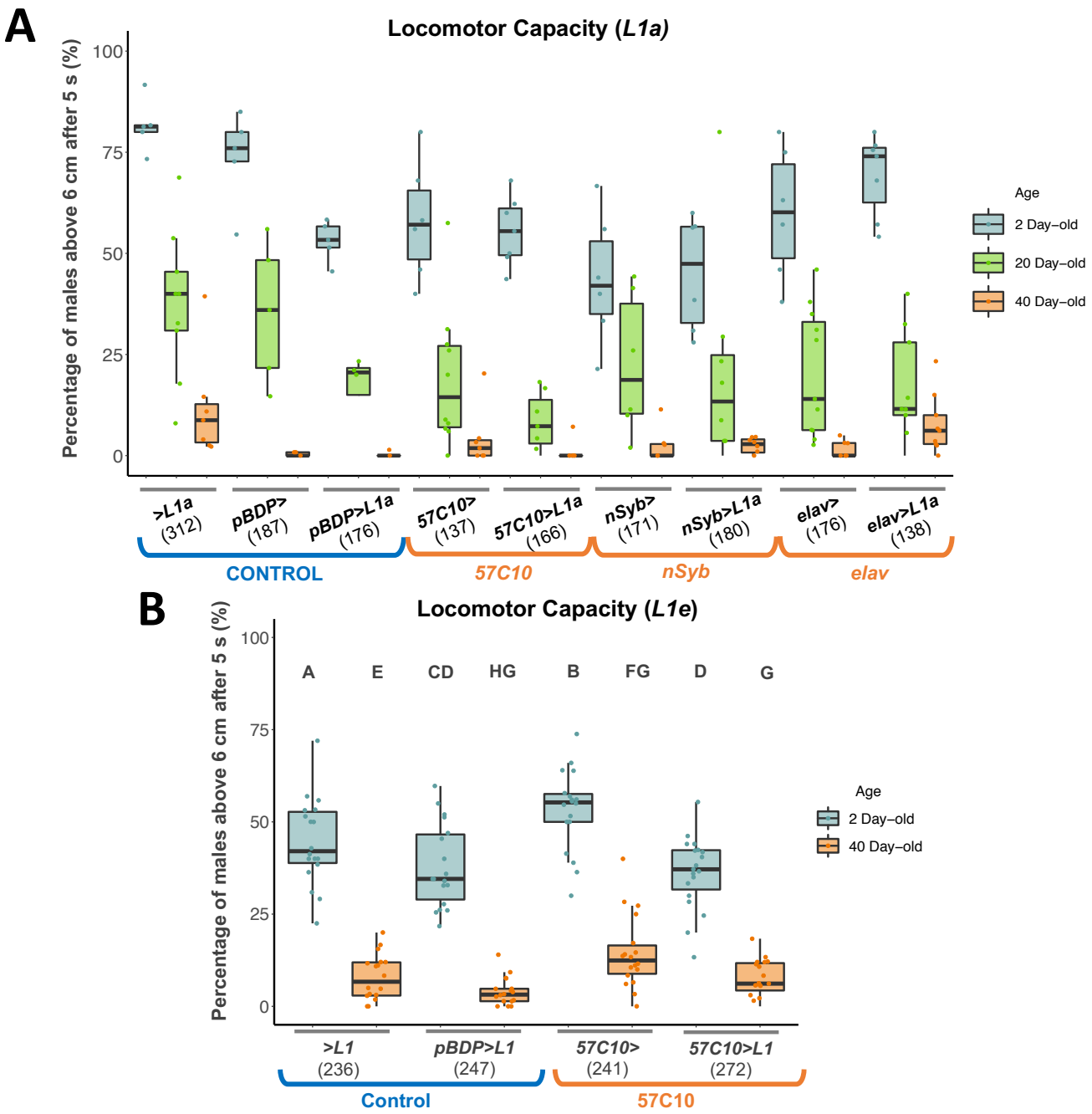

Figure 3.2: Forced expression of *L1* in *Drosophila* neurons throughout lifespan does not affect neuronal function. (A) *UAS-L1a* flies were crossed to three pan-neuronal GAL4 drivers *57C10>*, nSyb> and *elav>* or the negative control driver (*pBDP>*) or the background control (*w[1118]*). The GAL4 drivers were also crossed to *w[1118]*. The flies locomotor behavior was scored as the percentage of males above 6 cm 5 seconds after initiating the assay. (B) *UAS-L1e* flies were crossed to *57C10>*, *pBDP>* and *w[1118]*. A GAL4 driver control included the cross of *57C10>* to *w[1118]*. (A-B) Box plot showing the anti-gravitational response of male flies at 2, 20, and 40 days after eclosion or at 2 and 40 days after eclosion for A and B, respectively. Numbers below the genotype represent the number of individuals (N) tested for each genotype. Whiskers extend to the smallest and the largest value that are at most 1.5 times the IQR (Tukey's method). Black bars represent the median. Dots beyond whiskers are outliers. $p < 0.05$, Tukey's multiple comparisons test. Genotypes sharing the same letter (black) are not statistically different at $\alpha = 0.05$.

line using the *57C10>* pan-neuronal driver and assaying for locomotor behavior 2 and 40 days after eclosion (Figure 3.2.B). Again, no statistically significant reduction in locomotor activity was detectable upon *L1* expression under the control of the *57C10* driver, when compared to all controls.

Taken together, these results demonstrate that expression of human *L1* in the fly nervous system does not lead to a detectable loss of locomotor capacity. However, to conclude that increased human *L1* retrotransposition does not alter neuronal function in flies, we must demonstrate that human *L1* is capable of retrotransposition in fly neurons.

3.3 Human *L1 ORF2p* is translated in *D. melanogaster*

In order to mediate retrotransposition, *L1* must be transcribed and the peptides it encodes, ORF1p and ORF2p, translated (Moran *et al.*, 1996). Our results above demonstrate that human *L1* is transcribed in *D. melanogaster* neurons upon GAL4-dependent activation (Figure 3.1.B). Nevertheless, whether or not *Drosophila* cells are capable of translating *L1* ORFs remain to be tested. Differences in codon usage preferences or capability to recognize ribosomal entry sites could compromise the ability of the *D. melanogaster* translation machinery to translate human *L1* ORFs, especially the second ORF, *ORF2*, of the *L1* mRNA, which is already translated at a very low relative efficiency (~30% relative to *ORF1*) in human cells (Dmitriev *et al.*, 2007; Taylor *et al.*, 2013).

To test if human *L1 ORF2* is translated into ORF2p in *D. melanogaster* cells, we expressed full-length human *L1* in *Drosophila* cells *in vitro* and *in vivo* and monitored *L1 ORF2* expression by immunofluorescence assays using a specific mouse monoclonal antibody raised against the human ORF2p (Sokolowski *et al.*, 2014). The *in vitro* experiments consisted in co-transfecting DL2 cells with a plasmid containing *pUAS-L1* and a plasmid containing a constitutively-active *GAL4* (*pAct-GAL4*, hereafter *pAct-GAL4*) and collecting the cells 48 h later for the immunofluorescence assays (Figure 3.3.A). Negative controls consisted in untransfected cells and cells transfected with either plasmid alone in equimolar concentrations, as described in detail in Chapter 2, Section 2.11. The results obtained showed positively-stained cells for anti-ORF2p, albeit infrequent, only in the condition where both *pAct-GAL4* and *pUAS-L1* plasmids were co-transfected (Figure 3.3.B, Figure 3.3.C). The low frequency of ORF2p-positive cells was not due to problems related with transfection efficiency, because when we repeated the whole experiment with a transfection control plasmid, *pUAS-GFP*, we saw the same low frequency of anti-ORF2p-positive cells, despite plentiful *GFP* expression (data not shown). These results demonstrate that ORF2p can indeed be translated from full-length bicistronic *L1* mRNA in

Drosophila cells.

To test if we could detect ORF2p expression *in vivo*, we performed immunofluorescence assays with the anti-ORF2p antibody on dissected CNSs of 3rd instar *57C10>L1* larvae, and compared them to the CNS from control *pBDP>L1* larvae (Figure 3.4.A). In contrast to the *in vitro* experiments, no anti-ORF2p positive staining was detected in any condition, as shown in Figure 3.4.B. These results could either suggest that human *ORF2* translation is less efficient in neurons or that our CNS immunofluorescence conditions were not optimal for ORF2p detection.

3.4 L1 does not induce DNA damage in *Drosophila melanogaster*

Both successful and failed L1 retrotransposition attempts produce DNA damage, such as DNA DSBs (Gasior *et al.*, 2006; Belgnaoui *et al.*, 2006). We therefore considered that if *L1* was competent for retrotransposition in *Drosophila*, its expression in *Drosophila* cells should lead to a detectable increase in DSBs. To quantify DSBs formation *in vitro*, we stained *Drosophila* cells co-transfected with *pAct-GAL4* and *pUAS-L1* with a mouse monoclonal antibody raised against γ-*H2AV* and compared them with untransfected cells or a series of control transfections (Figure 3.5.A) (Lake *et al.*, 2013). γ-*H2AV* is a phosphorylated variant of the histone H2A in *Drosophila* that accumulates mainly in DSBs sites, but also in re-replication sites in endocycling cells (Rogakou *et al.*, 1998; Rogakou *et al.*, 1999; Lake *et al.*, 2013). Experimental studies in human cells have shown that retrotransposition frequency correlates with the number of γ-*H2AX* foci (H2AX is the human H2A variant involved in the process of DSB repair) (Farkash *et al.*, 2006). Figure 3.5.B shows no observable increase in the number of cells with foci, nor in the number of foci *per* cell when *pAct-GAL4* and *pUAS-L1* are co-transfected together. These results suggest that L1 proteins do not induce detectable levels of DSBs in *Drosophila* cells.

To assess DSBs formation *in vivo*, we performed immunofluorescence assays with the anti-γ-H2AV antibody on dissected CNSs of 3rd instar *57C10>L1* larvae, and compared them to the CNS from control *pBDP>L1*, *>L1*, and *57C10>* larvae (Figure 3.6.A). As demonstrated in Figure 3.6.B, the expression of *L1* in fly neurons (*57C10>L1*) is not associated with an increase in DSBs, because the anti-γ-H2AV stained CNSs of those animals show no noticeable differences when compared to the CNSs of *>L1* and *57C10>* control animals. Even though the complexity of the fly CNS represents an additional obstacle to the immunohistochemistry procedures, because the antibody has to diffuse and penetrate every cell in order to have a trustworthy staining, these results are consistent with the results obtained *in vitro* (Figure 3.5.B), and suggest that *L1* expression does not induce detectable levels of DSBs in the fly genome.

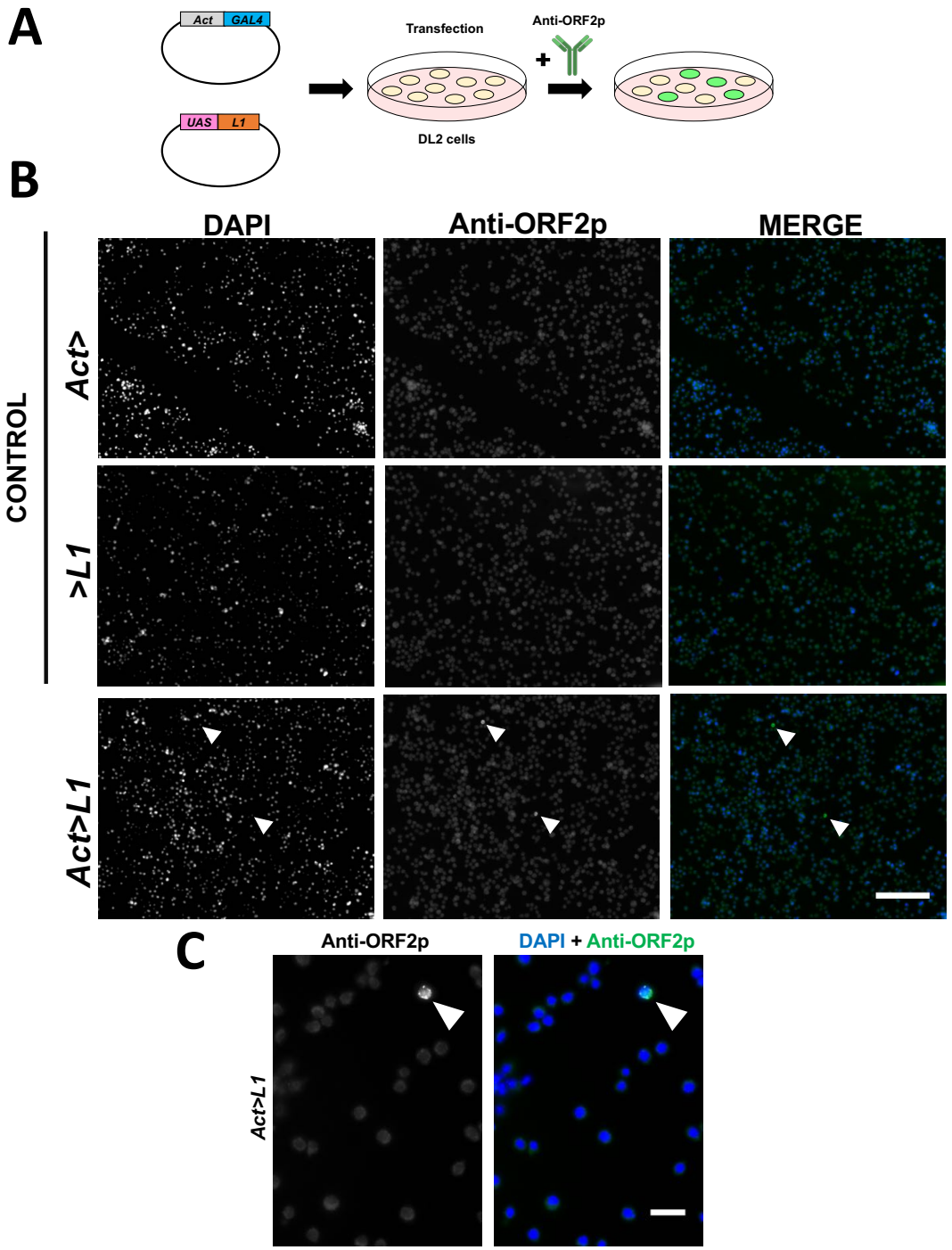

Figure 3.3: Human L1 ORF2p is translated in DL2 cells. (A) Representative scheme of the *in vitro* approach used to detect the L1 ORF2 protein (ORF2p). DL2 cells were co-transfected with equimolar concentrations of an *pActin-GAL4* (*pAct-GAL4)* and *a pUAS-L1* plasmids, and stained with a mouse monoclonal antibody raised against the human ORF2p (anti-ORF2p) followed by a secondary antibody coupled to Alexa Fluor 488 (hereafter, referred to as anti-ORF2p staining). (B) Representative fluorescence photomicrographs of cells co-transfected with the respective plasmids (left labels). Anti-ORF2p (green channel). Nuclei were counterstained with DAPI (blue). White arrowheads highlight anti-ORF2p positive cells. n = 2 technical replicates, i.e., 2 wells *per* condition. Scale bar represents 100 μm. The white arrowheads highlight the positive staining for ORF2p (cells producing ORF2p). (C) Detail of an ORF2p-positive cell. Scale bar represents 50 μm.

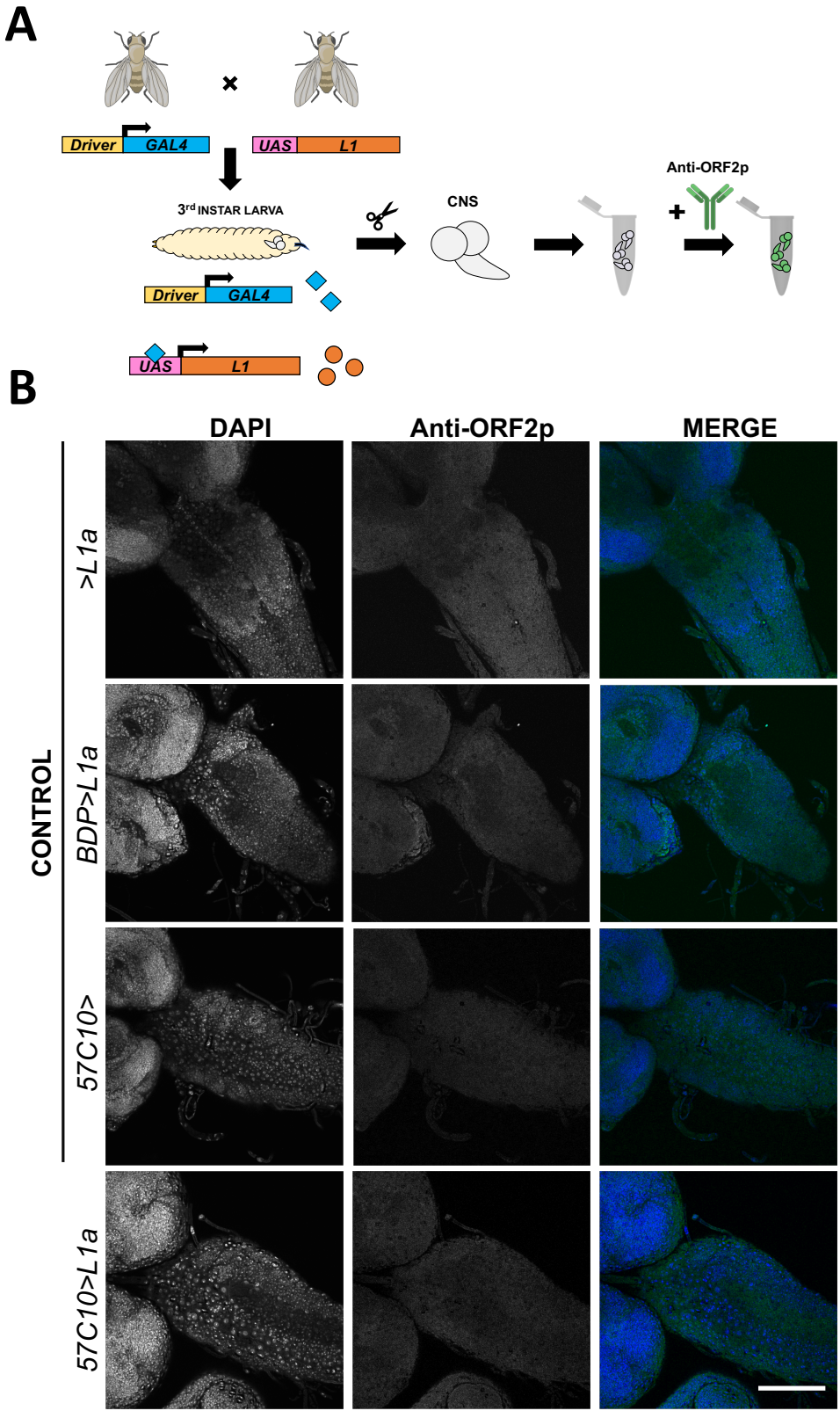

Figure 3.4: Human L1 ORF2p is not detectable *in vivo* in *Drosophila* CNS. (A) Schematic representation of the *in vivo* experiments used to detect the human L1 ORF2p. *UAS-L1a* flies were crossed to the pan-neuronal GAL4 driver *57C10>*, the control driver *pBDP>* and the background control *w[1118]*. *57C10>* crossed to *w[1118]* served as an additional driver control. The CNS of 3rd instar larvae from the previous crosses were isolated and stained with the anti-ORF2p antibody. (B) Sum of confocal Z-stack slices of 3rd instar larvae CNS with the respective labels (left), stained with anti-ORF2p (Green), and counterstained with DAPI (Blue). Scale bar represents 100 μm.

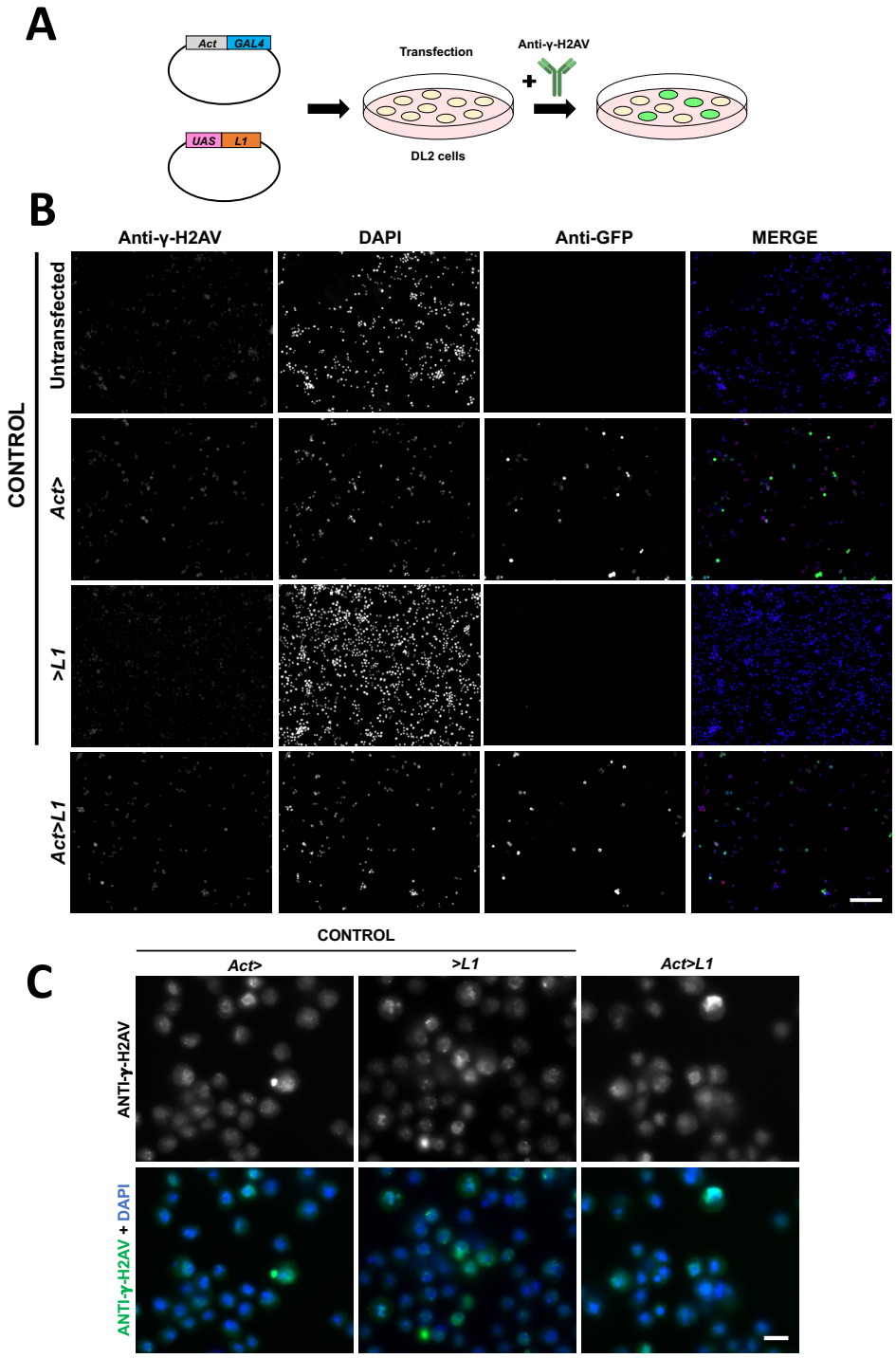

Figure 3.5: *L1* expression does not induce DSBs in DL2 cells. (A) Flow chart of the *in vitro* experimental design to detect DNA damage. Like before, DL2 cells were co-transfected with equimolar amounts of *pActin-GAL4* (*pAct-GAL4 pUAS-L1*, and *pUAS-GFP* plasmids, and stained with a mouse monoclonal antibody raised against γ-H2AV (used here as a biomarker for DSBs followed by a secondary antibody coupled to Alexa Fluor 594 (hereafter referred to as anti-γ-H2AV staining). (B) Representative fluorescence photomicrographs of cells transfected with the respective plasmids (left labels). *pUAS-GFP* serves as control for transfection (green) and was added in every condition except in the untransfected control. Anti-γ-H2AV (red channel). Nuclei were counterstained with DAPI (blue). n = 2 technical replicates. Scale bar represents 100 μm. (C) Detail of anti-γ-H2AV staining (green). DAPI (nuclei, blue). Scale bar represents 10 μm.

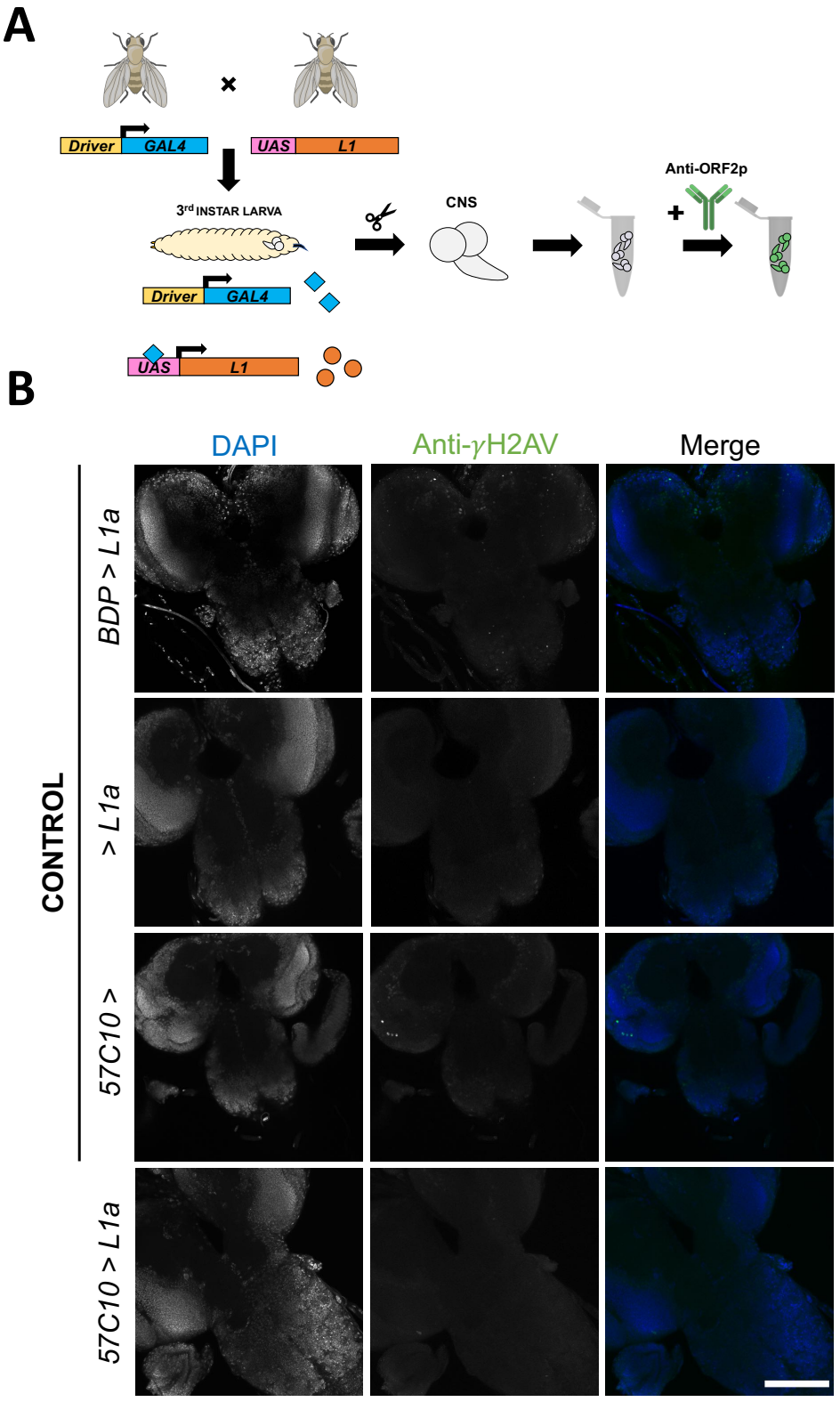

Figure 3.6: *L1* expression does not induce DSBs in the *Drosophila* larval CNS. (A) Representative illustration of the experimental design used to detect DNA damage *in vivo*. CNSs of 3rd instar larvae were collected and stained with the anti-γ-H2AV antibody. The genotypes analyzed were the same as Figure 3.4.B: *57C10>L1* and the controls *>L1*, *pBDP>L1*, and *57C10>*. (B) Sum of confocal z-stack slices of 3rd instar larvae CNS with the respective labels (left). anti-γ-*H2AV* (green), DAPI counterstain (nuclei, blue). Scale bars represent 100 μm.

3.5 Tub-Alu[Act-sfGFP] transgenic lines constitutively express *Alu*.

Besides mediating self-retrotransposition *in cis*, human *L1s* also non-autonomously retrotranspose SINEs (Dewannieux *et al.*, 2003, Dewannieux and Heidmann, 2005, Hancks *et al.*, 2011, Hancks *et al.*, 2012). Having this in mind, we generated a retrotransposition reporter element based on the human SINE, *Alu*, whose cellular readout is the production of GFP. As illustrated in Figure 3.7.3, we placed a GFP reporter cassette inside the *Alu* sequence at a position towards its 3' region, which should not interfere with *Alu* activity (Dewannieux *et al.*, 2003). The GFP reporter cassette comprises a *sfGFP* sequence preceded by the constitutive *Drosophila actin* promoter and followed by an eukaryote transcription termination signal, *SV40* (*Act-sfGFP)*. The *sfGFP* sequence is disrupted by the *MhcInt16* intron from *D. melanogaster*, which was placed in the opposite direction of the *Act-sfGFP* sequence (Figure 2.3). To ensure the production of the reporter element, *Alu[Act-sfGFP]*, we put it under the regulatory control of the constitutive *Drosophila Tubulin* promoter (*Tub-Alu[Act-sfGFP]*). Therefore, retrotransposition can be monitored by direct inspection for GFP activity in transfected cells *in vitro*, or in cells of transgenic animals *in vivo* (Figure 3.9).

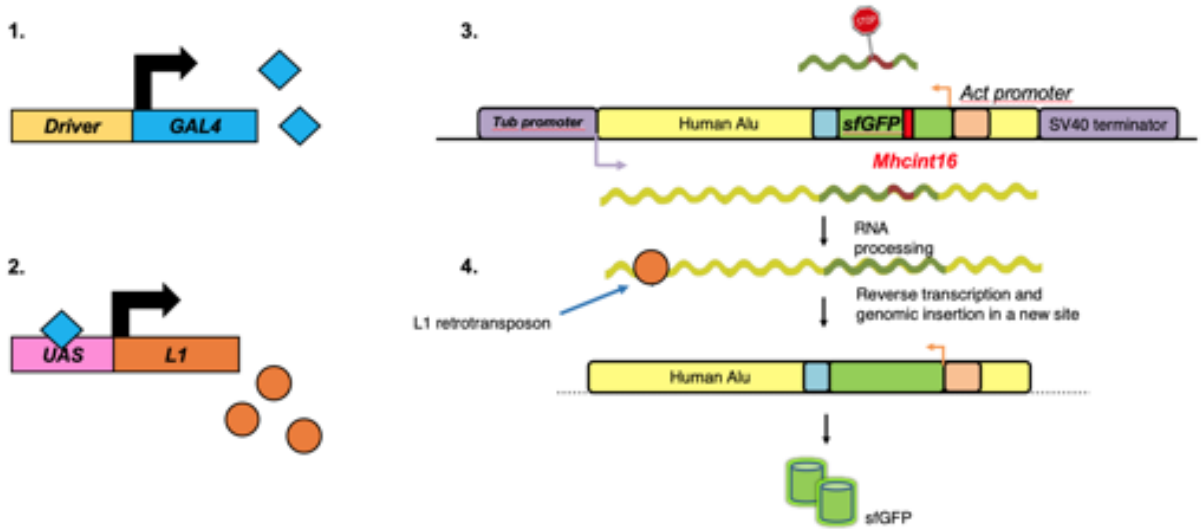

Figure 3.7: Schematic illustration of the human LINE/SINE system used in the retrotransposition assays. (1) A promoter drives the expression of *GAL4* (blue diamond), which (2) binds to an *UAS* sequence, leading to the expression of L1 proteins (orange circles). (3) A *Drosophila Tubulin* promoter (*Tub*) regulates the expression of the human SINE, *Alu* (*Tub-Alu[Act-sfGFP]*), which has a *sfGFP* cassette in the opposite sense. The *sfGFP* cassette comprises the constitutive *Drosophila Act5c* promoter followed by a *sfGFP* sequence which is interrupted by the inverted *Drosophila MhcInt16* ([*Act-sfGFP*]). (4) Only after splicing and a successful round of retrotransposition (mediated by L1 proteins), the sfGFP protein (green cylinder) will be produced.

We started by testing the system *in vitro* by co-transfecting DL2 cells with equimolar amounts of *pTub-Alu[Act-sfGFP]*, *pUAS-L1*, and *pAct-GAL4* plasmids. However, we were unable

to detect the production of intronless *sfGFP* in a reproducible manner, neither by immunocytochemistry procedures, nor via amplification of the gDNA extracted from transfected cells (data not shown). While a positive GFP-stained cell would have been proof of retrotransposition, there is less confidence to conclude that the absence of GFP-positive cells means that there is no retrotransposition *in trans* between L1 and *Alu* in *Drosophila* cells. Many factors can affect this result, such as the limited frequency of triply co-transfected cells and the fact that successful retrotransposition of full-length elements is not expected to be a highly frequent event.

The limitation of co-transfections could be surmounted *in vivo* by generating transgenic animals. Thus, we generated and characterized transgenic flies carrying the *Tub-Alu[Act-sfGFP]* construct. As shown in Figure 3.8, *Alu[Act-sfGFP]* transcripts were detected in all tested lines, although their abundance varied 5 orders of magnitude. This variation in expression level could be due to positional effects of the transgene insertions.

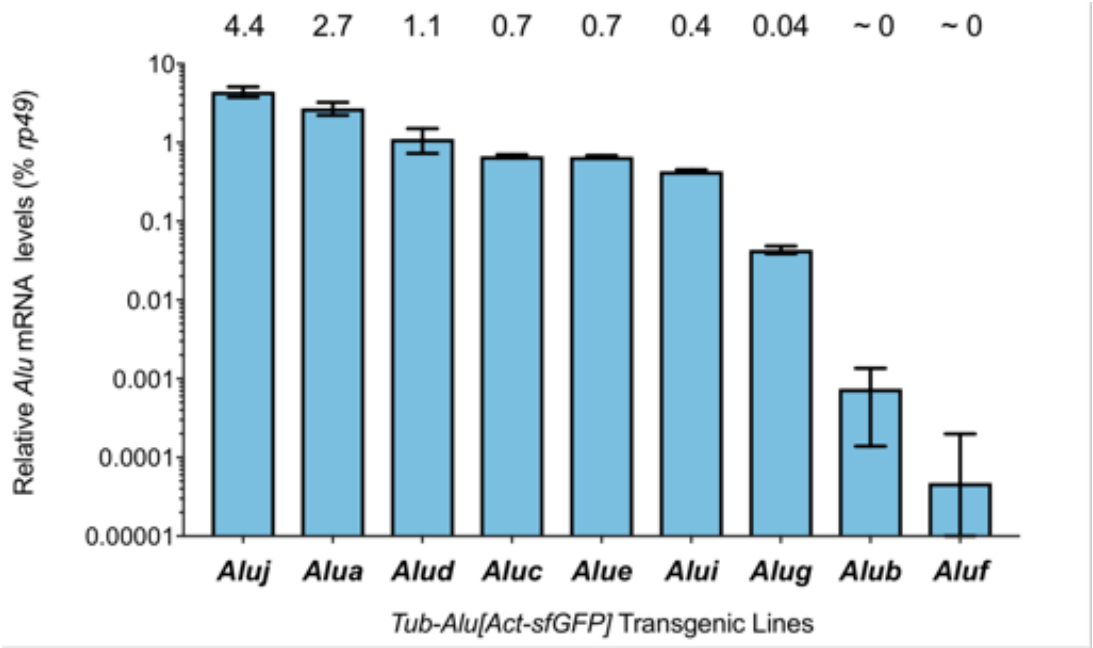

Figure 3.8: *Alu* expression levels in *Tub-Alu[Act-sfGFP]* transgenic lines. Bar plot with the relative *Alu* mRNA levels (determined by RT-qPCR) in adult heads of nine transgenic fly lines carrying independent insertions of the *Tub-Alu[Act-sfGFP]* transgene. Values represent the geometric mean ± SD of three technical repeats of estimated *Alu* mRNA levels relative to *rp49* mRNA levels(*rp49* = 100 %). Data was sorted in a descending order according to the relative *Alu* mRNA levels (% *rp49*, above the bars).

3.6 Human L1 does not retrotranspose *Alu* in fly neurons.

To directly test the *L1/Alu* system *in vivo*, we generated a line expressing the *UAS-L1a* and *Tub-Alu[Act-sfGFP]* transgenes (by crossing UAS-L1a flies to Tub-Alub flies; here referred to

as *UAS-L1a* and *Tub-Alu[Act-sfGFP]*) (Table 2.1), which we crossed to a stock carrying the pan-neuronal GAL4 driver, *57C10>*, or to the negative GAL4 driver control, *pBDP>*. Then, the CNS of 3rd instar larvae and the brain of 3-7 day-old adult flies resulting from the previous crosses were isolated and stained with a rabbit polyclonal antibody raised against GFP, anti-GFP (Figure 3.9.A).

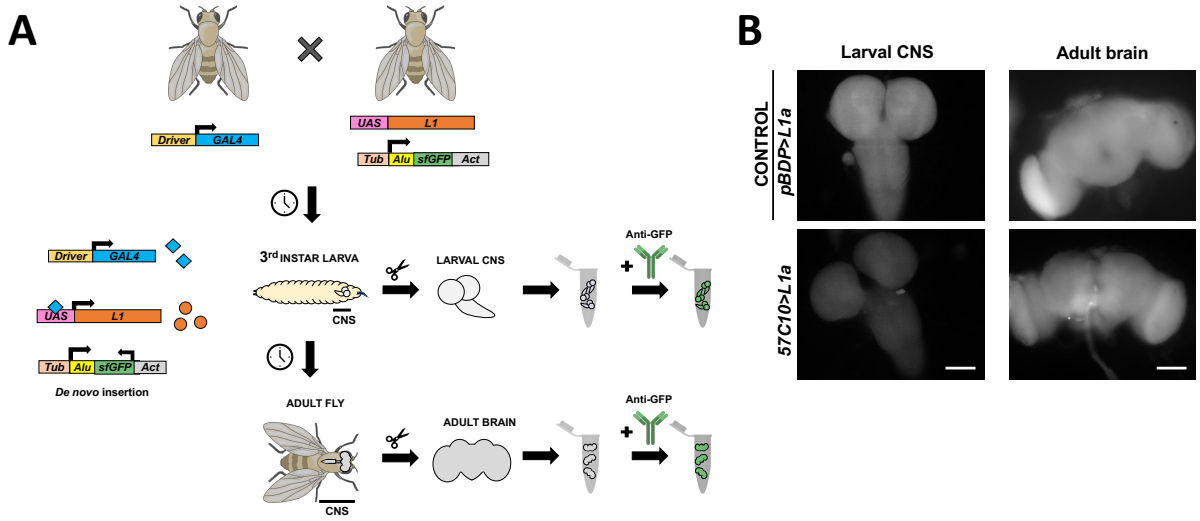

Figure 3.9: Human L1 does not mediate *Alu* retrotransposition *in trans* in fly neurons. (A) Schematic diagram of the retrotransposition assays performed *in vivo*. Flies containing both *UAS-L1a* and *Tub-Alu[Act-sfGFP]* transgenes were crossed to the pan-neuronal GAL4 driver line *57C10>* or to the negative driver control line *pBDP>*. The CNS of 3rd instar larvae and the brain (CNS without the ventral nerve cord) of adult individuals (aged between 3-7 days) resulting from the previous crosses were stained with a rabbit polyclonal antibody raised against GFP, anti-GFP, followed by a secondary antibody coupled with Alexa Fluor 488 (hereafter referred to as anti-GFP staining). (B) Representative fluorescence photomicrographs of 3rd instar larvae CNS (left panel) and adult brains (right panel) of the respective genotypes (labels on the left). Anti-GFP staining (green). Scale bars represent 100 μm

We detected no difference in anti-GFP staining in the larval CNS or adult brain of *57C10>L1a* animals compared to the CNS and adult brains of *pBDP>L1a* control animals (Figure 3.9.B). Consistently, the same results were observed in *nSyb>L1a* animals (data not shown). These results suggest that L1 is not able to mediate *Alu* retrotransposition *in trans* in *Drosophila* neurons.

3.7 ORF2p cannot retrotranspose a human *Alu* in *D. melanogaster*.

We next sought ways to overcome the inefficient translation of the human ORF2p from the *L1* bicistronic mRNA in *Drosophila*. The rationale was that a higher efficiency of ORF2p translation could lead to higher, and therefore, detectable, retrotransposition rates. According

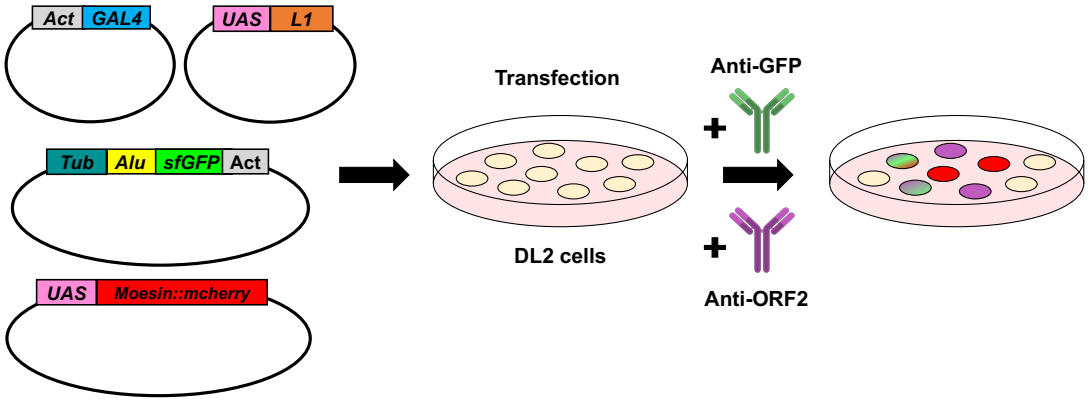

Figure 3.10: Rationale of the retrotransposition assay performed in DL2 cells. Representative scheme of the *in vitro* approach used to detect the successful retrotransposition events using the LINE/SINE reporter system. DL2 cells were co-transfected with equimolar concentrations of *pActin-GAL4*, *pUAS-ORF2*, *Tub-Alu[Act-sfGFP]*, and *pUASp-moesin::mCherry* plasmids, and stained with a rabbit polyclonal antibody raised against GFP (anti-GFP) followed by a secondary antibody coupled to Alexa Fluor 488 and with anti-ORF2p staining as described in Figure 3.3.

to Dewannieux *et al.* (2003), the human L1 ORF2p is sufficient to mediate the non-autonomous retrotransposition of human *Alu*. Therefore, we attempted to increase ORF2p levels by facilitating its translation by replacing the full-length *L1* (*pUAS-L1*) by the *ORF2* sequence alone (*pUAS-ORF2*) (Figure 3.10). The idea is that higher ORF2p translation rates would occur if the *ORF2* was the first ORF to be translated, rather than being second in the bicistronic *L1* mRNA.

We found that the number of DL2 cells stained with anti-ORF2p was higher and similar to the number of membrane-organizing extension spike protein (moesin)::mcherry-positive cells when *pAct-GAL4* was co-transfected with *pUAS-ORF2* instead of *pUAS-L1* (Figure 3.11.A - last panel row, Figure 3.3.B). Thus, with this strategy we were able to overcome the human *L1 ORF2* low translation efficiency observed before (Figure 3.3.B and 3.3.C). Very rarely, we found one or two cells positive for anti-ORF2p when DL2 cells were co-transfected with *pUAS-ORF2* and *pTub-Alu[Act-sfGFP]* but without *pAct-GAL4* (Figure 3.11.A, second row in the panel). This is interpreted as leakiness of the pUAS-plasmid.

As regards the *pTub-Alu[Act-sfGFP]* retrotransposition reporter, results of the transfection experiments demonstrate similar anti-GFP staining in cells regardless of the presence or absence of *pUAS-ORF2* in the co-transfection mixture (middle vs. bottom panel rows; Figure 3.11.A and Figure 3.11.B). Similar results were obtained when cells were collected 96 h after transfection (data not shown). Furthermore, we were unable to detect the production of intronless sfGFP via amplification of the gDNA extracted from transfected cells (data not shown). These results suggest that ORF2p is insufficient to retrotranspose *Alu* in *Drosophila* DL2 cells.

Curiously, co-transfected cells with both *pUAS-ORF2* and *pAct-GAL4* displayed a reduction

in the intensity of moesin::mcherry (Figure 3.11.B). We also noticed that the number and the length of DL2 cells projections *per se* appear to be reduced when ORF2p is produced, but it is difficult to exclude that this is an impression caused by the reduced expression of moesin::mcherry *per se*. Regarding these observations, we speculate that ORF2p might somehow interfere with moesin::mcherry production or stability.

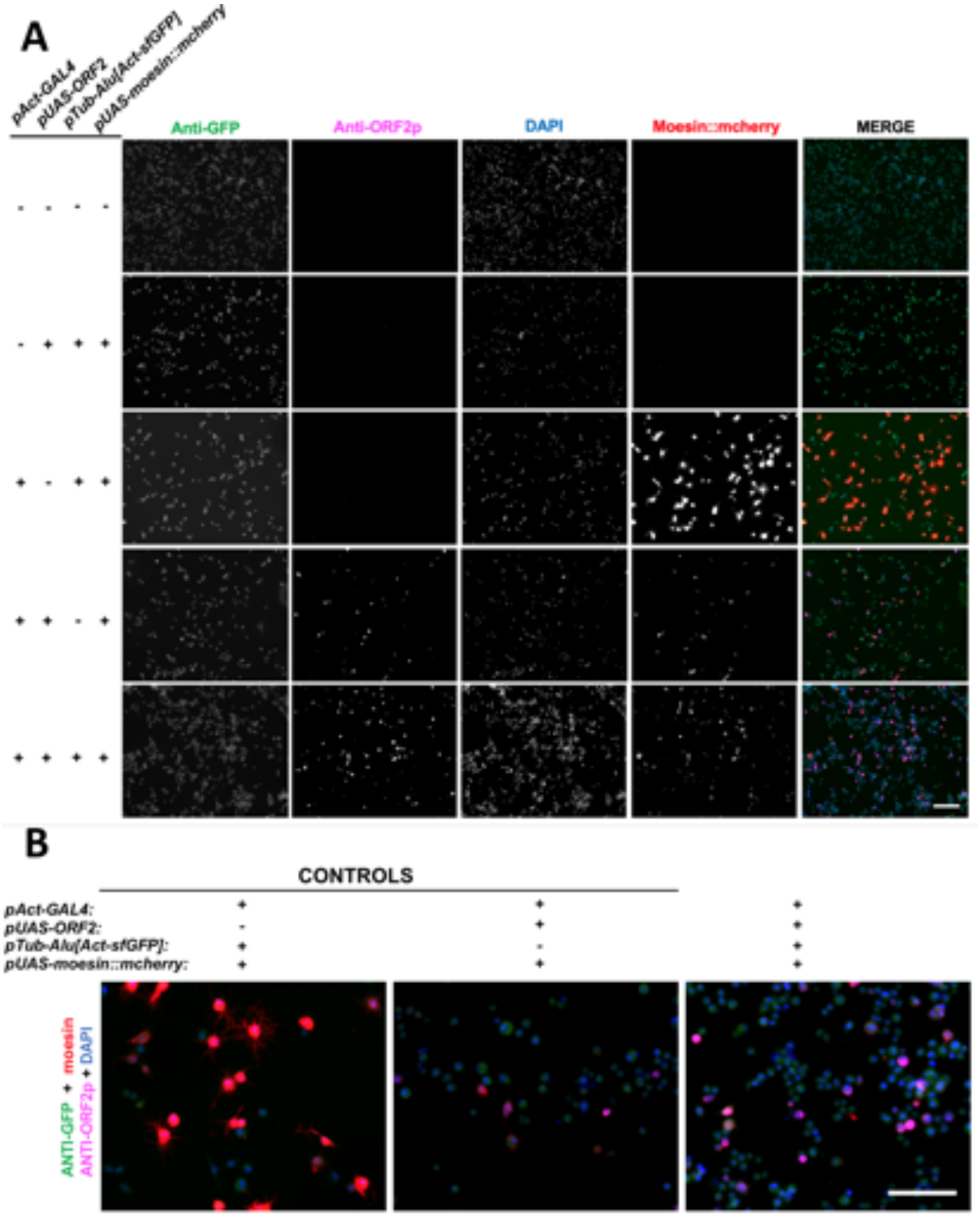

Figure 3.11: Human ORF2p is not sufficient to retrotranspose *Alu* in DL2 cells. (A) Representative fluorescence photomicrographs of the retrotransposition assay performed in DL2 cells to detect GFP production. Expression of ORF2p instead of full-length *L1* results in a higher efficiency of translation (magenta) but it is not sufficient to retrotranspose *Alu* in *D. melanogaster* (green). *pUASp-moesin::mcherry* (in red) is a transfection control plasmid that highlights the actin cytoskeleton of DL2 cells. The presence of *L1* leads to a decrease in moesin fluorescence intensity. n = 2 technical and 2 biological replicates per condition. +,- represent the presence or absence of the plasmid, respectively. Scale bars represent 100 μm (B) Fluorescence photomicrographs detailing transfected DL2 cells more closely. Scale bars represent 100 μm.

Chapter 4

Discussion

Ageing is the main risk factor for developing neurodegenerative diseases. Previous studies have suggested that during ageing, the RE repressing mechanisms tend to fail and REs become activated (De Cecco *et al.*, 2013a; De Cecco *et al.*, 2013b; Li *et al.*, 2013; The *et al.*, 2017; Van Meter *et al.*, 2014). In addition, the RE-mediated genomic instability has been associated with the age-dependent decline in neuronal function seen in neurodegenerative diseases (Li *et al.*, 2013; Tan *et al.*, 2011; The *et al.*, 2017; reviewed in Dubnau, 2018; Erwin *et al.*, 2014; Gorbunova *et al.*, 2014). However, it is still unclear whether RE expression is a causal agent or a consequence of the age-dependent decline in neuronal function. In an attempt to clarify the relationship between RE expression and the age-dependent decline in neuronal function, we have constructed an unique heterologous RE system that allows the expression of a human *L1* in a tissue-specific manner in *D. melanogaster*. We found out that forced expression of human *L1* in the fly neurons throughout lifespan does not affect neuronal function. However, we did not find any evidence supporting the ability of L1 to retrotranspose itself *in cis*, or a human *Alu in trans*, in either DL2 cells or the fly CNS. Recent studies have shown that both human and mice *L1* remain capable of mediating retrotransposition when introduced into chicken cells (Suzuki *et al.*, 2009; Wallace *et al.*, 2008a). Despite being warm-blooded vertebrates, birds are evolutionary distant from mammals and do not encode homologues of the human *L1* in their genomes. As highlighted by Wagstaff *et al.* (2011), the work developed by Wallace *et al.* (2008a) suggests that *L1* (and *Alu*) retrotransposition would not require species-specific interaction with additional host factors. Therefore, we hypothesized that if flies were able to

transcribe and translate the bicistronic *L1* mRNA into correctly folded ORF1p and ORF2p, then L1 retrotransposition would occur similarly in flies. In fact, previous research has demonstrated that *Drosophila* cells are able to transcribe and translate human genes into functional proteins (Krug *et al.*, 2017). Furthermore, because *L1* is extraneous to the surveillance mechanisms that repress RE expression in *D. melanogaster*, *L1* would not be repressed by these mechanisms. As seen in Figure 3.1, Figure 3.3, and Figure 3.11, flies are indeed capable of transcribing and even translating ORF2p, the second protein from the human *L1* bicistronic transcript. However, the forced expression of *L1* in the fly neurons had no reproducible effect on neuronal function as determined by the negative geotaxis assay (Figure 3.2), thereby suggesting that this heterologous system is not completely functional in *D. melanogaster*. This idea of an impaired system was further corroborated by both *in vitro* retrotransposition assays (Figure 3.5 and Figure 3.11) and *in vivo* experiments (Figure 3.6 and Figure 3.9). Taken together, the results herein obtained suggest that the system built to study retrotransposition is nonfunctional and impaired at a post-translational level since we were able to detect both *L1* transcripts and ORF2p.

Several possible explanations for why the system does not work include piRNAs cross-reaction, the high complexity of the developed system, gain of resistance against *L1* and *Alu* elements by the flies, acquisition of a mutation in any of the *ORF1* and *ORF2* sequences, and requirement of additional factors in the retrotransposition process.

A reasonable explanation would be the cross-reaction of piRNAs. Albeit flies do not have a *L1* homologous element, they do have LINE-like elements that are targeted by piRNAs. Therefore, it is possible that *L1* and some of the LINE-like elements share conserved sequences and as such, piRNAs targeting that conserved region could be cross-reacting, targeting *L1*, and mediating its silencing. However, as stated by Siomi *et al.* (2008), piRNAs are not detected in DL2 cells, and ergo, piRNA-mediated silencing would not explain why we were not able to detect evidence of L1 activity *in vitro*.

Acquisition of resistance against the REs used in the retrotransposition system would be an improbable justification for the system's malfunction due to many reasons. Firstly, the main mechanism through which *L1* is repressed in the human is through DNA methylation of the CpGs present in its promoter, however this mechanism of RE repression is absent in the flies. Secondly, although studies have shown that *Drosophila* is able to acquire resistance against TEs, that resistance is mediated by piRNAs (Vanssay *et al.*, 2012). Therefore, for flies to acquire resistance against *L1* (or *Alu*), L1 elements would have to retrotranspose and reintegrate within a piRNA cluster in the fly genome. Since L1 shows no preference for integration sites

and the insertion in the genome is random, re-integration in a piRNA cluster would be infrequent. Besides, given that the *UAS-L1* transgenic lines are maintained without GAL4 (Table 2.1), the reintegration of *L1* in a piRNA cluster in these lines is highly inconceivable because only residual, if any, *UAS-L1* is transcribed in the absence of GAL4.

Another possible explanation to consider is the occurrence of a mutation in either *ORF1* or *ORF2* genes, thereby interfering with the protein functions. The idea of a mutated *ORF1* sequence can be rejected because it would not explain why the retrotransposition assay with the *pTub-Alu[Act-sfGFP]* as a reporter failed, given that ORF2p has been reported to be sufficient to mediate Alu retrotransposition (Figure 3.11) (Dewannieux *et al.*, 2003; Wallace *et al.*, 2008a). Regarding ORF2p coding sequence, it is known that both endonuclease and reverse transcriptase domains are crucial to mediate retrotransposition of both *L1* and *Alu* (Moran *et al.*, 1996). Spontaneous mutations in *D. melanogaster* are rare and it would be even rarer to have two mutations in both domains given their proximity (less than 1 kb) (Keightley *et al.*, 2013). Although the occurrence of a mutation in the endonuclease domain could explain why we did not see an increase in the H2AV staining in DL2 cells or flies expressing *L1* (Figure 3.5.B and Figure 3.6.B), it is known that endonuclease-independent retrotransposition events do occur and L1 can integrate itself (or *Alu* elements) in sites where there was a DSB (Morrish *et al.*, 2002). However, a mutation in the reverse transcriptase domain would also render the system nonfunctional, hindering the retrotransposition of both *L1* and *Alu*. Without this functionality, the system would not function due to the incapability of reverse transcribing the retrotransposons. Notwithstanding that mutations do occur at a very low frequency, it would be very unlikely that all the plasmids, as well as the different flies used throughout the experiments, would have accumulated mutations that would render them all nonfunctional. In fact, since we are evaluating somatic transposition, all the cells in a fly would have a mutated *L1*, which implicates that this mutation would have occurred in the fly germline and then transmitted to the progeny, and by chance all the flies of the population would have inherited the mutated allele. This would require many generations to occur, which makes it very unlikely given the time of their existence.

Therefore, the results seem to point towards the idea that other factors besides L1 proteins themselves might be involved in the retrotransposition process. Such factors would be present in human, mouse, and chicken, but not in flies. It is becoming clearer that different host factors are involved in *L1* retrotransposition (Suzuki *et al.*, 2009; Taylor *et al.*, 2013; Taylor *et al.*, 2018). In the last two decades, several studies have been trying to unravel the L1 interactome and it has been shown that the host factors seem to interact and interfere mostly with the L1

RNP (Peddigari *et al.*, 2013; Suzuki *et al.*, 2009 Taylor *et al.*, 2013; Taylor *et al.*, 2018). Recent research on L1 activity has shown that in order to successfully retrotranspose, L1 requires phosphorylation of ORF1p (Cook *et al.*, 2015). Cook *et al.* (2015) showed that although the non-phosphorylation of ORF1p does not affect the binding capacity of the protein, retrotransposition of *L1* is prevented. In the same study, the authors showed that phosphorylated ORF1p interacts with a protein involved in cellular phosphorylation regulatory cascades (Cook *et al.*, 2015). In a different study performed with human cells, it was shown that L1 ORF2p requires the binding of a specific host protein, polymerase-delta-associated sliding DNA clamp (PCNA) (Taylor *et al.*, 2013). By mutating *ORF2* to prevent ORF2p of binding to PCNA, the authors noticed that retrotransposition was decreased significantly. Downregulation of the protein also led to the downregulation of *L1* (Taylor *et al.*, 2013). Given that different proteins interact with L1 throughout retrotransposition, it is conceivable that some of the interacting proteins might not be present in *D. melanogaster*, resulting in the non-functionality of the system and explaining why we were not able to detect successful retrotransposition events.

The reason why this work stands out from the other RNAi- and inhibitor-based studies on REs and neurodegeneration is the fact that this system theoretically does not affect the function of other host proteins (Krug *et al.*, 2017; The *et al.*, 2017). However, this system also has limitations. Firstly, flies and humans (or mammals in general) are evolutionarily very distant, which might be translated in differences in what concerns cellular factors between these two organisms. In addition, there are no *L1* nor *Alu* homologs in flies. Therefore, if *L1* retrotransposition requires additional host factors, these might be lacking in flies. Secondly, the *L1* construct used in this work was codon-optimized for humans by Wagstaff *et al.* (2011). Hence, differences in the codon usage could hamper the translation efficiency of our constructs, namely when in full length. Lastly, retrotransposition events are already rare in humans and with the introduction of a human-optimized *L1* into *D. melanogaster* genome, chances are that the retrotransposition frequency decreases even more. Additionally, it could be that the absence of detection of retrotransposition events in the fly CNS was due to the complexity and density of the tissue, which would hinder the access of the antibody to the cells and, consequently, to the target proteins. However, this is very unlikely since the samples were incubated with the antibodies for at least 2 days to avoid that problem. In what concerns *in vitro* experiments, the complexity of the LINE/SINE reporter system represents a great challenge to DL2 cells. The system requires the use of three different plasmids (*pAct-GAL4*, *pUAS-L1*, and *pTub-Alu[Act-sfGFP]*, Table A.1, Appendix A) that must be incorporated by the same cell in order to be able to detect a successful retrotransposition event. At the same time, the cells need to incorporate

sufficient number of plasmids to guarantee a detectable production of the plasmid products (GAL4, *Alu* mRNA and L1 ORF2p).

In conclusion, throughout this dissertation we have generated and used an inducible heterologous system to study the causal relationship between RE expression and neurodegeneration in *D. melanogaster*. Our results revealed that forced expression of *L1* may not be a cause of the neuronal function decline associated with age. However, despite the several attempts, we failed to show evidence supporting the functionality of our experimental system. Nevertheless, this study showed that flies are able to transcribe and translate human-optimized *L1* sequences (reinforcing the idea that flies are a good alternative model - and more ethically recommended) to study human gene expression. This study raised several questions and challenges for the future such as: why is *L1* unable to mediate retrotransposition in flies? Why can *L1* retrotranspose in chicken, but not in fly cells? How could we increase ORF2p translation efficiency while maintaining ORF1p production? Can L1 retrotranspose in fish (zebrafish) or amphibians (e.g., frogs)? Is *L1* retrotransposition trully dependent on additional host factors? If so, what are they? One thing is clear, *L1* and TEs in general, are becoming more and more implicated in the ageing process and in the development of sporadic form of neurodegenerative diseases. Therefore, it is important to understand their role in these deteriorating processes and try to find ways , if possible, to prevent, slow down, or mitigate the progress of these diseases.

In the future, we intend to clarify whether *L1* is or not able to retrotranspose in *D. melanogaster*. Therefore, it is important to guarantee that all the components of the built system function as expected. Extraction of gDNA from UAS-L1 flies and sequencing of the *L1* sequence, as well as the use of an antibody that recognizes specifically a different region of the *ORF2p*, downstream of the endonuclease domain as for instance an anti-reverse-transcriptase, would help in determining whether the proteins are truncated, mutated or fully functional. Although we have used an anti-ORF2p which specifically binds to the endonuclease domain of the ORF2p, we are aware that *L1* has several weak transcriptional stop signals throughout its coding sequences, which can often result in the formation of shortened transcripts carrying only the endonuclease domain (Kines *et al.*, 2014). It would also be important to investigate the *L1 ORF2/Alu in vivo*, perform a negative geotaxis assay, and look for any phenotypic effect in the neuronal function decline associated with age. At the same time, the construction of a *pUAS-L1* reporter element would be important because it would lower the complexity of the reporter assay by removing one plasmid, the SINE reporter element (*pTub-Alu[Act-sfGFP]*), from the transfection conditions.

In order to test our new hypothesis, that *L1* requires additional factors to undergo retrotransposition, research on the differences between the interactomes of *L1* in human and DL2 cells via immunoprecipitation procedures would help determining whether there are factors required for *L1* retrotransposition. We have seen that when cells were co-transfected with *pUAS-ORF2* and *pAct-GAL4*, the intensity of moesin decreased. It would be interesting to see if different concentrations of the *pUAS-ORF2* plasmid have any effect in the amount of moesin (or actin) that is produced in the cell. Quantification of the protein could be achieved by performing a Western Blot to quantify protein concentration. Moreover, we intend to inspect whether L1 ORF2p interacts or not with any of the actin cytoskeleton components, therefore disturbing its dynamics.

Finally, to further investigate the role of REs in the neurofunctional decline associated with age, it would be interesting to incorporate another organism (other arthropods, which are evolutionarily close to *D. melanogaster*, or zebrafish) TEs in our system and look for evidence of retrotransposition, and, if possible, look for an effect in the neuronal function decline associated with age.

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Appendix A

Supplemental Material

Table A.1: List of the plasmids used to inject the flies and to transfect DL2 cells.

Short name (ID)	Name	Size (bp)	Origin
pAct-GAL4	*pAC-Gal4*	9094	Maria Dominguez's lab (CSIC-UMH)
pAct-sfGFP	*EcoRI-XbaI-AgeI-Act5pro-AgeI-sfGFP-InvMhcInt16-XhoI-SV40polyA-NcoI-NotI_in_pUC57*	4549	Alisson Gontijo's lab (CEDOC)
pTub-Alu[Act-sfGFP]	*tub_prom-KpnI-AluYb8-AgeI-Act5pro-AgeI-sfGFP-InvMhcInt16-XhoI-SV40polyA-NcoI-AluYb8-AvrII-AgeI-PolyA-NotI_in_pCaSpeR4*	12800	Alisson Gontijo's lab (CEDOC)
pUAS-GFP	*pJFRC-10xUAS-IVS-myr::GFP*	8800	Addgene # 26222
pUAS-L1	*pUASTN_L1(ORF1+2)*	13832	Alisson Gontijo's lab (CEDOC)
pUAS-L1PA1	*pBS-L1PA1-CH-mneo*	11342	Addgene # 21588
pUAS-ORF2	*pUASTN2-L1_ORF2*	12843	Alisson Gontijo's lab (CEDOC)
pUASp-moesin	*pUASp-moesin::mCherry*	11061	Alisson Gontijo's lab (CEDOC)
pUAST	*pUAST-empty*	8900	Addgene # 24344
pUASTN	*pUASTN-empty*	8900	Alisson Gontijo's lab (CEDOC)

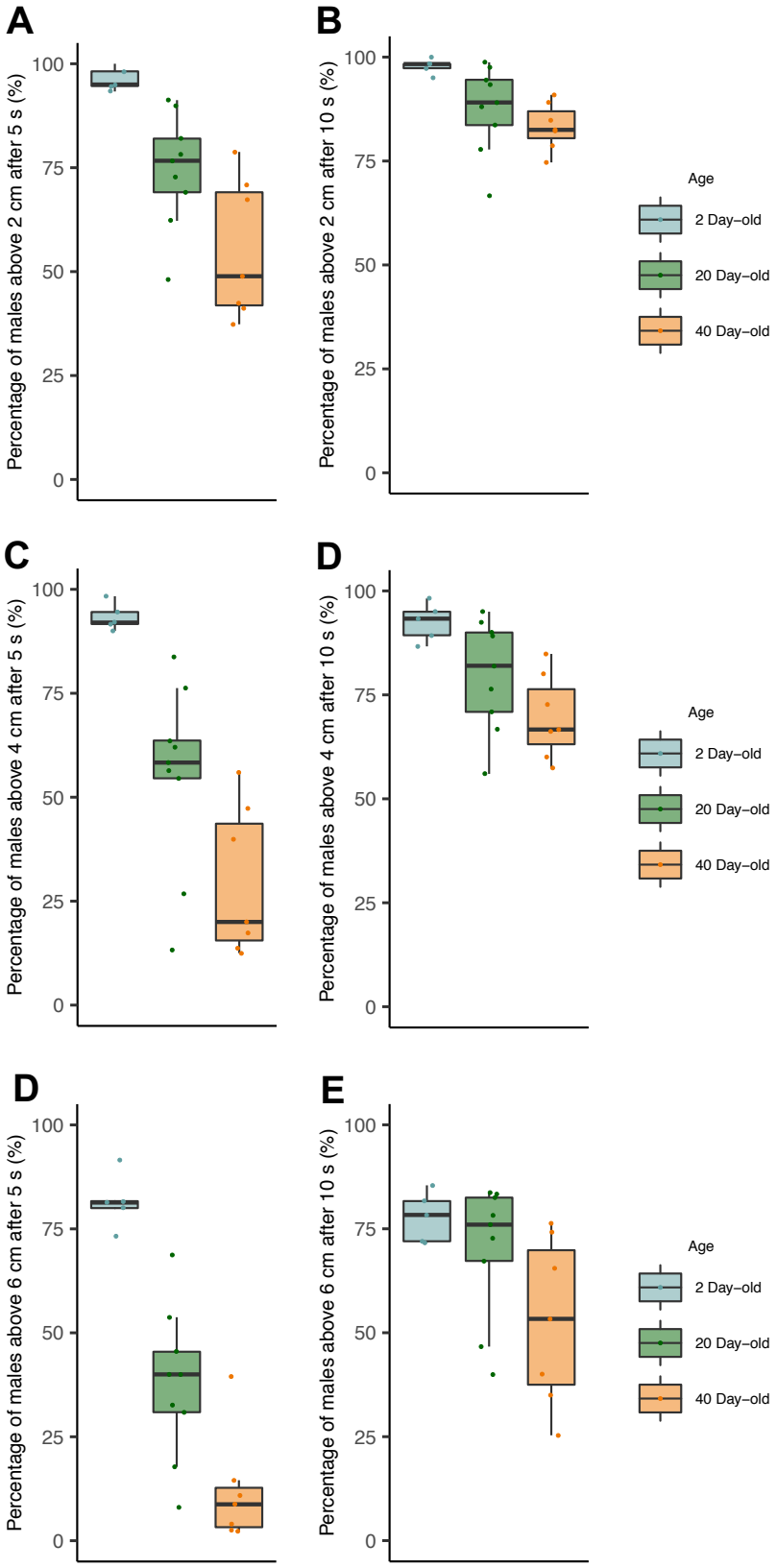

Figure A.1: Box plot with the anti-gravitational response of male control flies at 2, 20, and 40 days after eclosion. The anti-gravitational response was scored as the percentage of males above (A, B) 2, (C, D) 4, and (D, E) 6 cm after (A,C,D) 5 s and (B, D, F) 10 s of the beginning of the assay. Whiskers extend to the smallest and the largest value that are at most 1.5 times the IQR (Tukey's method). Black bars represent the median. Dots beyond whiskers are outliers.

Table A.2: Statistical significance between the anti-gravitational response of different conditions (genotype and age) in the negative geotaxis assay performed with *UAS-L1a* transgenic lines.

Genotype & Age (days)	***>L1*: 2 d**	***>L1*: 20 d**	***>L1*: 40 d**	***pBDP>*: 2 d**	***pBDP>*: 20 d**	***pBDP>*: 40 d**	***pBDP>L1*: 2 d**	***pBDP>L1*: 20 d**	***pBDP>L1*: 40 d**	***57C10>*: 2 d**	***57C10>*: 20 d**	***57C10>*: 40 d**	***57C10>L1*: 2 d**	***57C10>L1*: 20 d**	***57C10>L1*: 40 d**	***nSyb>*: 2 d**	***nSyb>*: 20 d**	***nSyb>*: 40 d**	***nSyb>L1*: 2 d**	***nSyb>L1*: 20 d**	***nSyb>L1*: 40 d**	***elav>*: 2 d**	***elav>*: 20 d**	***elav>*: 40 d**	***elav>L1*: 2 d**	***elav>L1*: 20 d**	***elav>L1*: 40 d**
>*L1*: 2 d																												
>*L1*: 20 d	****																											
>*L1*: 40 d	****	*																										
***pBDP>*: 2 d**	ns	***	****																									
***pBDP>*: 20 d**	****	ns	ns	**																								
***pBDP>*: 40 d**	****	***	ns	****	**																							
***pBDP>L1*: 2 d**	ns	ns	****	ns	ns	****																						
***pBDP>L1*: 20 d**	****	ns	ns	****	ns	ns	**																					
***pBDP>L1*: 40 d**	****	***	ns	****	*	ns	****	ns																				
***57C10>*: 2 d**	ns	ns	****	ns	ns	****	ns	***	****																			
***57C10>*: 20 d**	****	ns	ns	****	ns	ns	**	ns	ns	****																		
***57C10>*: 40 d**	****	***	ns	****	*	ns	****	ns	ns	****	ns																	
***57C10>L1*: 2 d**	ns	ns	****	ns	ns	****	ns	**	****	ns	****	****																
***57C10>L1*: 20 d**	****	**	ns	****	ns	ns	****	ns	ns	****	ns	ns	****															
***57C10>L1*: 40 d**	****	***	ns	****	**	ns	****	ns	ns	****	ns	ns	****	ns														
***nSyb>*: 2 d**	***	ns	**	*	ns	****	ns	ns	****	ns	ns	****	ns	***	****													
***nSyb>*: 20 d**	****	ns	ns	****	ns	ns	*	ns	ns	**	ns	ns	**	ns	ns	ns												
***nSyb>*: 40 d**	****	****	ns	****	**	ns	****	ns	ns	****	ns	ns	****	ns	ns	****	ns											
***nSyb>L1*: 2 d**	**	ns	***	ns	ns	****	ns	ns	****	ns	*	****	ns	***	****	ns	ns	****										
***nSyb>L1*: 20 d**	****	ns	ns	****	ns	ns	**	ns	ns	***	ns	ns	***	ns	ns	ns	ns	ns	ns									
***nSyb>L1*: 40 d**	****	****	ns	****	**	ns	****	ns	ns	****	ns	ns	****	ns	ns	****	ns	ns	****	ns								
***elav>*: 2 d**	ns	ns	****	ns	ns	****	ns	***	****	ns	****	****	ns	****	****	ns	***	****	ns	****	****							
***elav>*: 20 d**	****	ns	ns	****	ns	ns	**	ns	ns	****	ns	ns	***	ns	ns	ns	ns	ns	ns	ns	ns	****						
***elav>*: 40 d**	****	****	ns	****	**	ns	****	ns	ns	****	ns	ns	****	ns	ns	****	ns	ns	****	ns	ns	****	ns					
***elav>L1*: 2 d**	ns	***	****	ns	**	****	ns	****	****	ns	****	****	ns	****	****	ns	****	****	ns	****	****	ns	****	****				
***elav>L1*: 20 d**	****	ns	ns	****	ns	ns	**	ns	ns	****	ns	ns	***	ns	ns	ns	ns	ns	ns	ns	ns	****	ns	ns	****			
***elav>L1*: 40 d**	****	***	ns	****	*	ns	****	ns	ns	****	ns	ns	****	ns	ns	***	ns	ns	****	ns	ns	****	ns	ns	****	ns		

The locomotor capacity is scored as the % of males above the 6 cm mark, 5 s after initiating the assay. *ns - $p > 0.05$*; * - $p \leq 0.05$; ** - $p \leq 0.01$; *** - $p \leq 0.001$; **** - $p \leq 0.0001$*; Tukey's multiple comparisons test. *>L1 - UAS-L1a*; *pBDP> - pBDP-GAL4*; *57C10> - 57C10-GAL4*; *nSyb> - nSyb-GAL4*; *elav> - elav-GAL4*