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The Retrotransposon Storm and the dangers of a Collyer's genome

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Abstract

Like the famous Collyer's mansion in NY, our genomes have accumulated vast quantities of sequences that have been referred to as 'junk DNA', much of which consists of retrotransposons. A recent literature establishes the phenomenology that many retrotransposons become expressed at progressively higher levels during the course of normal aging. This seems to reflect gradual loss of heterochromatin in old age. In addition, retrotransposons appear to be precociously expressed in brains of younger animals that are experiencing neurodegenerative decline. Although it is difficult to distinguish cause from consequence, several recent studies support the contention that retrotransposon expression, and even perhaps transposition, causally contribute to both the normal deterioration seen with age and to the precipitous decline in some neurodegenerative disorders. This may reflect a two hit model in which normal age-dependent loss of heterochromatin synergizes with a disruption to post transcriptional silencing of RTEs caused by genetic and environmental stress.

Introduction

In March of 1947, Homer and Langley Collyer were found dead in their Harlem mansion, surrounded by 120 tons of rubbish. The trash that was later removed from the Collyer brothers' home included defunct baby carriages, piles of old food, glass chandeliers, inoperable camera equipment, sections of a horse-drawn carriage, rusted bicycles, huge piles of old newspapers, the jaw bone of a horse and a decrepit X-ray machine. In the folklore of New York City, Collyers Mansion Syndrome refers to a psychological obsession with hoarding all manner of items to the point where an apartment is packed from floor to ceiling with junk. A Collyers Mansion is every fire fighters nightmare because the sheer volume of refuse present provides a dangerous source of fuel that can be ignited by any errant spark. Eukaryotic genomes have become a Collyers Mansion of sorts as a byproduct of the evolutionary battle with selfish genetic elements. Indeed our chromosomes are packed floor-to-ceiling with the refuse from ancient viral infections and from the detritus from the replication of mobile elements. Nearly 50% of the human DNA content consists of such

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accumulated refuse. This is not to say that all of this accumulated sequence is useless. Indeed, evolution has tinkered with this trash and in many cases has turned it to good use. But in this essay I will focus on the potential danger of the repetitive elements in our genomes. I will outline the "retrotransposon (RTE) storm" [7] hypothesis of age-dependent neurodegeneration, and will argue the case that the natural process of aging synergizes with inherited genetic predispositions and with environmental triggers to overwhelm the cellular systems that normally silence RTE expression. This systemic failure of RTE control mechanisms then ignites a storm of toxic RTE expression and perhaps even mobilization, leading to myriad downstream toxic effects, ultimately leading to cell death. I speculate that cell death caused by RTEs may exhibit non-cell autonomous toxic effects, thereby impacting neighboring cells in the brain.

RTE driven genetic heterogeneity: A function for the junk?

Before discussing evidence that unconstrained RTE expression contributes to age-dependent neurobehavioral decline, it is important to acknowledge the hypothesis that regulated RTE expression during developmentally prescribed time windows has the potential to contribute meaningful impact to normal brain function. L1 elements were first established to be capable of replicating and inserting de novo copies within the genomes of hippocampal neurons during development [1]. This startling observation raised the possibility that L1 driven genetic heterogeneity could provide a sort of genomic plasticity to the brain. A series of subsequent publications provided additional evidence that L1s and probably other RTEs are capable of replicating in neurons and glial cells [2–12]. The rate of such de novo events as measured by genomic sequencing experiments remains highly controversial [13–15] and it seems likely that some fraction of reported de novo insertions are artifacts from chimeric sequence reads generated during multiple displacement amplification (MDA) based library preparation [13]. Yet this sort of artifact cannot explain all of the findings. First, the rate of such false positives likely depends on both the analysis pipeline and library preparation method (e.g. not all reports used MDA). But more importantly, there also is overwhelming evidence from a wide array of methods that converge on the conclusion that RTEs are capable of replicating in somatic tissues, including the brain, and even in post-mitotic neurons and glial cells [1,2,4,5,7–10,16–21].

Importantly, this literature includes findings from DNA sequencing using more than one library preparation method, from bulk tissue as well as from single cells, and from several different species and brain region [3–5,10]. In addition, the findings in support of this conclusion include data that is wholly independent of sequencing.

In the case of R2, a LINE like element that inserts specifically in 28S ribosomal RNA genes, a simple but elegant genomic PCR approach was used to document de novo insertions in somatic tissues in *Drosophila* [21]. In the case of mammalian L1 mobilization, a series of studies used a now classic L1-GFP reporter cassette in which the mRNA encoding a GFP fluorescent protein can only be transcribed after removal of an intron by splicing of the L1 genomic RNA that is then transcribed from the opposite strand after re-insertion {e.g. [1,2,19]}. Thus GFP expression can only take place after the L1 passes through an RNA intermediate, is copied back into a cDNA, and integrates into a new chromosomal location.

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A second reporter has been developed to reveal de novo transposition of the gypsy LTR retrotransposon/ERV in *Drosophila* [7]. And as with the L1-GFP reporter, this so-called "gypsy-trap" reporter has been used by several research groups to reveal age-dependent somatic transposition, including brain and other tissues [7,16–18]. Finally, rare L1 mobilizations in neurons also have been validated using somatic cell nuclear transfer to clonally amplify neuronal genomes from adult mice [8,9]. This provided the means to sequence the entire genome of a cloned cell line or mouse strain in which the de novo insertion was incorporated. Together, the above literature provides conclusive evidence that RTEs replicate in normal somatic tissues, including neurons. The rate at which this occurs per neuron, whether the new insertions are essentially random or selective, how it varies across brain regions, cell types, species and individuals still is unresolved, as are the functional consequences.

RTE expression: a hallmark of aging?

Irrespective of whether regulated RTE transposition impacts normal brain development and function, there is accumulating evidence that dysregulated RTE activation contributes to age-dependent neurophysiological decline. Early attempts to characterize RTE expression in aging somatic tissues lead to the discovery that IAP LTR retrotransposons are increasingly expressed in liver from aged mice [22,23]. Although functional consequences were not established, the activity of these potentially mutagenic agents evoked a popular aging hypothesis: that accumulation of DNA damage might contribute to biological aging. More recently, a series of studies have corroborated the idea that expression of some RTEs increases markedly with age in a variety of species and cell types [7,16–18,22,24–30]. This phenomenology has now been documented in yeast, worms, flies, and mammals. And it appears to occur in the germline as well as a variety of somatic tissues including stem cells, fat, liver, and brain. In the case of brain, the new insertions are presumably occurring in non-dividing cells, consistent with ongoing de novo insertions rather than expansion of a lineage of cells that contain a de novo event.

Age dependent expression, and indeed replication, of the Ty1 LTR RTE has been observed in the context of a chronological aging model in S. cerevisiae [24] in which cells are maintained in stationary growth phase. Ty1 elements also are activated in a yeast model of replicative aging in which the number of times a mother cell has divided is progressively increased [29,30]. Similarly, expression of both LINEs and non-autonomous RTEs become aggressively expressed in a mammalian fibroblast culture model of replicative senescence [27] as well as in somatic tissues of aging mice [22,23,28]. Ty3/gypsy family LTR elements also are reportedly active in C. elegans germline in an age dependent manner[25]. Also, both LINE like and LTR retrotransposons become increasingly expressed in adult *Drosophila* somatic tissues, including adipose/liver [16,17,26], intestinal stem cells[18] and brain [7].

Importantly, in the case of the gypsy ERV/LTR element, age-dependent expression also correlates with de novo replication leading to activation of the gypsy-trap insertion reporter. This reporter makes use of a genomic hotspot for gypsy integrations, which is fused to a Gal80 repressor protein such that de novo integrations disrupt Gal80 expression. This activates Gal4 driven expression of GFP, revealing individual cells in which an RTE has

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inserted in the Gal80 cassette. Unlike the L1-eGFP reporter, this gypsy-trap reporter offers the advantage that it detects replication of endogenous elements rather than ones that are transgenically supplied. On the other hand, it suffers from the disadvantage that it does not distinguish which gypsy family member may have replicated. Despite this caveat, the gypsy-trap reporter has been used by several groups to detect age dependent increases in de novo transposition events in brain[7], in adipose/liver tissue[16,17], and in intestinal stem cells[18].

Overall, the picture that is emerging is one in which the progressive de-silencing of RTE transcription is a hallmark of aging that is conserved across species and tissues. This phenomenology of apparent activation of RTEs implies a failure of mechanisms that normally stifle such expression (Fig. 1). Indeed, in both germline and somatic tissues, expression of mobile elements usually is suppressed by multi-layered silencing systems [31–35]. In fission yeast, plants, worms and fly germline, where the silencing systems are better delineated, several general themes have emerged [31–34,36,37]. First, genomic regions that are rich in transposon sequences normally are kept transcriptionally quiescent by packaging into repressive heterochromatin. This primary silencing system likely includes recruitment of repressive histone marks by the RNA induced transcriptional silencing system (RITS). Second, post-transcriptional 'backup' silencing systems exist in which transposon RNAs are degraded by the RNA induced silencing complex (RISC). Both RITS and RISC make use of small RNAs with sequence complementarity to mobile elements, loaded onto members of the argonaute protein family (Fig. 1). The source of small RNAs, the mechanisms of their biogenesis, and the identities of argonaute family members vary between species and tissues. Importantly, few details are known about small RNA regulation of RTEs in brain aside from the known expression and requirement of several argonaute proteins[7,38,39] and the presence of various pools of transposon targeted small RNAs. Nevertheless, the observed age-dependent expression of RTEs in brain and in other somatic tissues points to a collapse of the mobile element silencing systems.

RTE expression: cause or consequence of aging?

The literature is replete with phenomenological changes that correlate with age. As always, the key question is whether a given cellular effect is a cause or consequence of the aging process, and establishing such causality is not a trivial matter. In the case of RTE expression, the jury is not yet in. But three types of observation from the *Drosophila* system make it clear that contribution of RTE activity to age dependent decline deserves serious consideration. First, the expression of RTEs not only correlates with age, but also is impacted by dietary perturbations that alter the kinetics of aging. Chronic dietary restriction (DR) has a documented lifespan extending impact in a variety of organisms [40–44]. In flies, DR not only extends lifespan, it also significantly delays the onset of age-related RTE expression in adipose/liver tissue [16]. This DR regimen also apparently reduces the rate of de novo RTE insertional mutations, as measured using the gypsy-trap reporter [16]. Thus the kinetics of RTE expression, replication and of aging appear correlated even when the kinetics of aging is altered.

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Secondly, the integrity of heterochromatic gene silencing, the primary cellular mechanism responsible for preventing RTE expression, is progressively eroded with age in a variety of organisms including yeast, worms, flies, mice and human cells [45–49]. Here too, genetic manipulations that delay the kinetics of heterochromatin loss result in a concomitant delay in the onset of RTE expression and an extension of lifespan. For example, increasing the efficacy of heterochromatin in old age by expression of Sir2, Dicer-2 or Su(var)3–9 or reducing the gene dosage of ADAR [16] were sufficient extend silencing of transposons in older animals. These same genetic manipulations also extended lifespan.

Although it is not yet clear what causes repressive histone modifications to be lost with age, a hint comes from genetic manipulations of the RNA editing enzyme adenosine deaminase acting on RNA (ADAR), a protein expressed almost exclusively in neurons [26]. In addition to its well-known role in specific editing that changes amino acid coding potential in mRNAs, ADAR (it turns out) also has a promiscuous editing mode in which it attacks long double-stranded RNA templates and copiously edits up to 50% of adenosines. The ADAR gene is able to switch into this promiscuous editing mode by specifically auto-editing its own transcript, leading to production of an ADAR protein isoform that favors promiscuous editing. This regulatory switch provides a brake on chromatin silencing of RTEs (Fig. 1), probably by competing with Dicer-2 for double stranded RNA substrates[26]. This ADAR switch also is associated with elevated levels of expression of heterochromatic sequences, including mobile elements. The levels of ADAR editing thus may provide a critical balance that determines the functional efficacy of RNAi mediated RTE silencing at the transcriptional level (and potentially at the post transcriptional level as well). Importantly, ADAR levels also modulate lifespan[26]. This provides a mechanistic hypothesis to explain the observation that RTEs are progressively de-repressed [16,26–28] and provides further evidence that such de-repression may impact longevity. And as with the case of components of the heterochromatin silencing apparatus, perturbations that alter the balance of ADAR editing simultaneously impact the efficacy of heterochromatin based silencing, the kinetics of mobile element expression during age and lifespan.

Genetic perturbations of argonaute family members that are crucial for RTE silencing also reveal functional impact on aging phenotypes. For example, mutations in *argonaute-2* in *Drosophila* cause precocious expression of several RTEs in brain [7] and other tissues[50–52]. Such mutations also cause rapid age-related memory impairment and shortened lifespan[7]. Similarly, mutations in piwi clade of argonaute proteins lead to increased expression of several RTEs in both brain [38] and adipose/liver [17] of young fruit flies. And as with argonaute-2, mutations in piwi or in the flamenco locus from which many of the piwi loaded small RNAs are derived, cause a dramatic shortening of the animals lifespan[17]. These findings also are consistent with a report that piwi mediated repression of RTEs is required to protect somatic stem cells from age-dependent apoptotic cell death[18]. Thus, although the details of small RNA biogenesis in various somatic tissues and the division of labor between different branches of the argonaute family for somatic RTE suppression are still murky, there is evidence that both siRNAs loaded onto argonaute-2 and piRNAs loaded onto PIWI may each participate in protecting some somatic tissues during aging.

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Taken as a whole, the available evidence reveals that RTE expression is progressively increased with age in a variety of species and tissues. In yeast and in *Drosophila* brain, adipose/liver tissue, and somatic stem cells, the rate of de novo transpositions of Ty1/gypsy family elements also are increased [7,16,18,24]. The effectiveness of heterochromatic silencing of RTEs appears also to fail in older animals, providing a potential mechanistic explanation [16,26,27,53]. Finally, in the face of perturbations to the rate of aging, or to the effectiveness of RTE silencing mechanisms, the correlation between RTE expression and aging are maintained.

Precocious RTE expression: a hallmark of neurodegenerative disorders?

Although age dependent RTE expression and even mobilization has been documented in a variety of somatic cell types and tissues, the potential for impacts in the nervous system is potentially most severe because virtually all of the cells are post-mitotic. This has two major implications. First, with the exception a relatively small percentage of cells in a few brain regions originating via adult neurogenesis [54], the overwhelming majority of neurons cannot be replaced if lost. This is in stark contrast to the situation in most somatic tissues. Second, in non-dividing cells, homologous mechanisms to repair DNA double strand breaks are offline. In post-mitotic neural tissue, such DNA damage thus is more likely handled by non-homologous end joining mediated repair, or by cell death. Hence the DNA damage that replicating RTEs contribute in brain may drive programmed cell death of an irreplaceable pool of cells. The observation that age results in RTE expression and in de novo insertions in brain therefore immediately invoked the possibility of a connection to neurodegeneration [7]. Indeed, the largest known risk factor for onset of neurodegeneration by far, is age. Likewise, while RTE expression appears to be progressively increased in advanced normal aging, there also is accumulating evidence that levels of RTE transcripts are precociously elevated in connection with a suite of neurodegenerative disorders.

For instance, several RTE sequences are elevated in expression in patients with sporadic forms of Creutzfeldt-Jakob, a transmissible prion disease [55] as well as in a hamster scrapie model [56]. Defective dicer-1 and RNA toxicity from expression of non-autonomous elements also has been functionally connected to macular degeneration [57,58]. Expression of the gypsy ERV has been implicated in the toxicity of an rCGG expanded repeat in a *Drosophila* model of fragile-X tremor ataxia[59]. Tau-related neurodegeneration in fly and mouse models as well as in brain tissue from Alzheimer's subjects, exhibit a loss of heterochromatin accompanied by expression of heterochromatic transcripts, which include RTEs [60]. Conditional deletion during cortical development of the Uhrf1 gene, which regulates DNA methylation of RTE sequences, also leads to severe postnatal neurodegeneration that correlates with activation of IAP family ERVs [61]. There also is considerable evidence that expression of the HERV-W Env gene is associated with multiple sclerosis, an inflammatory disease leading to CNS demyelination and ultimately to neurodegenerative leasions [see [62] for review].. And finally, a series of recent studies[63–68] establish that activation of ERVs and perhaps other classes of RTEs are associated with disorders that involve protein aggregation pathology of the TAR DNA and RNA binding protein 43 (TDP-43). These disorders include amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). As with normal aging, it is not yet clear whether

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RTE expression is a cause or a consequence of neurodegenerative toxicity. But in the case of TDP-43 disorders, there is accumulating evidence in support of a causal contribution of RTEs.

A causal role for RTEs in neurodegeneration? Evidence from ALS and FTLD

Protein pathology of TDP-43, an aggregation-prone RNA and DNA binding protein, is a hallmark of a suite of neurodegenerative diseases including ALS and FTLD [see [69] for review]. TDP-43 is a member of the hnRNP family that binds to single stranded RNA and DNA with UG/TG-rich motifs. TDP-43, which was originally identified as a transcriptional repressor that binds to the TAR element of the HIV-1 retrovirus[70], is normally found predominantly in the nucleus in healthy cells. But in cells that are experiencing TDP-43 protein dysfunction, the protein accumulates in dense cytoplasmic inclusions. The functional implications of TDP-43 proteinopathy still are not fully clear, but the disorders are thought to involve some combination of toxicity caused by the cytoplasmic aggregates, generalized interference with normal cytoplasmic functions, and depletion of normal nuclear TDP-43 stores leading to a loss of its normal nuclear function. Such cytoplasmic inclusions of TDP-43 are seen in the vast majority of ALS subjects and in approximately half of FTLD. While rare familial mutations in the TDP-43 protein cause this pathology in some patients, approximately 90% of ALS and a large fraction of FTLD cases are sporadic, meaning that no causal genetic lesion has been identified. Thus in most subjects, TDP-43 protein of apparently normal amino acid sequence forms these abnormal cytoplasmic inclusions in affected neural tissue.

Why does TDP-43 proteinopathy occur in apparently genetically normal individuals with sporadic forms of several neurodegenerative diseases? A hint comes from the observation that TDP-43 contains a low complexity domain in its C-terminal region. This protein motif in fact is a common feature of RNA binding proteins that exhibit aggregation pathology in a variety of neurodegenerative disorders. An emerging literature has established that various forms of cellular stress induce such low complexity domain proteins to undergo concentration dependent phase separation, leading to formation of liquid droplets that over time may drive fibrillization [71–73].

Animal models of TDP-43 related disorders have taken advantage of the concentration dependence of low complexity domain protein aggregation[74]. Using transgenic overexpression to increase protein concentration above endogenous levels, it is possible to reproduce many of the signatures of human disease, including aggregation of TDP-43 protein in cytoplasmic inclusions and downstream neurological defects[69,75,76]. Although such animal models are imperfect representations of what is largely a sporadically occurring disorder, they have enabled the delineation of a myriad of cellular roles for TDP-43 and have provided the means to uncover genetic interactions between TDP-43 and other genes that are implicated in neurodegenerative disorders. TDP-43 pathology in animal models is now understood to cause global alterations in mRNA stability and splicing, de-repression of cryptic splicing, and biogenesis of some microRNAs [69,75–78]. A recent literature

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indicates that such TDP-43 pathology also may globally disrupt RTE surveillance systems [64], leading to toxic over-expression of ERVs and other RTEs[63–67].

It has been known for some time that ALS subjects and blood relatives often contain elevated levels of reverse transcriptase enzymatic activity in both serum and cerebrospinal fluid [79,80]. The observation that some ALS subjects exhibit elevated levels of HERV-K within affected neural tissue [65,67] suggested the possibility that this reverse transcriptase activity could derive from an endogenous retrovirus rather than from an exogenous source. Although a causative role for HERV-K in ALS has not been established, all major open reading frames encoded by HERV-K appear to be expressed in post mortem cortical tissue from some patients, and expression of HERV-K Env protein is neurotoxic and is sufficient to cause motor neuron degeneration when expressed in mouse [67]. The expression of HERV-K also may be mechanistically linked to TDP-43 by the observation that TDP-43 protein binds to the HERV-K LTR DNA sequences [67]. These findings raise the possibility that HERV-K per se may be functionally relevant to disease progression in some patients. But there also is accumulating evidence that TDP-43 proteinopathy seen in ALS and FTLD may induce widespread derepression of RTEs -- HERV-K may be the tip of the iceberg

Two types of observation suggest the idea that TDP-43 pathology may initiate a full-scale collapse of the RTE surveillance systems. First are phenomenological observations from deep sequencing datasets [63,64,66] and second are observations from functional studies in model organisms [64,68]. A number of groups used RNAseq to characterize transcriptional profiles in post mortem tissue from ALS subjects [81] or mouse [82,83] or fly[64] TDP-43 neurodegeneration models. Several publications also reported the profile of RNA targets that co-purified with the TDP-43 protein from mouse, rat or human brain tissues[82,84,85]. In each case, these studies identified many hundreds of direct binding targets of TDP-43 and also described many transcripts whose levels were altered either in patient tissue or in animal models. Because of the challenges of mapping repetitive sequences from short read technology, the early studies did not describe effects on RTEs [81–85]. Subsequent re-analysis of these repetitive reads [63], however, uncovered a strong connection between TDP-43 pathology and RTEs. In each of the RNA binding datasets from mouse, rat and human tissue, analysis of the multi-mapping sequences revealed that TDP-43 binds broadly to transcripts derived from RTEs. This included not only LTR retrotransposons/ERVs (such as HERV-K), but also many LINE and SINE elements. Second, in tissue from FTLD patients, the association between TDP-43 and RTE derived sequences appeared to be almost completely lost. This was in contrast to the mRNA targets of TDP-43, which were largely unchanged. Finally, levels of RTE sequences were found to be broadly increased in each of two mouse TDP-43 models [63]. This finding, that TDP-43 pathology is correlated with broad upregulation of RTEs has since been confirmed in a fly model [64] and has been observed in post mortem cortical tissue from ALS/FTLD subjects [66]. Taken together, the above findings raise the possibility that TDP-43 protein dysfunction somehow circumvents RTE silencing, leading to their broad up-regulation. Evidence from a *Drosophila* model provides mechanistic support for this hypothesis and also demonstrates for the first time that an endogenous RTE causally mediates toxic impacts of TDP-43 expression [64].

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Overexpression of TDP-43 in *Drosophila* neurons or glial cells reproduces many pathological hallmarks seen in human subjects and other animal models, likely through dominantly inducing formation of cytoplasmic inclusions[75,76,86,87]. Such expression also causes progressive neurodegenerative effects, cell death, neurobehavioral decline and shortened lifespan. RNA sequencing from flies that express pathological levels of human TDP-43 reveals that such expression also causes a dramatic increase in transcripts from of a broad panel of RTEs, including LINE-like elements, LTR/ERV elements, and non-autonomous elements as well [64]. The broad expression of RTEs in response to pathological levels of TDP-43 expression results from a collapse of the argonaute-2/siRNA mediated gene silencing [64]. The disruptive effects on siRNA mediated silencing was visualized using a reporter system in which an RNAi transgene was used to target either GFP or an eye pigmentation gene.

The toxic expression of TDP-43, either in glial or neuronal cells, was shown to interfere with reporter silencing in an age-dependent manner. The mechanism by which TDP-43 pathology impairs siRNA silencing is not clear, but may involve disruption of small RNA biogenesis pathways [64,68,77,88] (Fig. 1).

Unfettered expression of RTEs can be highly detrimental for a variety of reasons. First, expression of high levels of RTE proteins, RNAs or extrachromosomal cDNA copies can have toxic cellular effects including activation of inflammatory response pathways. Such expression also provides the potential for functional replication of the RTEs, which can lead to insertional mutagenesis and activation of the DNA damage response leading to programmed cell death. In the case of TDP-43 pathology in *Drosophila* glial cells, expression of the gypsy ERV/LTR element has been demonstrated to contribute to the underlying toxicity from the TDP-43 protein because knocking down the expression of gypsy was sufficient to significantly ameliorate the effects of TDP-43 on lifespan of the animals as well as on cell death [64]. Moreover, the effects of TDP-43 in this context appear to be caused at least in part by DNA damage mediated programmed cell death because knockout of loki, the fly ortholog of Chk2, results in a near complete suppression of the detrimental effects of TDP-43 on both neuronal and glial cell death and on lifespan [64]. This is consistent with a model in which the toxicity of TDP-43 aggregation pathology is mediated at least in part by its disruptive impact on siRNA mediated silencing, leading to a storm of RTE expression.

These findings from the *Drosophila* model establish a mechanistic connection between TDP-43 proteinopathy and disruption to the RTE silencing systems. Early reports from post mortem brain tissue provides some support for the idea that TDP-43 related disorders exhibit RTE expression [63,65–67], although larger sample sizes are needed as are mechanistic investigations and functional validation to test whether the findings from *Drosophila* are predictive of the impact in human patients.

Thoughts and Perspectives: A two hit hypothesis

Age is the predominant risk factor for all neurodegenerative disorders. But neurodegeneration is not an inevitable outcome of healthy aging. Importantly the subset of

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individuals who succumb to neurodegenerative disorders also are of normal health until the point of diagnosis. The onset of neurodegenerative disease appears as an inflection point in a lengthy pre-symptomatic stage, after which the rate of decline increases dramatically. The kinetics of normal aging and of decline after diagnosis of neurodegenerative disorders suggests a two hit model (Figs 1 and 2) in which RTEs might play a contributory role. In this model, normal aging progressively erodes the primary RTE silencing systems, which act at the level of chromatin to block transcription. This leads to some expression of RTEs, an increased background of de novo transposition events, and possibly accumulation of toxic products from RTE expression, which may drive inflammation. On this background of age-dependent heterochromatin decline, any genetic or environmental perturbation that accelerates the effects of age on the primary system [60] or undermines the integrity of the secondary post-transcriptional silencing systems[64], may synergize to unleash toxic RTE expression. In plants, there in fact is evidence for such a synergy between primary and secondary silencing systems. Double mutant combinations in *Arabidopsis thaliana* of ddm1 and rdr6, which disrupt both transcriptional and post-transcriptional silencing respectively, provide a synergistic effect on transposon expression [89] and on phenotypic consequences [90].

As outlined above, normal aging erodes the efficacy of heterochromatin based gene silencing [47,91,92]. Recent work from *Drosophila* demonstrates that TDP-43 proteinopathy disrupts RNAi mediated silencing through argonaute-2 [64]. This likely perturbs the secondary RISC pathway, although there may also be cross-talk with transcriptional silencing. Any such disruption to both primary and secondary RTE silencing pathways could explain the observed synergy between age on the one hand and genetic, environmental or stochastic events on the other. When both primary and secondary surveillance systems fail, a given cell crosses a threshold after which expression of RTEs may overwhelm any residual cellular defenses. The ensuing storm of awakened RTE expression then contributes to the detrimental impact of TDP-43 proteinopathy, perhaps via toxicity of accumulated RTE RNAs and extrachromosomal cDNA copies and/or DNA damage, leading programmed cell death within that cell. But TDP-43 dependent neurodegeneration is also known to exhibit a fundamental non-cell autonomous toxicity [93] in which glial cells become toxic to nearby neurons (Fig. 2). Given the evolutionary relationship between exogenous retroviruses and ERVs such as gypsy and HERV-K, it is tempting to speculate that such elements may also contribute to these non-cell autonomous effects. Indeed there is some evidence that both gypsy and HERV-K [94–97] retain the ability to move genetic material between cells.

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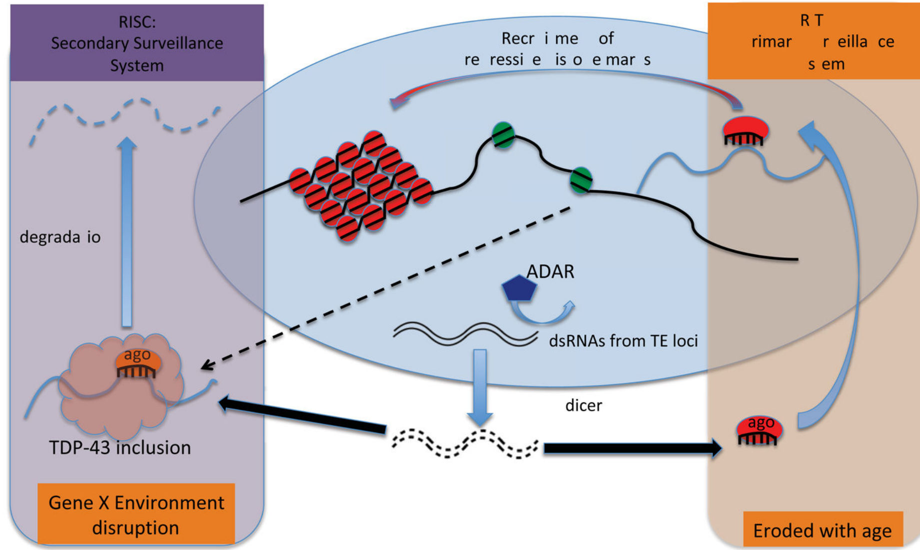

Figure 1. Can two hits ignite a fire?

RTE are normally silenced by overlayed systems of repression. First, genomic regions that are rich in RTE sequences are packaged into silent heterochromatin. Heterochromatin formation and maintenance depends on repressive histone modifications, which can be recruited by small RNAs loaded onto an argonaute family member. This loaded RITS complex targets RTE sequences in nascent transcripts. This primary chromatin level silencing appears to be eroded with age [16,27,47,49,53,91,92], perhaps via competition between ADAR and Dicer enzymes for double stranded RNA templates[26]. A secondary system of RTE silencing relies on post transcriptional targeting of RTEs by the RISC complex. This system appears to be stable in the face of normal aging, but is disrupted by TDP-43 proteinopathy[64].

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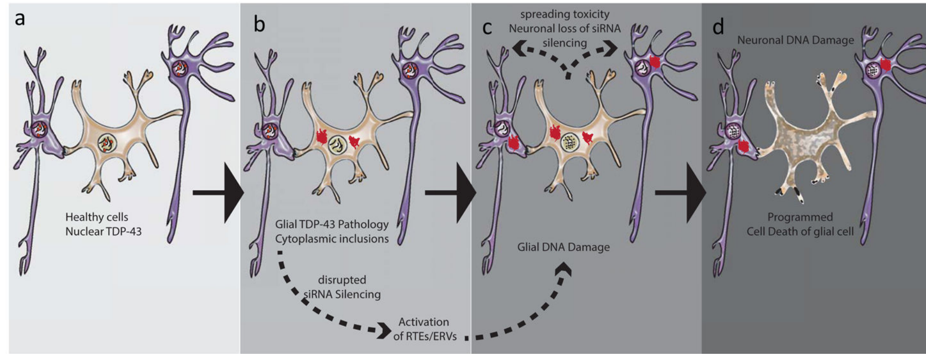

Figure 2. Can the fire spread between glia and neurons?

In healthy glia and neurons, TDP-43 is mostly nuclear in localization (a). For reasons that are not understood, pathological TDP-43 proteinopathy can be initiated, perhaps stochastically in response to cellular stressors (b). When this occurs, TDP-43 is cleared from the nucleus and aggregates in cytoplasmic inclusions that may interfere with argonaute-2 mediated post transcriptional silencing. This leads to a storm of awakened RTEs and ERVs, resulting in DNA damage mediated programmed cell death (c). Non-cell autonomous toxicity of glial cells has been observed in co-culture (see [93] for review). While the mechanisms of such toxicity is not fully explored, the fundamental homology between ERVs and exogenous retroviruses raises the specter of a self amplifying toxic mechanism.