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REVIEW

Transposable elements, circular RNAs and mitochondrial transcription in age-related genomic regulation

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ABSTRACT

Our understanding of the molecular regulation of aging and age-related diseases is still in its infancy, requiring in-depth characterization of the molecular landscape shaping these complex phenotypes. Emerging classes of molecules with promise as aging modulators include transposable elements, circRNAs and the mitochondrial transcriptome. Analytical complexity means that these molecules are often overlooked, even though they exhibit strong associations with aging and, in some cases, may directly contribute to its progress. Here, we review the links between these novel factors and age-related phenotypes, and we suggest tools that can be easily incorporated into existing pipelines to better understand the aging process.

KEY WORDS: Aging, CircRNAs, Mitochondria, Transposable elements

Introduction

The field of geroscience seeks to understand and characterize the mechanisms that contribute to aging and that may directly or indirectly drive aging-associated pathologies (Kennedy et al., 2014). Over the past few decades, a number of evolutionarily conserved pathways that modulate lifespan in yeast, nematodes, flies and mammals have been identified, such as the insulin signaling, mTOR and sirtuin pathways (Bareja et al., 2019; Bishop et al., 2010; Pan and Finkel, 2017).

One of the major limitations the field has encountered is that the relative magnitudes of lifespan changes that genetic and pharmaceutical interventions exhibit do not linearly translate between organisms. For example, dietary restriction can increase lifespan in worms two- to threefold, but this intervention may only increase mouse lifespan between 30 and 50%, and its long-term effects on humans are not well characterized (Fontana et al., 2010). Thus far, there is no known intervention that can bridge the gap between the average human lifespan (~79 years in the USA) and the maximal, recorded human lifespan (~122 years). This suggests that there may be complex underlying molecular factors important during the aging process that are still poorly understood.

Experimental studies of the aging process can be carried out at several levels in the laboratory: (1) chronological aging (time since the birth of an organism, which is the most intuitive definition of aging); (2) replicative aging [the number of times a cell can divide (Mortimer and Johnston, 1959)]; (3) cellular senescence [the endpoint of replicative aging, corresponding to irreversible cell cycle arrest once a cell population reaches its 'Hayflick limit' (Hayflick and Moorhead, 1961)], the burden of which on multi-cellular animals increases with age (Dimri et al., 1995) and which may underlie aspects of organismal aging and age-related diseases (Baker et al., 2016; Bussian et al., 2018); and (4) premature aging syndromes [e.g. Hutchinson-Gilford progeria syndrome (Burtner and Kennedy, 2010)]. Moreover, aging is characterized by several 'hallmarks', including genomic instability, cellular senescence, altered intercellular communication/pervasive inflammation, loss of protein homeostasis, mitochondrial dysfunction, deregulated nutrient sensing, stem cell exhaustion, telomere attrition and epigenetic alterations (Kennedy et al., 2014; López-Otín et al., 2013). Importantly, a central hypothesis in the geroscience field is that aging itself is a major risk factor for a number of chronic 'age-related' diseases, such as Alzheimer's disease (AD) and other dementias, cancer, etc. (Franceschi et al., 2018a). Finally, many features of the aging process can be fine-tuned, even within a species, based on environmental cues, inter-individual genetic variations and even sex (Fischer et al., 2016).

Accumulating evidence across experimental models of aging suggests that complex underlying molecular factors are important during aging, many of which are still poorly understood. Changes in traditional gene expression features such as general transcription levels (Lai et al., 2019), epigenetic regulation (Booth and Brunet, 2016; Pal and Tyler, 2016; Sen et al., 2016) and alternative splicing (Bhadra et al., 2020) are reviewed elsewhere. In this Review, we focus on three emerging factors of interest: transposable elements, circular RNAs (circRNAs) and the mitochondrial transcriptome. The analysis of the first two factors can be readily incorporated into existing data analysis pipelines and may provide important insights into the aging process. We provide an introduction to these emerging factors, highlight known relationships and current gaps in knowledge with respect to aging and, when available, indicate tools that are available for their analysis. In the future, a more systematic incorporation of these factors into aging studies will help provide a more comprehensive view of the changes that occur, suggest novel molecular mechanisms underlying aspects of aging, and provide new therapeutic targets for pro-longevity interventions.

Transposable elements as drivers of aging and aging-related pathologies

Transposable elements (TEs; informally referred to as 'jumping genes') are mobile genetic elements within host genomes that may impact several aspects of the aging process. Transposons constitute a significant fraction of eukaryotic genomes, ranging anywhere from ~3% in yeast (*Saccharomyces cerevisiae*) to ~80% in the frog

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Box 1. Emerging tools to quantify genomic regulation of TEs

From a data analysis perspective, the inherent repetitive nature of TEs makes many analyses complicated and computationally intensive, notably because short-read NGS technologies are limited in their ability to distinguish different instances of the same element (Treangen and Salzberg, 2012). Thus, sequencing reads from TEs are most frequently ignored and discarded in genomic analyses (e.g. RNA-seq, ChIP-seq and ATAC-seq), and only the expression profiles of annotated genes are considered (Slotkin, 2018). This analytical pragmatism means that researchers often have an incomplete representation of genome regulation associated with their manipulation or intervention of interest. To attempt to address this deficit, a growing number of pipelines are being developed to discover, classify and quantify TEs from high-throughput sequencing experiments (Table 1; reviewed by Goerner-Potvin and Bourque, 2018; O'Neill et al., 2020). As these tools are still relatively young and are based on different algorithmic assumptions, it will be important to benchmark them in order to understand which ones are most useful in the study of aging genomic-regulation networks. Ultimately, systematically incorporating TE analyses in bioinformatic pipelines, especially in the contexts of aging research, will help us gain a more comprehensive view of transcriptional landscapes, which may implicate TEs in previously unconnected phenotypes or disease states.

Rana esculenta (Biémont and Vieira, 2006). Of greater relevance, the proportion of TEs has been reported to be ∼38% in the mouse genome and ∼46% in the human genome (Lander et al., 2001; Waterston et al., 2002). TEs are highly diverse with regards to DNA sequences, mechanisms of mobilization and genomic regulation (reviewed by Levin and Moran, 2011; Rebollo et al., 2012). Transposition occurs through two main mechanisms: class I transposons are RNA mediated: their RNA is reverse transcribed into cDNA, which then integrates into a new genomic site (i.e. a 'copy-and-paste' mechanism). Class II transposons are DNA mediated, encoding enzymes that cleave the original TE DNA from its original site and integrate it into a different genomic site (i.e. 'cut-and-paste' mechanism) (Kidwell, 2002).

A key biological process where TEs can become more active is during aging and also in certain age-related diseases. Importantly, age-related reactivation of TEs is observed across species, including *C. elegans* (Dennis et al., 2012), *Drosophila* (Chen et al., 2016; Li et al., 2013; Wood et al., 2016) and mouse (Benayoun et al., 2019; De Cecco et al., 2013b). Altered activity of specific TEs is also associated with multiple age-associated pathologies, including cancer (Di Ruocco et al., 2018; Iskow et al., 2010; De Luca et al., 2015; Rodić et al., 2014), AD (Guo et al., 2018; Sun et al., 2018) and TDP-43-mediated ailments (e.g. amyotrophic lateral sclerosis, frontotemporal lobar degeneration, etc.) (Krug et al., 2017; Li et al., 2012; Prudencio et al., 2017). Broadly, this re-activation has a number of implications for gene expression. Transposition-competent TEs may drive mutation and, if inserted into genes, drive production of non-functional gene products (Kidwell and Lisch, 1997). TEs may also alter expression of nearby genes. Indeed, TEs harbor regulatory elements that can alter gene expression in *cis*, although an additional possibility is that they may sequester transcriptional machinery away from nearby genes (Elbarbary et al., 2016; Jordan et al., 2003). Ultimately, whether altered TE activity is a mere by-product or an actual driver of aging and age-related diseases remains mostly unclear. To fully understand the contributions of TE mis-regulation to the aging process, the field will need to determine which hallmarks are modulated by TE deregulation and characterize any underlying mechanisms (Box 1; discussed below). We indicate a selection of bioinformatic tools that can easily be integrated in existing analytical pipelines to interrogate TE regulation (Table 1).

In the human genome, the two most abundant families of TEs are RNA mediated: the long interspersed element 1 (LINE-1 or L1) and Alu elements, which account for ∼16-17% and ∼9-11% of the genome, respectively (Lander et al., 2001; Venter et al., 2001) (Fig. 1A). Part of their high prevalence may be explained by the facts that L1s appear to be the only currently active autonomous TEs in the human genome, and that Alu elements can hijack the L1 machinery for their own transposition (Dewannieux et al., 2003). Other TEs, such as endogenous retroviruses (ERVs), fall within the long-terminal repeat (LTR) group of TEs, which makes up ∼8.3% of the human genome (Lander et al., 2001). Interestingly, these are expressed as part of normal physiology (Schmitt et al., 2015), although they do exhibit age-dependent expression patterns (Balestrieri et al., 2015; Nevalainen et al., 2018) and links to immune system stimulation (Hurst and Magiorkinis, 2015; Manghera and Douville, 2013), cancer (Bannert et al., 2018; Li et al., 2017a; Zhou et al., 2016) and neurodegenerative disease (Tam et al., 2019). Given their abundance and activity in the human genome and in human disease, as well as the functional diversity of different families of TEs, we primarily focus on L1 and Alu elements (Lander et al., 2001; Sudmant et al., 2015). The biogenesis and general functions of L1 and Alu are reviewed elsewhere

Table 1. Tools to analyze and quantify TE-derived sequences in ultra-high-throughput sequencing experiments

Genomic data type	Tool	Link to software	Reference
RNA-seq (transcription)	HOMER	http://homer.ucsd.edu/homer/index.html	Heinz et al. (2010)
	LIONS	https://github.com/ababaian/LIONS	Babaian et al. (2019)
	piPIPES	https://github.com/bowhan/piPipes	Han et al. (2015)
	RepEnrich	https://github.com/nskvir/RepEnrich	Criscione et al. (2014)
	SalmonTE	https://github.com/LiuzLab/SalmonTE	Jeong et al. (2018)
	SQuIRE	https://github.com/wyang17/SQuIRE	Yang et al. (2019)
	TEcandidates	https://github.com/TEcandidates/TEcandidates	Valdebenito-Maturana and Riadi (2018)
	TEtools	https://github.com/l-modolo/TEtools	Lerat et al. (2016)
	TEtranscripts	https://github.com/mhammell-laboratory/TEtranscripts	Jin et al. (2015)
	TEtools	https://github.com/l-modolo/TEtools	Lerat et al. (2016)
	TeXP	https://github.com/gersteinlab/texp	Navarro et al. (2019)
ChIP-seq or ATAC-seq (Epigenome)	HOMER	http://homer.ucsd.edu/homer/index.html	Heinz et al. (2010)
	piPipes	https://github.com/bowhan/piPipes	Han et al. (2015)
	RepEnrich	https://github.com/nskvir/RepEnrich	Criscione et al. (2014)
	TEpeaks	https://github.com/mhammell-laboratory/TEpeaks	*Under development*

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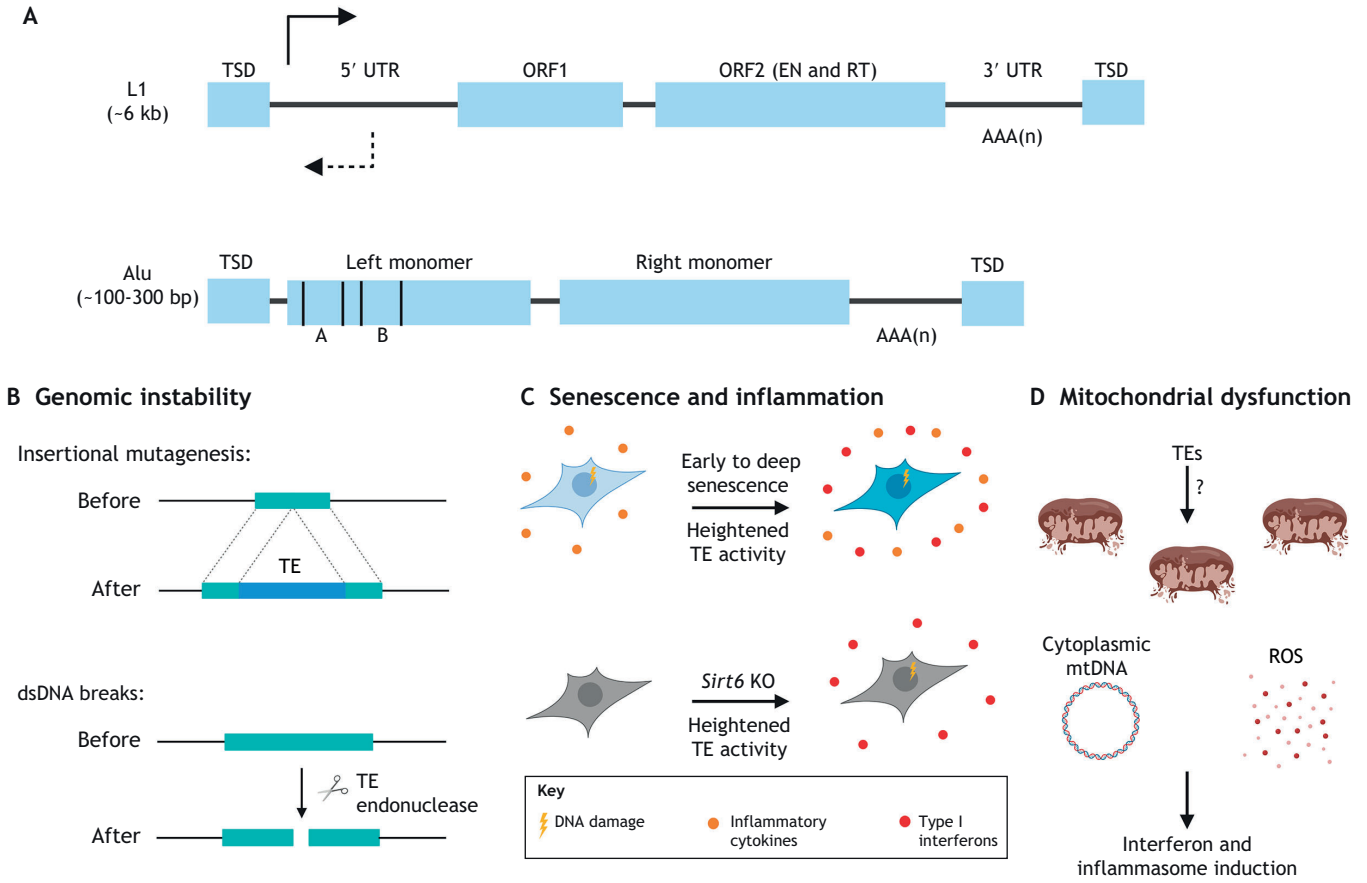

Fig. 1. Retrotransposons may influence multiple pillars of aging. (A) L1 and Alu retro-elements are the most abundant transposable elements (TEs) in the human genome. A stereotypical L1 (top) and Alu (bottom) are displayed. (Top) Human L1s are typically ∼6 kb long TEs with flanking target site duplications (TSDs). There is a bidirectional promoter in the 5′ UTR, as well as two open reading frames (ORFs): ORF1 and ORF2. ORF1 encodes a RNA-binding protein with nucleic acid chaperone activity; ORF2 encodes an endonuclease (EN) and reverse transcriptase (RT). An additional ORF in the antisense orientation, ORF0, is present in the 5′ UTR of primate L1. For simplicity, ORF0 is not depicted here. (Bottom) Alu elements are typically 100-300 bp, with flanking target site duplications (TSDs). They usually contain a left Alu monomer with A- and B-box promoters, and a right Alu monomer. Alu elements are non-autonomous and use the L1 machinery for transposition. (B) TE activity can lead to genomic instability through insertional mutagenesis or double-stranded DNA breaks. (C) TE upregulation in senescent cells (and in the absence of *Sirt6*) correlates with an inflammatory secretory phenotype, notably with the production of type I interferons. (D) Emerging evidence suggests that TEs can interact with mitochondria, which may lead to mitochondrial dysfunctions.

(Richardson et al., 2015). Below, we review the mechanisms by which Alu/L1s are regulated and can drive cellular dysfunction, and we tentatively categorize them with respect to the hallmarks of aging to promote further research into their roles within an aging context.

Molecular regulation and control of TEs

Eukaryotic hosts have evolved several, canonical pre-transcriptional and post-transcriptional modes of TE regulation, which are reviewed elsewhere (Levin and Moran, 2011; Loreto and Pereira, 2017; Nätt and Thorsell, 2016; Rebollo et al., 2012; Yang and Wang, 2016). Briefly, pre-transcriptional mechanisms include repression by CpG DNA methylation and heterochromatinization (Levin and Moran, 2011; Yang and Wang, 2016). Post-transcriptional mechanisms include the piRNA and siRNA pathways, as well as post-transcriptional RNA editing (Levin and Moran, 2011; Yang and Wang, 2016). Indeed, both L1- and Alu-derived endogenous siRNAs have been identified (Soifer et al., 2005; Xia et al., 2012; Yang and Kazazian, 2006), and targeting of Alu elements for editing by adenosine deaminase acting on RNA (ADAR) is a key area of research focus (Ahmad et al., 2018; Chung et al., 2018).

Type I interferon response

Another way that L1s can be regulated is by type I interferons (IFNs), which represent a central family of cytokines (Yu et al., 2015) that may contribute to pathological levels of inflammation with age. Broadly, type I IFNs play important roles in defense against viral infections by inducing a gene expression program that leads to an ‘antiviral state’ and sensitizes infected cells to apoptosis, among other functions (Stetson and Medzhitov, 2006). In the context of TEs, L1s can induce expression of IFNβ, which restricts L1 mobility in human and mouse cell lines (Yu et al., 2015). Moreover, Type I IFN production can be induced by pattern recognition receptors (PRRs) that recognize a multitude of foreign and foreign-like products (Motwani et al., 2019; Stetson and Medzhitov, 2006). For example, TEs can be detected by the double-stranded RNA sensor, melanoma differentiation antigen 5 (MDA5), which interacts with mitochondrial antiviral-signaling protein (MAVS) to activate IFN signaling (Stetson and Medzhitov, 2006), and by the cytosolic dsDNA sensor cyclic GMP-AMP synthase (cGAS), which signals through the stimulator of interferon genes (STINGs) (Motwani et al., 2019).

Therapeutically, nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) inhibit L1 reverse transcriptase (Dai et al.,

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2011; Jones et al., 2008) and ameliorate L1-driven IFN signaling and inflammation (De Cecco et al., 2019; Simon et al., 2019); however, some have highlighted the negative health effects NRTIs can pose and thus the need to find alternative, L1-specific reverse transcriptase or RNP inhibitors (Simon et al., 2019). This may, for example, necessitate researchers to carry out small-molecule screens for specific L1 inhibitors in the future (Simon et al., 2019).

Candidate regulators for future investigations

A number of candidate regulators of L1 transposition in human cancer cell lines have been revealed in a recent genome-wide CRISPR-based screen (Liu et al., 2018). These candidate regulators have diverse biological functions that include chromatin and transcriptional regulation, RNA processing, DNA replication, homologous recombination, non-homologous end joining and genes involved in the Fanconi anemia complex (Liu et al., 2018). As cancer cells are known to misregulate a number of key processes that safeguard genomic integrity, it will be crucial to determine whether the genes identified in this screen play similar roles – if any – throughout life in non-cancerous somatic cells, including in post-mitotic cells. Although it is likely that the repressive or permissive functions of a subset of genes will be conserved across cell types, it is also likely that a large proportion of these regulators may have cell type- and cell state-dependent effects. With regards to aging, it will be important to determine which of these genes may be responsible for age-related changes in L1 regulation and which may also play roles in Alu regulation. Finally, there are intriguing differences in L1 regulation when using codon-optimized versus non-optimized L1 transposition reporters (Liu et al., 2018), indicating that some L1 transposition regulators may rely on specific sequence patterns, or that codon-optimization of the reporter may obscure or enhance subtle physiological effects. These caveats should be kept in mind when using these reporters to probe the relationships between L1 and aging.

TE links to the hallmarks of aging

L1 and Alu element activity promotes genome instability

Genomic instability – the tendency of genomes to accumulate alterations – has long been thought to be one of the central drivers of aging (Vijg and Suh, 2013). These alterations encompass DNA chemical damage, mutation and epimutation (Vijg and Suh, 2013). There are multiple mechanisms by which L1 and Alu elements can drive genome instability, but how these alter aging trajectories remains unclear. Most obviously, TE activation can increase mutation frequency through insertional mutagenesis (Fig. 1B). Indeed, L1 copy number increases in aging mouse liver and muscle (De Cecco et al., 2013b) and in senescent human fibroblast cultures (De Cecco et al., 2013a). Alu copy number is significantly more variable in white blood cells derived from elderly (>75 years) versus younger (<30 years) individuals, although no significant differences in mean levels have been detected (Morgan et al., 2017). However, to our knowledge, changes in health or lifespan of transgenic animals with hyperactive L1 or Alu elements has not yet been fully explored. Some tools already exist, such as mice harboring synthetic, codon-optimized L1s that exhibit high levels of transposition across multiple tissues (An et al., 2006). These mice do not show obvious fitness deficits when aged for over 18 months (An et al., 2006), although additional studies would need to be conducted with direct comparison with wild-type aging mice, or even mice carrying an inactive form of the transposon transgene. If, indeed, no differences are detected in these contexts, this could suggest that animals are relatively robust to certain levels of somatic L1 transposition, at least in the absence of external challenges.

Another source of TE-induced genomic instability resides in L1-induced double-stranded DNA breaks (DSBs), even without successful transposition (Fig. 1B). Overexpression of L1 in human and mouse cell lines induces DSBs, indicated by γH2A.X chromatin marks (Rogakou et al., 1998). However, the number of γH2A.X foci observed in these studies exceeds the expected number of successful insertion events (Belgnaoui et al., 2006; Farkash et al., 2006; Gasior et al., 2006). As L1 ORF2 encodes a protein that has both reverse transcriptase and endonuclease functions, it would be informative to generate animal models that overexpress only the endonuclease region of ORF2 to assess the impact of ORF2-mediated DSBs on health and lifespan, in the absence of successful transposition events. Such a model would help distinguish the relative impact of L1-driven DSB generation, from that of PRR activation by TE viral-like genomes, or from that of successful insertion events.

Finally, L1 and Alu elements can promote other types of mutations: L1s are implicated in large genomic deletions (Gilbert et al., 2002; Symer et al., 2002), 5′ and 3′ TE flanking sequence co-mobilization (Moran et al., 1999; Symer et al., 2002), chromosomal inversions and recombination with other L1s (Symer et al., 2002). Indeed, L1-driven rearrangements are believed to directly drive cancer, such as through the deletion of tumor-suppressor genes (Rodriguez-Martin et al., 2020). Perhaps they play similar roles during aging, removing genes that regulate the aging process. Correspondingly, Alu elements can generate deletions and duplications via non-allelic homologous recombination or stimulate non-homologous end-joining-related genome alterations (Bailey et al., 2003; Boone et al., 2014; de Smith et al., 2008; Faiz et al., 2013; Li et al., 2006; Strout et al., 1998; White et al., 2015; Witherspoon et al., 2009). Alu-mediated deletions cause a number of diseases (Kolomietz et al., 2002), although their actual contributions to aging phenotypes are still unclear. Though TEs are frequently associated with genome instability and aging, it will be important for future work to characterize to what extent L1/Alu elements drive aging through this mechanism.

Role of L1 and Alu in cellular senescence and age-related inflammation

A key feature of cellular senescence is the senescence-associated secretory phenotype (SASP), whereby senescent cells secrete ‘numerous proinflammatory cytokines, chemokines, growth factors, and proteases’ (Campisi, 2013). This altered secretome facilitates changes in cell-to-cell communication and is believed to contribute to chronic, low-grade, sterile (i.e. in the absence of an infectious agent) inflammation with age through stimulation of the innate immune system, a phenomenon sometimes referred to as ‘inflamm-aging’ (Campisi, 2013; Franceschi et al., 2018b). This chronic inflammatory response is believed to be damaging to the organism, as mouse models that deplete senescent cells from aging mice show improved health and lifespan (Baker et al., 2016; Demaria et al., 2014). TEs become de-repressed following cell senescence (De Cecco et al., 2013a; Wang et al., 2011) (Fig. 1C), but whether they can drive entry into senescence or progression into deeper senescence has been largely unexplored.

Early research has focused on the relationship between TEs and senescence entry and maintenance. Indeed, L1 (or L1 ORF2) expression can induce a senescence-like state even in cancer cell lines such as MCF7 or in HeLa cells (Wallace et al., 2008). Similar results have been obtained in normal human fibroblasts and adult mesenchymal stem cells (Belancio et al., 2010). As chemical

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inhibition of L1 RT in more recent studies does not alter senescence entry (De Cecco et al., 2019), these results together suggest that the endonuclease domain (and not the RT domain), specifically, may modulate entry into senescence.

Another study proposed that knockdown of Alu transcripts might promote exit of adult adipose-derived mesenchymal stem cells from senescence (Wang et al., 2011). This suggests that there are biological contexts where Alu transcription may be necessary for maintenance of the senescent state. It will be important to determine whether the converse is true, i.e. whether Alu element overexpression is sufficient to drive senescence, and whether other TEs can similarly act as 'checkpoints' for senescence entry/exit.

Recently, others have shown that L1s become de-repressed in the late stages of replicative or chemically induced senescence in human fibroblasts (De Cecco et al., 2019) (Fig. 1C). Importantly, this de-repression is coupled with upregulation of the type I IFN response (De Cecco et al., 2019). L1s directly contribute to this upregulation, as the type I IFN response is reduced by shRNA-mediated knockdown of L1 in senescent cells (De Cecco et al., 2019). A similar response is observed with an NRTI without altering the level of L1 transcripts, implicating L1 cDNA as a driver of the type I IFN response (De Cecco et al., 2019). Continuous treatment with the drug as cells progress into senescence reduces the later SASP response without altering the timing of senescence entry or affecting the early SASP response (De Cecco et al., 2019). *In vivo*, administering NRTIs to 26-month-old mice reduces the expression of IFN-I and SASP markers, without altering the senescent cell burden, and restricts aging phenotypes (De Cecco et al., 2019). This landmark study highlights the importance of L1s in the establishment of the senescent state and implicates them in SASP-mediated, altered cell-to-cell communication. Additionally, the authors demonstrate the viability of NRTIs in targeting the RT aspect of the L1 life cycle and the pathological states it can promote.

A similar relationship between TEs and IFN response induction has been noted in aged mice and *Sirt6*-deficient mouse models of aging (Simon et al., 2019). Sirt6 is a NAD-dependent deacetylase homologous to yeast Sir2, which has a robust link to mammalian longevity: *Sirt6*-deficient mice exhibit progeroid phenotypes (Mostoslavsky et al., 2006), whereas male *Sirt6* transgenic mice are long lived (Kanfi et al., 2012). Interestingly, *Sirt6*-deficient mice exhibit extensive genome instability, DNA-damage and unscheduled activation of L1s (Mostoslavsky et al., 2006; Van Meter et al., 2014). The predominant source of DNA damage in *Sirt6*-deficient mouse embryonic fibroblasts (MEFs) may be due to L1 activity, because RNAi knockdown of L1 reduces γH2A.X foci to wild-type levels (Simon et al., 2019). Moreover, NRTI treatment efficiently restricts L1 transposition, consistent with their role as RT inhibitors, and reduced transposition is associated with decreased markers of DNA damage (Simon et al., 2019). These mice also exhibit elevated levels of cytoplasmic L1 DNA and expression of cGAS and type I IFN across tissues and/or cells (Simon et al., 2019). Supporting the idea that cytoplasmic L1 DNA may activate cGAS signaling and interferon induction, immunoprecipitation of cGAS from MEFs and bone marrow dendritic cells shows enrichment for the binding of LINE sequences (Gentili et al., 2019; Simon et al., 2019). NRTI treatment reduces cytoplasmic L1 DNA and type I IFN levels in tissues and primary cell cultures, an effect mirrored by L1 siRNA/shRNA knock-down in MEFs (Simon et al., 2019). Functionally, NRTIs improve the health (as assessed by body weight, bone density, muscle mass, etc.) and lifespan of *Sirt6*-deficient mice. These relationships appear to be biologically relevant for normal aging, because NRTI treatment reduces interferon levels and L1 DNA content in old wild-type mouse tissues (Simon et al., 2019). Finally, it is worth noting that plasma concentrations of SASP factors in old wild-type mice trend downwards upon NRTI treatment, consistent with the previous study (De Cecco et al., 2019). Because of the negative impact of senescent cells on aging mice (Baker et al., 2016; Demaria et al., 2014), there has been increasing enthusiasm for senolytics (i.e. drugs that selectively induce the death of senescent cells) as a potential therapy for age-related ailments. Given the link between senescence and TE activity, it will be interesting to evaluate whether senolytic drugs may also ameliorate TE-associated pathological inflammation and whether they promote an overall reduced transposon load.

TE activity may promote mitochondrial dysfunction

The mechanisms by which TEs contribute to cellular dysfunction are multifaceted, targeting the central 'information system' (i.e. the genome) of a cell through novel transposition or mutagenic events and its 'communications system' through induction of pro-inflammatory signaling molecules. Another vital system whose relationship with TEs has not been thoroughly explored is the 'energy system' of the cell, which is heavily dependent on mitochondrial function. Alterations in mitochondria function, and thus cellular metabolism, are known to occur with aging (López-Otín et al., 2013), and in age-associated pathologies, including cancer [i.e. the Warburg effect (Hanahan and Weinberg, 2011)]. Emerging evidence suggests that TE activity may promote mitochondrial dysfunction, but future work will need to determine the relevance of this to the aging process.

Consistent with the multi-pronged mechanisms of TE cellular disruption, studies suggest that Alu elements can disrupt mitochondrial organellar integrity and function (Fig. 1D). These relationships have been characterized within the context of an advanced form of age-related macular degeneration (Kerur et al., 2017; Tarallo et al., 2012). In one case, accumulation of Alu RNA, such as under conditions of DICER1 deficiency, induces production of mitochondrial reactive oxygen species (ROS), which contributes to activation of NLRP3 inflammasome and IL-18/MyD88-dependent apoptosis (Tarallo et al., 2012). Moreover, in human cell culture and mouse models, Alu RNA promotes opening of the mitochondrial permeability transition pore, release of mtDNA into the cytosol, activation of cGAS, induction of IFN-β and NLRP3 inflammasome activation (Kerur et al., 2017). Whether these mechanisms are specific to age-related macular degeneration or are more broadly applicable to aging cells remains unclear.

Non-specific effects of Alu (i.e. effects that mirror those of 7SL RNA, from which Alu is believed to be derived) are observed (Baryakin et al., 2013; Herbert, 2019). Specifically, MCF-7 cells transfected with either Alu or 7SL RNA do not robustly induce interferon-inducible gene expression, but do show modulation of pro-apoptotic changes in expression of genes such as *NUPR1*, *DDIT3* and *FOXRED2* (Baryakin et al., 2013). Interestingly, the last two genes play roles in endoplasmic reticulum (ER) stress, and the *DDIT3* gene product can also alter the mitochondrial transmembrane potential, generate ROS and induce apoptosis (Riemer et al., 2009; Tabas and Ron, 2011). The signal recognition particle (SRP), which normally contains 7SL RNA and is involved in delivery of proteins to the ER for co-translational translocation, is likely to play a role in these cellular responses. SRP loss induces RNA/protein mistargeting to mitochondria and mitochondrial dysfunction (Costa et al., 2018). As Alu RNA binds to SRP components SRP9/14 (Bovia et al., 1995; Chang and Maraia, 1993;

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Chang et al., 1994), some have proposed that Alu-induced protein mistargeting may itself cause ER stress (Baryakin et al., 2013) or mitochondrial damage (Herbert, 2019). Future work should seek to clarify the upstream molecular mechanisms driving ER stress and mitochondrial damage, whether it be through protein mistargeting or through alternate mechanisms. It would be informative to assess whether Alu elements drive ER stress and mitochondrial dysfunction under conditions of higher Alu expression, such as during aging.

Data also suggest that L1-derived molecules and mitochondria may physically interact, although it is unclear whether these interactions are indicative of a healthy or unhealthy cellular prognosis. Through a series of co-immunoprecipitation and mass spectrometry experiments, one study has identified a number of proteins that interact with L1 proteins (Taylor et al., 2018, 2013). Among them, mitochondrial import inner membrane translocase subunit Tim13 (TIMM13) and translocase of outer mitochondrial membrane 40 (TOMM40) associate with ORF2p, and the mitochondrial GTPase and potential chaperone ERAL1 associates with ORF1p (Taylor et al., 2018, 2013). These interactions suggest that ORF1p/ORF2p may have cellular roles beyond binding, reverse transcribing and integrating L1/Alu (Taylor et al., 2018, 2013). Alternatively, it raises the intriguing possibility that L1 or Alu RNA or cDNA may exhibit mitochondrial localization and interact with mitochondrial DNA. Interestingly, Alu elements appear to be enriched in genes involved in mitochondrial pathways (Larsen et al., 2018), although whether these integrations occurred before or after transfer of most mitochondrial genes to the nucleus is an interesting question. Less hypothetically, high MDA5-MAVS-interferon signaling in response to Alu-dsRNA is a feature of the autoimmune disorder Aicardi-Goutières syndrome (Ahmad et al., 2018), and MAVS can restrict L1 transposition *in vitro* (Goodier et al., 2015). It may be informative to assess whether heightened L1 and Alu expression during aging activate MAVS-mediated autoimmune responses. If so, this may partially explain age-related mitochondrial metabolic changes, as PRR activation and binding to MAVS impairs glucose metabolism (Zhang et al., 2019). Although this may not be observed, it is also possible that mitochondrial failure during aging may drive MAVS dysfunction, blunted immune responses to TE activity and additional downstream consequences of less-restricted TEs. Given the sheer number and different classes of TEs within any given genome, these ideas deserve further exploration, especially in the context of aging, age-related pathologies and cellular senescence.

circRNAs: an emerging species of regulatory RNAs relevant to aging

Circular RNAs (circRNAs) are a class of RNA species without free 5′- or 3′-ends (Salzman et al., 2012). Although they were assumed to be artefacts when first described (Hsu and Coca-Prados, 1979), research over the past decade has transformed our understanding of circRNAs, and they are emerging as an important family of RNAs that are ubiquitously expressed in eukaryotes (Salzman et al., 2012), including *D. melanogaster*, *C. elegans*, mice and humans (reviewed by Patop et al., 2019). The biogenesis and general functions of circRNAs are reviewed elsewhere (Barrett and Salzman, 2016) (Fig. 2A). Interestingly, one important feature in the biogenesis of circRNAs is the presence of introns containing splice sites and short inverted repeats, such as Alu elements, flanking the exons that will circularize (Liang and Wilusz, 2014). This again highlights the involvement of TEs in alternative gene expression profiles, including the expression of circRNAs. Mechanistically, circRNAs are able to modulate gene expression through multiple mechanisms, including (1) regulation of miRNA availability (Capel et al., 1993; Hansen et al., 2013, 2011; Memczak et al., 2013; Sekar et al., 2018 preprint), (2) regulation of RNA-binding protein availability (Ashwal-Fluss et al., 2014; Conn et al., 2015; Du et al., 2016b), and (3) production of alternative protein isoforms derived from circRNAs (Legnini et al., 2017; Pamudurti et al., 2017; Yang et al., 2017).

circRNAs in aging and age-related phenotypes

Age-related regulation of circRNA levels was first described in *D. melanogaster*, where increased circRNA levels are detected in the head between 1 and 20 days of life. Consistent with the tissue analyzed, a Gene Ontology analysis of circRNA-producing genes showed enrichment for terms related to neural processes. However, a similar analysis of circRNAs in embryos and cells lacking neurons also produced terms related to neural function. These results suggest that genes producing circRNAs may more broadly be involved in nervous system function (Westholm et al., 2014). Additional studies support an age-related increase in circRNA expression in aging brain tissue across species (Gruner et al., 2016; Lu et al., 2018; Westholm et al., 2014; Zhou et al., 2018), although similar or higher numbers of circRNAs with age-related decreases in expression have been detected in the tree shrew (Lu et al., 2018). Indeed, Gruner and colleagues compared the levels of circRNAs in the cortex, hippocampus and heart between 1-month and 22-month-old mice (Gruner et al., 2016). Although developmental changes may be confused with aging effects because of the extremely young age of the ‘young’ group (1 month old, before sexual maturity), the majority of changes support a trend for age-related upregulation of circRNA expression across brain regions (Gruner et al., 2016). Intriguingly, this study also established that changes in circRNA expression are mostly independent of that of their linear counterparts (Gruner et al., 2016). A similar trend for age-associated accumulation of circRNAs in the brain has also been reported in the rat (Zhou et al., 2018), although, again, the study selected an extremely young age for the ‘young’ group (2 weeks, also before sexual maturity) (Zhou et al., 2018). Age-related increased circRNA expression is observed in *C. elegans* between the L4 stage and 10 days, although the tissue or cells of origin are unclear because the analysis was conducted on whole worms (Cortés-López et al., 2018). Changes in circRNA expression are not limited to the nervous system and can exhibit tissue- or cell-specific expression trajectories. In rats, for example, while increasing circRNA expression in the brain is observed between 2 and 104 weeks of age, decreased circRNA levels are observed in lung tissue (Zhou et al., 2018). Similarly, circRNA levels decreased in rat testis between 21 and 104 weeks of age (true ‘aging’), and the genes producing these circRNAs are associated with spermatogenesis (Zhou et al., 2018). Finally, a study on *Drosophila* photoreceptors focusing on loss of visual acuity identified upregulation of circRNAs between 10 and 40 days, in parallel with stress and DNA-damage responses (Hall et al., 2017).

One particular circRNA, Cdr1as, appears to be important for proper neural function, broadly, and sensorimotor gating and synaptic transmission, specifically. *In vivo*, *Cdr1as* (*Cdr1os*) knockout mice are defective in these phenotypes, and these defects correlate with decreased miR-7 expression and increased miR-7 target expression, suggesting that Cdr1as stabilizes miR-7 (Piwecka et al., 2017). An additional study expanded the molecular network of Cdr1as and miR-7, and showed that the long non-coding RNA cyrano is important for maintaining stable levels of Cdr1as.

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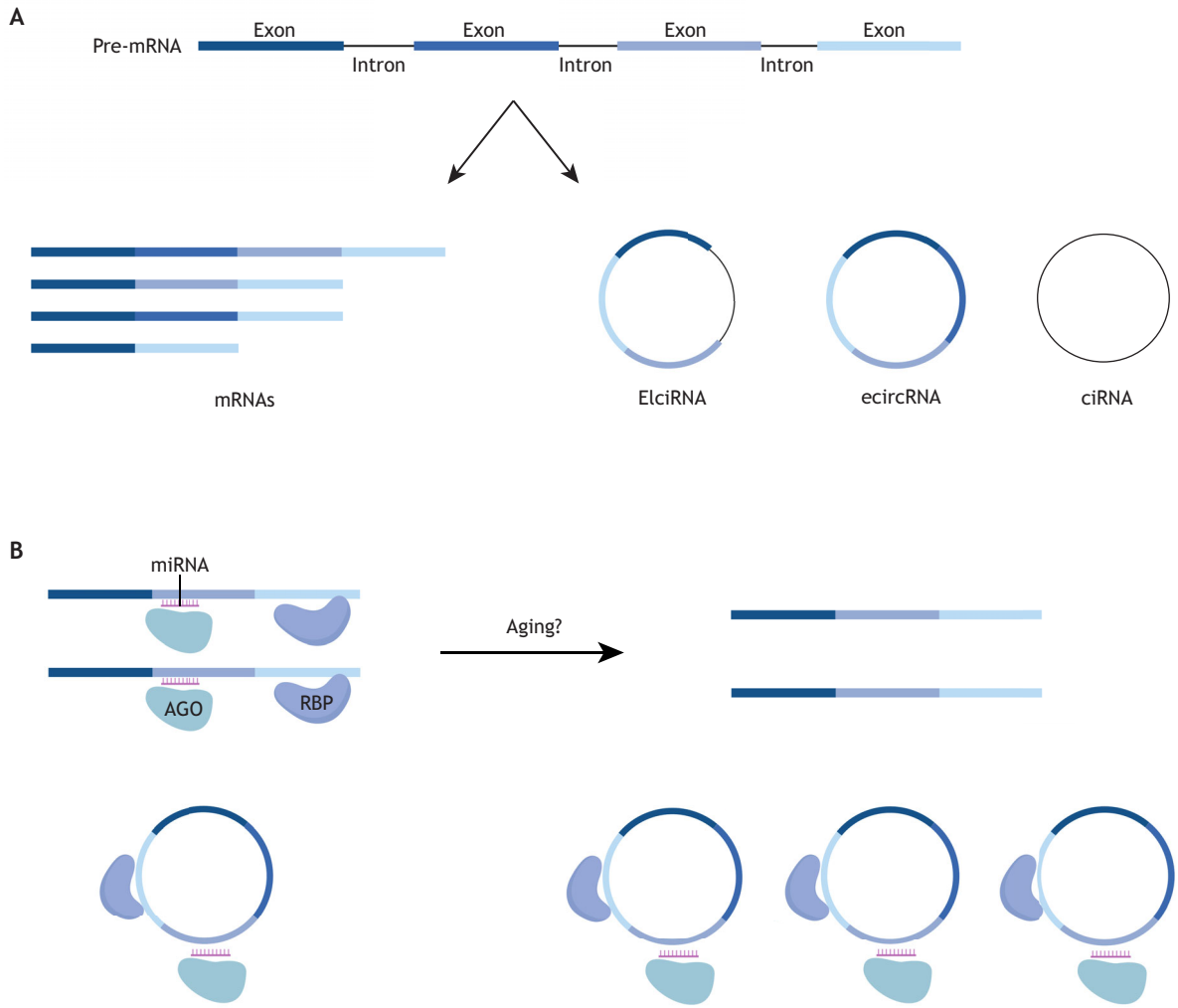

Fig. 2. circRNAs are circular alternatively spliced products that may accumulate with aging. (A) In addition to linear transcripts, three types of circular RNAs may be generated depending on the combination of exons and/or introns that are included: ecircRNA (exonic circular RNA), ElciRNA (exon-intron circRNA) and ciRNA (circular intronic RNA). (B) circRNAs can interact with the miRNAs and RNA-binding proteins (RBPs) that also bind to their linear counterparts. One hypothesis proposed here postulates that increased age-related circRNA expression may drive altered expression of linear isoforms by siphoning miRNAs and RBPs away from mRNAs. AGO, argonaute.

Specifically, cyrano knockouts exhibit high levels of mir-7, which enhances mir-671-mediated cutting of Cdr1as (Kleaveland et al., 2018). It is possible that Cdr1as has roles in age-related pathological conditions of the brain and in cancer, because miR-7 has also been found to regulate the expression of α-synuclein, a key marker of Parkinson's disease (Junn et al., 2009), and of cancer-related genes (Kefas et al., 2008; Reddy et al., 2008). With particular relevance to aging, analyses of the miRNAs targeted by 10 circRNAs present in astrocytes of elderly subjects has detected >14,000 unique interactions (Sekar et al., 2018 preprint). Thus, the role of circRNAs in the brain and in aging may go well beyond that of Cdr1as.

In a select example, additional relationships between circRNAs and brain health were identified by studying a mouse model of Alzheimer's disease (AD) [i.e. the senescence-accelerated mouse prone 8 (SAMP8) model, the learning and memory abilities of which spontaneously decline as a function of age]. Using this model, 235 significantly dysregulated circRNA transcripts were identified, compared with the senescence-accelerated resistant control strain (Zhang et al., 2017). Interestingly, a complex circRNA-miRNA-mRNA regulatory network may be involved in the pathogenesis of AD (Sekar et al., 2018; Wang et al., 2018; Zhang et al., 2017). Although much of the circRNA research focus has been placed on the role of circRNAs in the brain, increasing numbers of studies demonstrate that circRNAs can serve as markers for a number of diseases (reviewed by Haddad and Lorenzen, 2019; Lee et al., 2019; Zhang et al., 2018), including colorectal cancer (Huang et al., 2015; Zhang et al., 2013), diabetes mellitus (Fang et al., 2018), cholangiocarcinoma (Xu et al., 2019), cardiovascular diseases (Cai et al., 2019; Maiese, 2016) and other age-related conditions.

As a potential regulator for cardiovascular disease development or progression, *circFoxo3* is elevated in the hearts of aged individuals and mice, and can promote senescence in mouse embryonic fibroblasts (Du et al., 2016a). Conversely, downregulation of *circPVT1* is crucial to establish senescence in human fibroblasts (Panda et al., 2017b). More generally, functional consequences for circRNA misregulation are still largely unknown. Emerging research shows circRNAs may exert their effects through

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various signaling pathways, including modulating Wnt/β-catenin signaling to alter cellular growth (Kulcheski et al., 2016). Changes in the expression of circRNAs during aging and aging-associated diseases may thus promote sub-optimal cellular states, e.g. by promoting excessive senescence-mediated inflammation or altered cell signaling. Alternatively, changes in the levels of circRNAs produced by aging genes may alter linear isoform expression, such as by siphoning miRNAs or RNA-binding proteins, and thus contribute to altered aging trajectories. The field of circRNA biology is still very young and will benefit from systematic gain- and loss-of-function studies for circRNA genes to clarify their potential role in health and lifespan. Ultimately, a better understanding of the mechanisms and functions circRNAs partake in will allow the field to assess and better comprehend the roles these species play in aging (Fig. 2B).

Tools to probe changes in circRNA expression

Moving forward, it will be important to develop or improve experimental and analytical methods for a more efficient and systematic study of circRNAs. At the moment, generation of genetic tools involving circRNAs is highly challenging. Loss-of-function experiments involving siRNA-mediated circRNA knockdown may also knock down the linear transcripts. In contrast, gain-of-function experiments involving circRNA overexpression may also overexpress linear transcripts or induce an immune response, if the circRNA construct has elements that are not endogenous to the host (these limitations, as well as the role of circRNAs in aging, are discussed by Knupp and Miura, 2018). With regards to '-omics' approaches, routine RNA-sequencing library preparations may not be sufficient for thorough characterizations of circRNAs in samples of interest. As an example, rRNAs, which compose the majority of RNA in a cell, can be limited by enriching for polyadenylated transcripts. However, this is a restrictive method for circRNA analyses, as circRNAs do not contain polyA tails. Thus, preparations of total RNA libraries followed by rRNA depletion is an alternative option, but sequencing depth for circRNAs may still be too low. It is now common practice to digest samples with Ribonuclease R to enrich for circRNAs, which are more resistant to degradation compared with linear RNA (Jeck et al., 2013). Though effective, some circRNAs seem to be sensitive to this treatment (Jeck et al., 2013) and some linear RNAs are non-sensitive (Panda et al., 2017a). New methods addressing these limitations continue to be developed, such as RPAD (RNase R treatment followed by Polyadenylation and poly(A)$^+$ RNA Depletion) (Panda et al., 2017a). In any case, it will be crucial to incorporate analysis of these species into existing analytical pipelines for RNA-seq. A number of tools are now available to study circRNAs (Table 2; reviewed by Jakobi and Dieterich, 2019; Sharma et al., 2019; Zeng et al., 2017), including *de novo* identification of circRNA species and the quantification of circRNA expression levels. Incorporation of these tools into existing pipelines is readily feasible and should lead to more thorough characterizations and specific quantifications of organismal transcriptomes, as well as to the generation of novel links between these understudied RNAs and many phenotypes of interest.

Age-related transcriptional remodeling and mitochondria

Mitochondria are intracellular organelles primarily known for generating ATP. Mitochondria are also important players in a number of signaling pathways that control key cell processes, including cell growth (Antico Arciuch et al., 2011), apoptosis (Green, 1998) and host immunity (Breda et al., 2019). Although mitochondria are involved in a number of processes, the mitochondrial genome alone is insufficient to carry out these functions (Fig. 3A). For example, the zebrafish, mouse and human mitochondrial genomes are ∼16 kb (Anderson et al., 1981; Bibb et al., 1981; Broughton et al., 2001) and, until recently, were thought to harbor only 37 genes. In humans, these genes encode two rRNAs, 22 tRNAs and 13 proteins (Anderson et al., 1981). Consequently, mitochondria rely on the import of cytoplasmic molecules (i.e. proteins, RNAs and lipids) to establish and maintain proper function (Gebert et al., 2011; Sieber et al., 2011). Notably, nuclear-encoded mitochondrial proteins make up a significant fraction of imported molecules (Box 2; Fig. 3A). Thus, perhaps unsurprisingly, the mechanisms and targets of mitochondrial protein import machinery are the most characterized, with one study estimating over 1000 mouse or human genes producing mitochondria-localized proteins (Calvo et al., 2016). In contrast, the number of studies focusing on RNA import, its targets and its mechanisms, are sparse, leaving a huge gap in our understanding of not only basic mitochondrial function, but also phenotypes with a mitochondrial component, such as aging. Transcriptomic changes with age have been observed in a number of model organisms (Frenk and Houseley, 2018; Lai et al., 2019), but whether the nuclear-encoded mitochondrial transcriptome changes with age and whether this may impact mitochondrial function are still unanswered questions. Here, we review the prevalence of mitochondrial RNA import, its mechanisms, and age-related changes in MDPs.

Mitochondrial localization of nuclear transcripts

Mitochondrial localization has been observed for multiple, nuclear-encoded non-coding RNAs (Fig. 3A). Some examples of these include the 5S RNA in bovine mitochondria (Yoshionari et al., 1994), RNase mitochondrial RNA processing (MRP) RNA (Chang and Clayton, 1987), the RNA component of RNase P (Topper et al., 1992) and some nuclear-encoded tRNAs (Dörner et al., 2001; LeBlanc et al., 1999; Mercer et al., 2011; Rubio et al., 2008).

Table 2. Tools to analyze and quantify differential circRNA abundances in RNA-sequencing experiments

Tool	Link to software	Reference
CirComPara	https://github.com/egaffo/CirComPara	Gaffo et al. (2017)
circMeta	https://github.com/lichen-lab/circMeta	Chen et al. (2019)
CircTest	https://github.com/dieterich-lab/CircTest	Cheng et al. (2015)
CIRI2	https://sourceforge.net/projects/ciri/	Gao et al. (2015, 2018)
DCC	https://github.com/dieterich-lab/DCC	Cheng et al. (2015)
KNIFE	https://github.com/lindaszabo/KNIFE	Szabo et al. (2015)
NCLscan	https://github.com/TreesLab/NCLscan	Chuang et al. (2016)
PTESFinder	https://sourceforge.net/projects/ptesfinder-v1/	Izuogu et al. (2016)
Sailfish-cir	https://github.com/zerodel/Sailfish-cir	Li et al. (2017b)
STARChip	https://github.com/LosicLab/STARChip	Akers et al. (2018)

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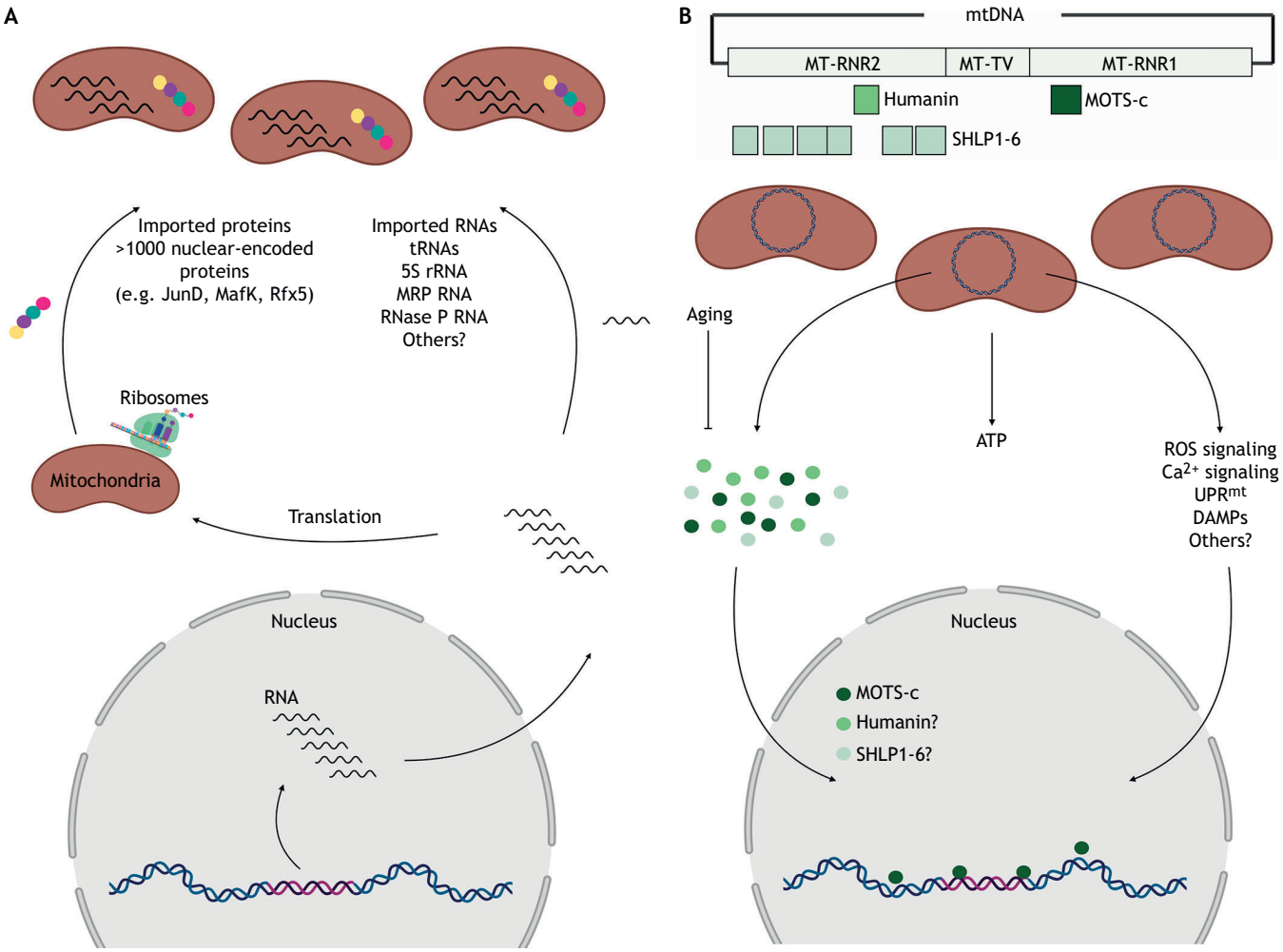

Fig. 3. Mito-nuclear communication is bidirectional and may be disrupted during aging. (A) Nuclear-encoded proteins and RNAs can be shuttled to mitochondria. Over 1000 nuclear-encoded proteins exhibiting mitochondrial localization have been documented in yeast and humans. Nuclear-encoded RNAs exhibiting mitochondrial localization are incompletely catalogued. (B) Mitochondria can signal to the nuclear compartment in a retrograde fashion. Small ORFs within the mitochondrial genome are believed to encode mitochondrial-derived peptides (MDPs), one of which, MOTS-c, has been shown to localize to the nucleus and to alter nuclear gene expression. Although other MDPs can also be exported from mitochondria, whether they also regulate nuclear gene expression has not yet been explored. However, there is a general trend for age-related decreases in the levels of MDPs. Other examples of retrograde communication from mitochondria include reactive oxygen species (ROS) and Ca^{2+} signaling, activation of UPR^{mt} and production of damage-associated molecular patterns (DAMPs). MT-RNR1: mitochondrially encoded 12S RNA; MT-RNR2: mitochondrially encoded 16S RNA; MT-TV: mitochondrially encoded tRNA valine.

Additionally, localization and enrichment of miRNAs in mitochondria have also been noted in rat and mouse livers, with some targets involved in apoptosis, cell proliferation, differentiation or mitochondrial function (Bian et al., 2010; Kren et al., 2009). Importantly, these findings are accompanied by the identification of the Ago2 protein, a key player in small RNA-mediated silencing, in mitochondria (Bian et al., 2010; Das et al., 2012), suggesting that Ago2-mediated RNA silencing may occur in this organelle. Indeed, miR-181c binds mt-COX1 mRNA, inhibits its translation and promotes mitochondrial respiration and reactive oxygen species (ROS) generation in rat cardiac monocytes (Das et al., 2012). Despite accumulating evidence demonstrating the localization and functional impact of nuclear RNAs in mitochondrial function, there have been few published efforts to characterize the mitochondrial transcriptome using unbiased, next-generation sequencing methods (Mercer et al., 2011; Sabharwal et al., 2018); to our knowledge, there have also been no well-controlled efforts to characterize its dynamics (Box 3).

Recently, an RNA-seq analysis of mitoplasts in a zebrafish model has led to the identification of 292 transcripts previously unknown to localize to mitochondria, one of which, *rpl11*, has been experimentally validated by qPCR and FISH (Sabharwal et al., 2018). This analysis suggests that the mitochondrial transcriptome has a prevalent fraction that is of nuclear origin and is largely being ignored (Box 3; Fig. 3A). Of particular interest, it is unknown how the mitochondrial transcriptome changes with age, how this change alters mitochondrial or cellular function, and how these alterations contribute to aging-related phenotypes. A characterization of mitochondrial age-related transcriptional remodeling is likely to lead to a new understanding of the mechanisms of aging and other phenotypes with a mitochondrial component. As both genomics datasets and our understanding of metazoan mitochondrial RNA-import mechanisms are limited, it is difficult to bioinformatically probe the contributions of the mitochondrial transcriptome to dynamic phenotypes.

Nevertheless, a few features of imported transcripts are known. A study using variants of the human 5S rRNA demonstrated that the α-

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Box 2. The genomics of mito-nuclear communication and its implications in aging

It is currently the standard practice to analyze genomic datasets (e.g. ChIP-seq) after filtering out repetitive (e.g. TE-derived) and mitochondrial sequences. This practice is based upon the tenet that nuclear-encoded proteins, and in particular transcription factors (TFs), should not have physiological roles in mitochondria. Thus, ChIP-enriched regions mapping to the mitochondrial genome are usually discarded as artefacts, or used to estimate background (Wu et al., 2010). However, accumulating evidence supports the notion that specific TFs may be imported inside mitochondria where they can bind mtDNA in a sequence-specific manner (e.g. JUN, JunD, MafK, Rfx5 and CEBPb) (Blumberg et al., 2014; Marinov et al., 2014). Supporting the biological relevance of these interactions, mtDNA sequences bound by nuclear-encoded TFs appear to be under negative selection (Blumberg et al., 2014), suggesting a functional role. Growing evidence also suggests that mammalian mitochondrial genomes can organize into nuclear chromatin-like structures (Blumberg et al., 2018; Marom et al., 2019). Together, these observations support the previously unsuspected existence of a complex bidirectional genomic communication between the nuclear and mitochondrial genomes, far more involved and active than previously surmised. Because of the central role of age-related mitochondrial dysfunction, it will be important to determine whether interactions between nuclear-encoded TFs and mtDNA, as well as their downstream consequences, are perturbed during aging and in age-related diseases.

and γ-domains are structural features necessary for import (Smirnov et al., 2008). Others conducted *in vitro* RNA import assays with mitochondria from yeast expressing polynucleotide phosphorylase (*Pnpase*) or mitochondria from the livers of mice with hepatocyte *Pnpase* knockout, and they show that PNPASE modulates import or stability of RNase P, 5S RNA and MRP RNAs (Wang et al., 2010). Importantly, import assays with full-length or truncated RNase P and MRP RNA implicate two stem-loop structures in the import process (Wang et al., 2010). More recently, silencing of TOMM40, TIMM23 and PNPase in HEK293 cells has shown to decrease the levels of mitoplast *RMRP*, again suggestive of their role in RNA import (Noh et al., 2016). A motif analysis of a subset of the 292 zebrafish transcripts identified three enriched motifs, suggestive of a sequence-dependent component involved in RNA import to the mitochondria (Sabharwal et al., 2018). These features of mitochondrially imported transcripts will be useful in generating hypotheses until more data are available. As an example, one can begin cataloguing whether other transcripts, in addition to the 292 zebrafish transcripts, contain the identified mitochondrial localization motifs, whether the corresponding genes are enriched in specific pathways, and whether similar motifs exist in other organisms. In any case, it is important to again highlight the scarcity of resources available and the gap in understanding regarding this topic. Future work should seek to clarify the biological significance of the entire mitochondrial transcriptome.

Mitochondrial-derived peptides (MDPs) in aging-related phenotypes

A second, emerging component of the mitochondrial transcriptome and proteome that necessitates further investigation are transcripts encoding mitochondrial-derived peptides (MDPs). In contrast to nuclear-encoded mitochondrial proteins, MDPs are believed to originate from small open reading frames (sORFs) within the mitochondrial genome and may be involved in retrograde communication with the nucleus (Kim et al., 2018a) (Fig. 3B). It is hypothesized that a decline in mitochondrial function may contribute to aging (reviewed by Sun et al., 2016), although the role of MDPs in this decline is unclear.

In addition to the canonical 13 mitochondrial-encoded proteins, a number of sORF-derived small peptides within the mitochondrial genome are being studied for their biological properties (Kim et al., 2017). There is ongoing debate over the origin of these peptides, due in part to the presence of nuclear mitochondrial sequences (NUMTs) that retain sORFs with the potential to produce MDP-like molecules (Bodzioch et al., 2009). Nevertheless, there is already evidence supporting the existence of eight different MDPs in humans (Fig. 3B). These include humanin (HN, 24 amino acids) (Guo et al., 2003; Hashimoto et al., 2001a,b; Ikonen et al., 2003), small humanin-like peptides 1-6 (SHLP1-6) (Cobb et al., 2016) and mitochondrial open reading frame of the 12S rRNA type-c (MOTS-c, 16 amino acids) (Lee et al., 2015) (Table 3). Humanin was the first peptide to attract attention, initially proposed when a functional screen of cDNA libraries from the occipital cortex of an individual with Alzheimer's disease identified genetic factors that repressed cell death (Hashimoto et al., 2001b). SHLP1-6 and MOTS-c have been subsequently discovered and characterized following *in silico* screening for potential sORFs within mitochondrial genes (Cobb et al., 2016; Lee et al., 2015). These peptides are believed to originate from sORFs within the 16S rRNA gene for humanin (Maximov et al., 2002) and SHLP1-6 (Cobb et al., 2016), and the 12S rRNA gene for MOTS-c (Lee et al., 2015). These properties have complicated their analysis in routine transcriptomic experiments. Indeed, because rRNA sequences represent >95% of total RNA in cellular transcriptomes, depletion of ribosomal RNA sequences of both nuclear and mitochondrial origins has become a cost-effective norm in the analysis of gene expression changes by RNA-sequencing (Herbert et al., 2018; O'Neil et al., 2013). Unfortunately, these experimental constraints have led to a dearth of information on the differential transcriptional regulation of

Box 3. Limitations in characterizing the mito-transcriptome

Impurity of mitochondrial fractions is a major barrier in accurate identification of RNAs that are present inside mitochondria. Contaminating cytoplasmic RNAs in mitochondrial fractions can be depleted through treatment with RNases (Mercer et al., 2011). Additional depletion can be achieved through further isolation and RNase treatment of the inner mitochondrial membrane (Mercer et al., 2011). Though effective in obtaining purer fractions, this treatment can also deplete any mitochondrial membrane-bound RNAs or RNAs present in the mitochondrial intermembrane space, limiting our ability to characterize them. Finally, cellular fractionation protocols may co-isolate mitochondria and other cellular components associated with them, such as mitochondria-associated endoplasmic reticulum membrane. Thus, protocols for reliable isolation of mitochondria need to be used in order to avoid misidentifying the locations of RNAs (Williamson et al., 2015). Profiling of the mitochondrial transcriptome poses unique challenges in the study of aging primary cells as the progressive loss of material during subcellular fraction leads to large required amounts of starting material (beyond the number of freshly sorted cells that can be obtained from a single animal). Thus, development of novel, more-sensitive techniques will be required to increase the feasibility of systematically studying the aging mitochondrial transcriptome. Although next-generation sequencing of mitochondrial fractions can aid in the identification of mitochondria-localized transcripts, localization of top candidates should be confirmed using orthogonal approaches, such as RNA-FISH (as has been carried out by Sabharwal et al., 2018) or its increased sensitivity and resolution variant – single molecule RNA-FISH (Raj, 2013).

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Table 3. MDPs have therapeutic potential in age-related conditions

Peptide	Relevant conditions	Major findings
Humanin	Aging	Decreased plasma HN levels with age in mouse and humans (Muzumdar et al., 2009).
		HN serum levels are negatively correlated with age in humans (Ramanjaneya et al., 2019).
		IGF1/GH treatment reduces HN in mouse plasma. GH treatment reduces HN in human plasma. Inverse relationship between plasma HN levels and GH/IGF status in mice harboring mutations in these genes (Lee et al., 2014).
		HNG treatment induces AKT and ERK1/2 phosphorylation in the hippocampi of old mice (Kim et al., 2016).
		HN and MOTS-c are elevated in doxorubicin-induced senescent primary human fibroblasts. Treatment with these peptides increases SASP factor production in non-senescent and senescent cells (Kim et al., 2018b).
	Neurodegenerative	Protects neuronal cells against amyloid-β or mutant APP-, PS1- and PS2-induced cell death (Hashimoto et al., 2001b).
		Reduces toxic effects of APP mutation (Hashimoto et al., 2001a).
		Represses apoptosis induced by IGFBP-3 in glioblastoma-A172 cells (Ikonen et al., 2003).
		In APP/PS1 transgenic mice, HNG improves cognitive abilities and increases levels of exhibited markers of enhanced insulin signaling, reduced mTOR activity and improved autophagy in the hippocampus (Han et al., 2018).
		HN improves cognition in middle-aged mice. A HN SNP is associated with lower circulating HN and older cognitive age in humans (Yen et al., 2018).
	Cardiovascular	HNG treatment restricts myocardial fibrosis and apoptosis in aging mouse heart and promotes AKT and GSK3β phosphorylation (Qin et al., 2018).
		In human aortic endothelial cells, HN reduces ROS formation and apoptosis induced by oxidized LDL (Bachar et al., 2010).
	Ocular	HNG reduces pro-apoptosis gene expression in AMD cybrid cell lines (identical nuclei, but mitochondria are from AMD or control age-matched subjects) (Nashine et al., 2017).
		HN protects against oxidative-stress induced cell death and decline in mitochondrial function (Sreekumar et al., 2016). Similarly, HN protects RPE cells against ER-stress induced cell death (Matsunaga et al., 2016).
	Metabolism	HN serum levels are lower in individuals with type 2 diabetes (Ramanjaneya et al., 2019).
SHLP1-6	Aging	SHLP2 plasma levels decrease with age, and intracerebral infusion promotes glucose uptake in rats. SHLP2/3 reduces apoptosis and ROS production, and improves mitochondrial metabolism (Cobb et al., 2016).
	Cancer	Low SHLP2 levels are associated with prostate cancer in white patients (Xiao et al., 2017).
	Metabolism	SHLP2 prevents amyloid formation of IAPP, which is involved in T2DM (Okada et al., 2017).
MOTS-c	Aging	Polymorphism is associated with longevity of Japanese individuals (Fuku et al., 2015).
		Serum and muscle levels decrease between 4 and 32 months in mice (Lee et al., 2015).
		Serum levels are negatively correlated with age in humans (Ramanjaneya et al., 2019).
	Metabolism	Japanese men with this polymorphism tend to have type 2 diabetes (Zempo et al., 2016).
		MOTS-c treatment promotes insulin sensitivity in muscles of older mice comparable to that of young mice. It also suppresses obesity and insulin resistance induced by a high-fat diet (Lee et al., 2015).
		Plasma concentrations are similar in lean and obese individuals, and are associated with insulin resistance only in lean individuals (Cataldo et al., 2018).
		D-galactose injection aging mouse models exhibit lipid accumulation, which MOTS-c alleviates in the liver. Treatment restores epidermal morphology and inflammatory markers closer to control levels (Li et al., 2019).
		MOTS-c prevents obesity and insulin resistance in ovariectomy mouse models of menopause (Lu et al., 2019).
		MOTS-c serum levels are lower in individuals with type 2 diabetes (Ramanjaneya et al., 2019).

AKT, protein kinase B; AMD, age-related macular degeneration; APP, amyloid precursor protein; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GH, growth hormone; GSK3β, glycogen synthase kinase 3 β; HN, humanin; HNG, humanin G; IAPP, islet amyloid polypeptide; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; LDL, low density lipoprotein; MOTS-c, mitochondrial ORF of the twelve S rRDNA type-c; PS, presenilin; ROS, reactive oxygen species; RPE, retinal pigment epithelium; SASP, senescence-associated secretory phenotype; SHLP2, small humanin-like peptide 2; SNP, single nucleotide polymorphism; T2DM, type 2 diabetes mellitus.

mitochondrial rRNA sequences that encode MDPs. Nevertheless, by treating cells or animals with these peptides or by overexpressing them using genetic constructs, they have been shown to affect a number of age-related phenotypes (reviewed by Benayoun and Lee, 2019) and may play important roles during physiological aging (Table 3).

Broadly, one important factor of cellular fitness is mitonuclear communication. Indeed, 'incompatibility' between nuclear and mitochondrial genomes, as in conplastic mice with identical nuclear genomes but distinct mitochondrial genomes, is associated with differences in lifespan, metabolism, telomere length, tumor incidence and other age-related traits (Latorre-Pellicer et al., 2016). Multiple mechanisms exist to signal mitochondrial perturbations to the nuclear genome in order to alter nuclear gene expression and allow cellular adaptation to changing environments (Fig. 3B). Prominent examples include: (1) the mitochondrial unfolded response (UPR^{mt}), whereby, under stress, activating transcription factor associated with stress 1 (ATFS-1 in *C. elegans*) is re-directed from the mitochondria to the nucleus (Nargund et al., 2012; Qureshi et al., 2017); (2) mitochondrial-derived ROS, which seem to be a feature of some pro-longevity interventions (Shadel and Horvath, 2015); and (3) mitochondrial-derived damage-associated molecular patterns (DAMPs), molecular by-products of mitochondrial damage (Galluzzi et al., 2012; Wilkins et al., 2017). Emerging evidence suggests that MDPs may be another avenue by which this communication may occur. Indeed, MOTS-c has recently been shown to translocate to the nucleus under conditions of metabolic stress and this translocation is associated with altered gene expression, including a number of inflammatory markers (Kim et al., 2018a). Whether other MDPs exhibit similar regulatory properties remains to be characterized. Nevertheless, it is common practice in *in vitro* and *in silico* screens to look for nuclear-encoded regulators of various phenotypes (Box 2). This study, in contrast, suggests a need to consider mitochondrial-derived transcripts and peptides as alternative sources of genomic regulation and phenotypic variation. Future work on the development of conditional mitochondrial-gene manipulation may allow the field to further probe the existence of MDPs, as currently there is no method to specifically knock them out and study their impact on gene expression.

Concluding remarks

We have discussed accumulating evidence highlighting the links between aging phenotypes, transposable elements, circRNAs and the mitochondrial transcriptome. However, the basic biology of the mechanisms by which TEs and circRNAs directly alter different pillars of aging will deserve further exploration, using tools that are already available for their analysis on most usual '-omic' data types. In addition, we also discussed the important gap in knowledge around unbiased profiling of mitochondrial organellar 'omics' data, and the need for increased understanding of the mechanisms by which the mitochondrial transcriptome is established or modulated over time.

Though each of these elements remains understudied even in homeostatic youthful conditions, we also want to highlight the need to characterize how misregulation of TEs, circRNAs and mitochondria may influence each other, as well as influence other better-studied levels of regulation (i.e. mRNAs, miRNAs, etc.). Indeed, hallmarks of aging are most likely established through the loss of robustness of biological systems, at multiple levels. For example, data already supports a role for Alu elements in circRNA biogenesis (Liang and Wilusz, 2014), and the increased presence of cellular Alu elements may drive mitochondrial dysfunction (see above). In addition, L1 proteins have been found to interact with mitochondrial membrane proteins (Taylor et al., 2018). These observations raise an intriguing and thus far unanswered set of questions: Are L1 proteins interacting with mitochondrial proteins in the cytoplasm away from mitochondria, or do the interactions occur at the surface or inside mitochondria? What roles are L1 proteins playing in these interactions, if not reverse transcription, and are they age dependent? Does L1 or Alu interact with the mitochondrial genome? Possessing a relatively primitive DNA repair system based on base-excision repair (Yakes and VanHouten, 1997), the mitochondrial genome appears to be a relatively unprotected target for TE-based mutagenesis. A major barrier, however, appears to be translocation of TE RNA or cDNA into the mitochondrial matrix, although the presence of nuclear-encoded RNAs in the mitochondria has been documented. Additionally, stem loops, which seem to be a feature of mitochondrially imported transcripts, are conserved features of both L1 and Alu elements (Grechishnikova and Poptsova, 2016), further lending substance to this idea. Given their potential to disrupt cellular information (genomic), energy (mitochondria) and communication (secretome) systems, TEs, especially, may be important contributors to aging. Finally, accumulating evidence suggests that MDPs, despite their unusual origins, may profoundly alter cellular biology.

When available, we highlighted current tools for studying exotic species and their biological impacts to some extent. Many of these tools are readily implementable in most bioinformatic pipelines, and we believe they should be more systematically analyzed. Moving away from analytical pragmatism and embracing new modes of genomic regulation will ultimately advance the field of aging research, by elucidating the multi-layer changes that accompany aging, and finally attempting to bridge the gap between the longest living individuals and current, average life expectancies.

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Competing interests

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