Review

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Transcript diversity in aging: cryptic transcription and splicing

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Abstract: Increased transcript diversity, which is caused in part by alternative splicing and cryptic transcription, is an underappreciated aspect of age-associated transcriptome remodeling. Recent work has revealed that structurally novel transcripts increase during aging in many tissues. Genes with cryptic and alternatively spliced transcripts with age are enriched for functional categories relevant to tissue function and aging, and have been implicated in cognitive decline, decreased muscle strength, reduced oocyte quality, immune aging, altered stem cell properties, and senescence. Indeed, there is emerging evidence that alternatively spliced transcripts and elevated cryptic transcription directly contribute to aging phenotypes in multiple tissues. The full impact of the increased transcript diversity on the aging process has yet to be explored. The increased transcript diversity engendered by alternative splicing and cryptic transcription is emerging as a potent driver of aging and aging phenotypes, adding another layer to our understanding of the transcriptional regulation of aging.

Keywords: aging; alternative splicing; cryptic transcription; epigenetics

Introduction

Aging is characterized by the functional decline of many tissues, which is driven by specific cellular and molecular mechanisms, collectively termed the “hallmarks of aging” [1]. While itself not a hallmark of aging, transcriptional dysregulation is thought to be the consequence of other processes that are disrupted during aging. Transcriptomic changes have been linked to the loss of cell identity and altered tissue function during aging [2–4]. However, such changes have largely been characterized at the gene expression level, and do not take into account the nature of the transcripts themselves. Through cryptic transcription (aberrant transcription initiated from intragenic promoter-like sequences) and alternative splicing, novel transcripts that have a different structure than a gene’s primary, endogenous transcript can be generated. However, such transcripts are discounted in most analyses of age-associated gene expression changes, even though the structural differences can affect RNA stability, translation efficiency, and the sequence of any encoded proteins [5], all of which can impact physiology. Indeed, numerous studies have revealed that mRNA processing is disrupted in many tissues during aging [6], suggesting that distinct transcript variants generated from the same gene may be present in old and young organisms. Evidence is accumulating that the diversity of transcripts produced from a given gene increases during aging, particularly through alternative splicing and intragenic cryptic transcription initiation [7, 8], though these phenomena have not received nearly as much attention as gene expression changes. Understanding the effects of these novel transcripts is essential for our understanding of the aging process.

Recently, considerable progress has been made in knowing how dysregulated splicing and cryptic transcription impact aging. For the first time, cryptic transcription has been shown to increase during mammalian aging, and several groups have explored what drives this age-associated transcriptional dysregulation and how these transcripts may contribute to various aging phenotypes, including senescence [9, 10]. Other groups have shown that perturbations that increase cryptic transcription mimic certain aspects of aging in a variety of tissues [11–14], further implicating cryptic transcription in the aging process. An even greater focus has been on understanding how splicing changes drive aging. In addition to characterizing age-associated splicing events in a variety of tissues, several studies have identified chromatin state and transcription changes that drive splicing dysregulation during aging [15–23]. Accumulating evidence links loss of

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splicing regulators and specific age-associated alternatively spliced transcripts to the functional decline of tissues during aging [24–38]. These exciting new results both highlight that increased transcript diversity is a driver of aging and show that we are only beginning to understand its impact on this process.

Generation of structurally diverse transcripts though alternative splicing and cryptic transcription

Mammalian gene structure is complex, and it is well established that multiple transcripts can be generated from a single gene. Two fundamental processes that make these novel transcripts are alternative splicing and cryptic transcription. Both of these processes generate transcripts that have a distinct structure from the primary transcript, lacking (or gaining) exons and having distinct 5′ or 3′ UTRs [5, 8]. Before exploring the effects that these structural differences can have on the transcripts and the proteins they encode, we briefly discuss alternative splicing, cryptic transcription and how they are regulated.

Alternative splicing

Splicing is a co-transcriptional process by which introns are excised from a nascent transcript and the flanking exons are ligated together. Briefly, the U1 snRNP binds the 5′ splice site at the end of an exon, while SF1 binds the branch point within the intron, and the accessory factors U2AF1 and U2AF2 bind the 3′ splice site and intronic polypyrimidine tract. This allows the U2 snRNP to displace SF1 from the branch point site and interact with the 3′ splice site through U2AF1. The U2 snRNP then recruits the U4/U5/U6 snRNP complex to form the precatalytic spliceosome. U1 and U4 are released, and the 5′ splice site is cleaved; in the second catalytic step, the 5′ and 3′ splice sites are joined, linking the flanking exons [39]. While many, if not most, splicing events are constitutive, *i.e.*, occur in all copies of a given transcript, others are regulated and occur in only a fraction of all transcripts from a given gene. These regulated splicing events are termed alternative splicing, and this is a common mechanism used to increase transcript and protein diversity, with up to 95 % of all human genes subject to this process [40, 41].

At its heart, alternative splicing is a question of whether or not a particular splice site is used during the processing of a given transcript. Given the complexity of the splicing reaction, many factors can influence splice site usage, including the splice site itself, binding of splicing factors (SFs), the transcription rate, and even chromatin state [42, 43]. The sequences recognized by the core splicing machinery are degenerate; thus, intrinsically, some splice sites recruit spliceosome components more efficiently than others. In the absence of other regulation, these stronger splice sites are more likely to be used than weaker ones. However, SFs also contribute to splice site choice. These proteins bind to splicing enhancers or silencers in the primary transcript in a sequence-specific manner and either promote or inhibit the binding of core spliceosome components to nearby splice sites. Many SFs are in the hnRNP and SRSF families; broadly speaking, the former inhibit splice site usage, while the latter enhance it. As splicing occurs co-transcriptionally in the context of the primary transcript, both chromatin and the rate of transcription can affect the association of particular SFs with a primary transcript. Splicing enhancers and silencers can be upstream or downstream of the splice site they regulate; intuitively, then, the rate of transcription will impact whether a downstream regulatory element is transcribed soon enough to influence a given splice site choice. Furthermore, SFs can be recruited to a gene through their interaction with the modified histone residue H3K36me3, pre-positioning them for rapid association with a nascent transcript. Thus, altering the chromatin state, transcription rate, and SF availability can change splice site usage and induce alternative splicing (Figure 1).

Cryptic transcription

In contrast to alternative splicing, which is a normal part of RNA processing, cryptic transcription is typically inhibited in young, healthy tissues. This type of dysregulated transcription occurs when RNA polymerase II (Pol II) binds to a promoter-like sequence within the body of an actively transcribed gene and initiates transcription from this site. Significantly, cryptic transcription is distinct from alternative promoter use; not only are cryptic promoters definitionally not annotated promoters, but also their sequence composition and chromatin state when active are distinct from those of annotated transcription start sites [9, 10, 44].

Cryptic transcription is inhibited by a repressive chromatin state that renders these cryptic promoters inaccessible, preventing Pol II entry [45–47] (Figure 1). However, chromatin is opened during transcription, thus allowing Pol II to interact with cryptic promoters within actively transcribed genes. In young organisms, in the wake of Pol II transit, a closed chromatin state is restored, thus inhibiting

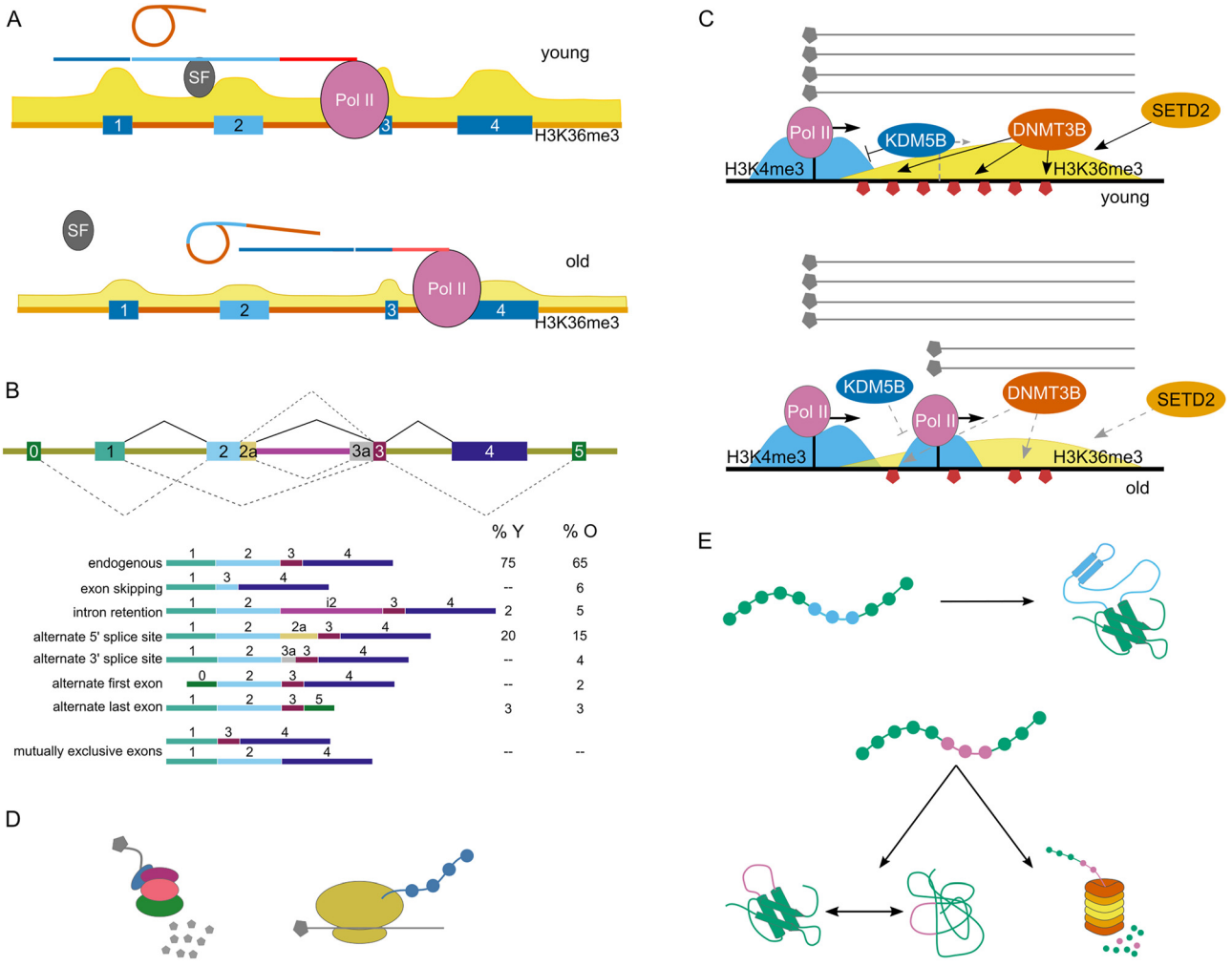

Figure 1: Regulation and effects of alternative splicing and cryptic transcription. (A) SFs bind to splicing enhancers and silencers in the nascent transcript and regulate whether a particular splice site is recognized by the spliceosome, and thus which alternative splicing event occurs. Splice site choices result in characteristic alternative splicing events: exon exclusion, intron inclusion, mutually exclusive exons, alternative 5' splice site, alternative 3' splice site, new first exon, and new last exon. SF availability and the presence/absence of splicing enhancers are further regulated by H3K36me3 levels on the gene and the rate of Pol II elongation. H3K36me3 levels, SF availability, and transcription rates change during aging, altering splicing patterns. (B) Intragenic cryptic transcription is typically inhibited by a repressive chromatin state. Co-transcriptionally conferred H3K36me3 recruits Kdm5b and Dnmt3b to the bodies of actively transcribed genes, where they erase H3K4me3 and methylate DNA, respectively, rendering cryptic promoters refractory to Pol II entry. As H3K36me3 is reduced with age, decreased DNA methylation and intragenic H3K4me3 allow transcription from cryptic promoters. (C) Cryptic and alternatively spliced transcripts can be targeted for degradation or translated. The resulting proteins may lack key domains or have/be novel sequences if the reading frame is altered. These peptides may be degraded, misfold and aggregate, or properly fold into functional proteins. Pol II, RNA polymerase II; H3K36me3, trimethylated lysine 36 of histone H3; H3K4me3, trimethylated lysine 4 of histone H3.

cryptic transcription [45]. The histone methyltransferase SETD2 associates with elongating Pol II and trimethylates histone H3 on lysine K36; H3K36me3 functions as a scaffold for the enzymes that generate a repressive chromatin state [8]. One such complex is FACT, which reassembles nucleosomes that are disrupted during transcription [48, 49]. H3K36me3 additionally recruits the DNA methyltransferase Dnmt3b to gene bodies to increase CpG methylation, which helps render cryptic promoters inactive [44]. Finally, H3K36me3 draws the histone demethylase Kdm5b to genic regions, where it functions to erase H3K4me3, which is co-transcriptionally conferred by MLL1 [50, 51]; this histone modification is associated with active promoters. Loss of any of these enzymes, which reduces H3K36me3 levels and DNA methylation and increases intragenic H3K4me3, allows cryptic transcription to occur.

Structurally diverse transcripts significantly impact cell function

It is difficult to detect and accurately quantitate the structural transcript variants generated by alternative splicing and cryptic transcription [9, 44, 48, 52–55]. As a result, although it is well established that a single gene can, and often does, produce multiple distinct transcripts, analysis of such variants is generally excluded from transcriptomic studies. Nevertheless, the diverse transcripts produced through alternative splicing and cryptic transcription, as well as their protein products, can have a significant effect on cellular function. Indeed, the cellular processes required to process these novel transcripts and their protein products (discussed below) are themselves disrupted during aging [1]. Therefore, it is especially important to understand the structural diversity of transcripts in this context.

There are seven widely accepted types of alternative splicing, categorized by the effects of each on the mature transcript: exon skipping, mutually exclusive exons, intron retention, alternative 5′ splice site, alternative 3′ splice site, alternative first exon, and alternative last exon (Figure 1) [56]. Alternative splicing thus produces transcripts that have distinct 5′ or 3′ UTRs, lack certain coding exons or parts thereof, or retain canonically intronic sequences relative to the standard spliceoform. In contrast, the mRNAs that result from cryptic transcription are less complex and simply lack some portion of the 5′ end of the full-length transcript, which can include the 5′ UTR and often a part of the coding sequence (Figure 1). In principle, depending on the location of the cryptic promoter, these transcripts may lack particular upstream splicing enhancers, and thus feed back and influence the exonic structure of the mature transcript.

These structural variants can have profound effects on both the RNA molecules themselves and the proteins translated from them. Changes to the UTRs can alter both transcript stability and translation efficiency, which in turn impacts the amount of protein produced [5]. Likewise, intron retention can drive nonsense-mediated decay and some cryptic transcripts are targeted for exosome-mediated clearance [44, 57]. Structural variants that alter the coding sequence can generate proteins with altered function due to inclusion or exclusion of regulatory, interaction, enzymatic, or localization domains; they can also produce entirely novel proteins if a different coding frame is used [8, 58]. These proteins, either novel peptides or structural variants, may also misfold, form aggregates, or be targeted for proteosomal or autophagic degradation [59] (Figure 1). As many RNA processing, proteostasis, and autophagy have reduced function in aging, increased cryptic transcription and alternative splicing will likely further aggravate an already overburdened system.

Increased alternative splicing contributes to aging phenotypes in a variety of tissues.

The regulation of splicing is dysregulated during aging

The complex regulation of splicing renders this process particularly vulnerable to age-associated dysregulation. Indeed, one of the first indications that splicing is altered during aging was the finding that SFs and genes associated with RNA splicing are among the most differentially expressed genes during aging. This has been observed in many tissues, including the brain [15, 60], cochlea [38], heart [18], bone marrow stem cells [25], leukocytes [61, 62], and muscle [63]. Decreased SF expression was also observed in aging *Drosophila* photoreceptors [28]. Proteomic analysis likewise revealed altered protein levels of SFs in muscle [19, 31] and oocytes [24] with age. With the exception of one of the studies in muscle [19], these genes and proteins were found to be downregulated during aging. SFs are also downregulated in fibroblasts from patients with the premature aging disease Hutchinson-Guilford progeria syndrome (HGPS) [64], as well as in senescent fibroblasts [62]. Moreover, a recent study in neurons transdifferentiated from old and young fibroblasts revealed that, in addition to a downregulation of genes involved in splicing, SNRNP70 and SNRNPA (components of the U1 snRNP); PRPF8 and SNRNP200 (part of the U4/5/6 snRNP); and the SFs TDP-43 and TIA1 become mis-localized to the cytosol during aging [36].

Several studies suggest a direct link between disrupted splicing and aging. Reduced expression of particular SFs in human blood correlates with aging pathologies, including cognitive decline and reduced muscle strength [31]. In mice, expression levels of several SFs in the spleen correlates with lifespan differences between different strains, with higher expression of several SFs associated with increased longevity [65]. Additional evidence is found in the expression of SFs in the naked mole rat, a rodent with an exceptionally long lifespan and few degenerative pathologies with age. These animals maintain consistent expression of SFs in several tissues during aging, in contrast to mice and humans, suggesting that maintaining youthful splicing patterns can delay aging and the onset of age-associated phenotypes [66].

In addition to SFs themselves being mis-regulated during aging, histone methylation patterns and transcription are also altered. A recent study has shown that the rate of Pol II elongation is increased during aging in a variety of animals and mammalian tissues; this is correlated with increased differential splicing in *Caenorhabditis elegans* and *Drosophila* [21]. Furthermore, the levels of H3K36me3, which can pre-position SFs for association with the nascent transcript, are reduced during aging [9, 67, 68]; this loss is associated with altered splicing patterns in flies [22]. Thus, the three major means by which splicing is regulated are disrupted during aging, which drives an increase of alternative splicing events with age.

Age-associated alternative splicing increases transcript diversity during aging

The fact that splicing is dysregulated with age implies that there should be an increase in the structural diversity of transcripts in aged tissues. Indeed, alternative splicing increases with age in many tissues. Perhaps the most attention has been given to brain tissue, due to the known association of alternative splicing with neurodegeneration, and peripheral blood, an easy source of tissue markers. In the mouse brain, several groups have observed altered splicing patterns in the temporal, pre-frontal, and cerebellar cortices, as well as the hippocampus [15, 23, 60, 69]. Likewise, in human leukocytes, age-associated changes in spliceoforms were detected in samples from the InCHIANTI study, in a cohort of subjects between 80 and 90 years old, and in a recent analysis of publicly available data [16, 61, 70].

Age-associated changes in splicing patterns have also been detected in the muscle of humans and rats, including through the GESTALT study [17, 19, 71]. Similarly, alternative splicing is increased with age in cardiac tissue from aged mice [18]. Splicing changes also occur during female reproductive aging; this has been observed in mouse oocytes, as well as in the ovaries of mice and *Drosophila* [24, 72, 73]. Likewise, differential splicing increases with age in mouse bone marrow stem cells [25].

Several groups have simultaneously examined splicing changes in multiple tissues during human and mouse aging. During mouse aging, skin, muscle, bone, thymus, and white adipose were found to have different levels of splicing aberrations. Bone showed the least differential splicing, while the greatest number of differentially spliced transcripts were observed in the skin [74]. Increased alternative splicing was also seen in human blood, skin, and adipose tissue [20]. Two independent analyses of RNA-seq datasets in the GTEx database uncovered increased alternative splicing with age in a variety of tissues [75, 76]. Senescent human fibroblasts and endothelial cells have increased differential splicing as well [35, 62].

In additional to mammals, splicing is dysregulated during aging in worms and flies. There is a progressive increase in intron retention during *Drosophila* aging. Interestingly, different genes and biological processes are affected at different timepoints, suggesting that dysregulated splicing contributes to different aspects of biology at different ages [77]. Furthermore, alternative splicing is increased in *Drosophila* photoreceptors during aging [28]. In *C. elegans*, differential splicing also increases with age, particularly in the intestine [26]. Likewise, in both worms and flies, increased rare splicing events, likely corresponding incorrect alternative splicing, were observed during aging [21]. Thus, increased alternative splicing is a common feature of aging throughout the animal kingdom.

Interestingly, there is tantalizing evidence that differential splicing, at least, progressively increases with increasing age. This trend is perhaps clearest in *Drosophila* heads, in which 68.7 % of differential splicing events are only observed in the oldest flies [77]. Similarly, alternative splicing is progressively elevated with increasing age in the skin, bone, and skeletal muscle of mice [74]. Work in human leukocytes indicates that differential splicing increased as subjects aged from 70 to 80 years old [16], which likely indicates that splicing, at least, is increasingly dysregulated with age. In contrast, a progressive increase in alternative splicing was not detected in mouse oocytes [24]. Nevertheless, the balance of evidence suggests that differential splicing continues to increase in a variety of tissues as individuals advance in age. This suggests both that splicing regulation itself is more disrupted in older individuals and that altered splicing can have a greater impact on cell and tissue function with increasing age.

Several trends are apparent throughout all these studies. First, in almost every comprehensive study in which both splicing and gene expression changes were assessed, little overlap was found between differentially expressed genes and those subject to age associated splicing changes [15–18, 23, 69–71, 75]; the one exception is in the *Drosophila* ovary, in which 22.5 % of genes with altered splicing are also differentially expressed [73]. Second, splicing is dysregulated during aging in a tissue-specific manner, which is most clearly evident in the multi-tissue analyses [20, 74, 75]. Additionally, largely unique genes are found to have increased transcript diversity in heart, muscle, and oocytes [17, 18, 24], though these studies were performed by different groups, making direct comparison less robust. Third, the genes undergoing age-associated alternative splicing are themselves related to aging, senescence, or tissue-essential

functions. In leukocytes, age-associated alternative splicing occurs in immune and inflammation-related genes [70]. In one study in muscles, differentially spliced genes are related to microtubule organization, calcium homeostasis, and muscle contraction [17], while in another, they are related to oxidative phosphorylation, adipogenesis, metabolism, and cell cycle regulation [71]. In the heart, they are involved in the sarcomere, metabolism, mitochondrial respiration, and translation [18]. In the brain, alternative splicing alters transcripts associated with mitochondria, DNA repair, protein oligomerization, isoprenoid biosynthesis, synaptic transmission, learning, and neurogenesis [15, 23, 60]. In *Drosophila* photoreceptors, they are associated with phototransduction, rhodopsin signaling, synaptic plasticity, and photoreceptor cell maintenance [28]. In oocytes, such genes are enriched for functional categories related to DNA damage and cell cycle regulation, which correlates with increased DNA damage and apoptosis in embryos developing from older oocytes [24], while in *Drosophila* ovaries, splicing impacts circadian rhythm and FOXO and MAPK signaling, all of which regulate aging [73]. Finally, in Bone Mesenchymal Stem Cells (BMSCs), alternative splicing drives the generation of transcripts that promote adipogenic differentiation at the expense of osteogenic differentiation during aging [25]. Altogether, this suggests that increased alternative splicing is an underappreciated factor in aging biology.

Altered splicing patterns drive aging phenotypes

While several alternative splicing events have long been associated with aging and senescence, namely splicing of lamin C and p53 [78, 79], recent studies have expanded our understanding of the role of splicing dysregulation in aging and aging phenotypes (Figure 2).

As discussed above, both SF expression and H3K36me3 are reduced in the photoreceptors of aged flies, which is correlated with increased alternative splicing [22, 28]. Importantly, photoreceptor-specific knockdown of select downregulated SFs, including Can; SC35; Caper; Saf-B; and the ortholog of the human LUC7-like protein, CG7564, both induces an aged splicing pattern and decreases visual acuity in young flies, directly implicating age-associated alternative splicing in this aging phenotype [28]. Furthermore, knockdown of *Set2*, which confers H3K36me3, drives a switch age-associated splicing patterns and vision loss in young flies, further implicating dysregulated splicing in visual aging [22].

One of the clearest pieces of evidence that age-associated alternative splicing regulates aging comes from *C. elegans*. Alternative splicing increases with age in worms, but older animals that maintain youthful splicing patterns tend to live longer. Furthermore, dietary restriction, which increases lifespan and healthspan, also maintains youthful splicing patterns in older worms, and disrupting splicing during dietary restriction blocks the beneficial effects of this intervention [26]. Interestingly, splicing patterns themselves are altered in young worms and the hippocampus of young mice undergoing dietary restriction; in both cases, affected genes encode proteins involved in metabolism, RNA processing, splicing, and translation or protein processing [27]. In *C. elegans*, the ability of dietary restriction itself to both extend lifespan and regulate splicing requires the SFs HRPU-1 (ortholog of human hnRNPU) and SFA-1 (human SF1) at a minimum. In the context of dietary restriction, SFA-1 is required for nutrient sensing downstream of TORC1 signaling, while HRPU-1-mediated splicing events are required to activate the nonsense-mediated decay pathway, both of which are necessary to extend lifespan [26, 27]. Reduced function of the SF RNP-6 (human PUF60) extends lifespan in worms by causing intron retention in *EGL-8* (a phospholipase Cβ ortholog) transcripts, reducing its protein levels; decreased EGL-8 protein inhibits TORC1 signaling [32]. Thus, there is a clear interaction between splicing and TORC1-mediated longevity in *C. elegans*.

Splicing dysregulation is also directly implicated in senescence, particularly the senescence-associated secretory phenotype (SASP). Treatment of senescent human fibroblasts with resveratrol or resverologs, which inhibit TORC1 signaling, decreases canonical senescence markers, including β-gal staining and SASP gene expression, while increasing SF expression. Importantly, this also alters the splicing patterns of several senescence-related genes [80]. This points to conservation of the interaction between splicing and TORC1 signaling in humans and worms. Likewise, inhibiting ERK or AKT signaling also increases SF expression and reduces senescence in senescent human fibroblasts and also decreases the senescence load of fibroblasts from HGPS patients, including reducing the SASP in both cases [64, 81]. In senescent human fibroblasts, reduced autophagy increases the levels of the SF SFPQ, thus promoting exon skipping in *EIF4H*, which encodes a short isoform of the protein. EIF4H-S promotes the interaction of EIF4A to its target mRNAs, including those of many SASP transcripts, increasing their translation [35], directly linking altered splicing to inflammaging. Additionally, downregulation of the SF SRSF7 is a feature of human fibroblast senescence; its loss drives senescence by promoting the alternative splicing of *MDM2* into the MDM2-C isoform, which is limited in its ability to ubiquitinylate p53 [34]. Similarly, in murine fibroblasts, an increase in the long

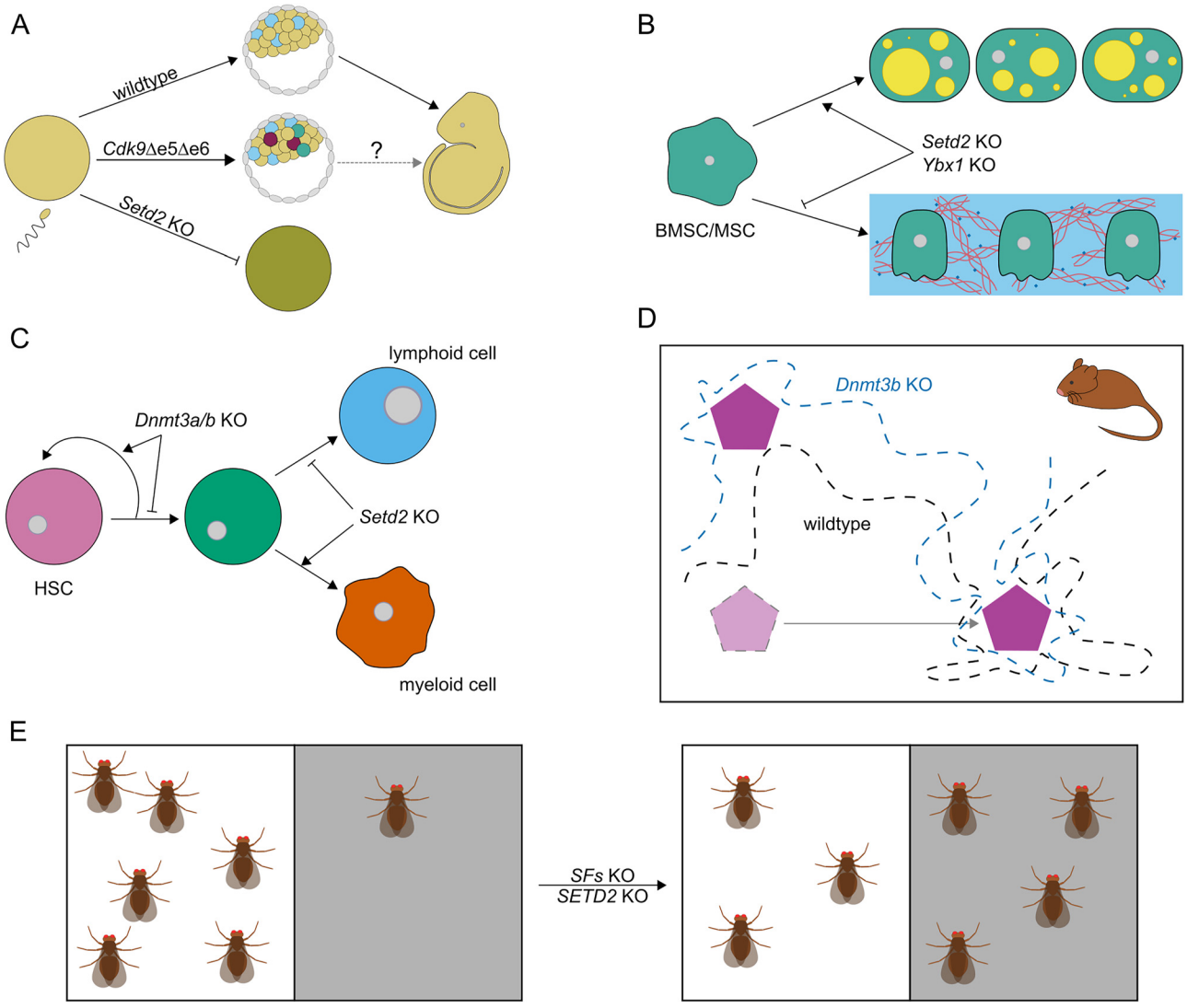

Figure 2: Perturbations that alter splicing and increase cryptic transcription mimic aging phenotypes. (A) In mouse oocytes, altered splicing of *Cdk9* to generate an age-associated spliceoform increases DNA damage and apoptosis in developing embryos. Similarly, loss of *Setd2*, which impacts splicing and elevates cryptic transcription, prevents progression to the 2 cell stage of development. (B) Loss of the splicing factor *Ybx1* in BMSCs or of *Setd2* in MSCs alters the differentiation potential of these cells, driving them towards an adipogenic fate. (C) HSCs that lack the paralogs *Dnmt3a* and *Dnmt3b*, which increases cryptic transcription, are driven to self-renew, expanding the progenitor pool. Loss of *Setd2* in these cells causes a myeloid bias during differentiation. (D) Knockout of *Dnmt3b* in the hippocampus of adult mice reduces recognition memory. KO, knockout; SFs, splicing factors. (E) Photoreceptor-specific knockout of several SFs or of *Set2* in *Drosophila* alters splicing patterns and impairs phototaxis.

variant of MRG15, which has reduced affinity for acylated H4, drives senescence through its lessened interaction with, and activation of, the *Cdk1* promoter [37].

Several studies link the loss of SF activity with increased alternative splicing in the brain. In the frontal cortex, the SF hnRNP K progressively mislocalizes from the nucleus to the cytosol during aging, and knockdown of *HNRNPK* in neuroblastoma cells increases differential splicing [29], similar to what is observed during brain aging. Protein levels of the long isoform of the SF hnRNP DL are reduced during aging in the mouse hippocampus, and loss of this protein in the brains of young mice increases alternative splicing, particularly of genes involved in synaptic function, cognitive processing, and synapse structure [30], which overlaps with the processes enriched for alternative splicing in the aged hippocampus [69]. In a model of neural aging, TDP-43 was found to bind 500 transcripts subject to age-associated alternative splicing; notably TDP-43 localizes to the cytosol in aged neurons, directly implicating the age-associated loss of this protein with splicing changes in neurons [36]. Alterative splicing of PDGFRβ is also implicated in the breakdown of the blood-brain barrier in the mouse brain. The expression of PDGFRβ-S, which has an

alternative first exon and does not code for the transmembrane domain of the protein, increases with age; this short isoform is translated into protein and is thought to disrupt PDGFR signaling, thus causing a functional decline in the pericytes that maintain the blood-brain barrier [33].

In BMSCs, *Ybx1* is one of many SFs with decreased expression during aging. Loss of this protein induces senescence and promotes adipogenic differentiation at the expense of osteogenic differentiation in BMSCs from young mice, which mimics the aging phenotype. Likewise, BMSC-specific knockout of *Ybx1* increases bone marrow adiposity in mice, another feature of aging. Significantly, differential splicing also increases in BMSCs that lack Ybx1, and Ybx1 directly binds to several alternatively spliced transcripts, including *Fn1*, *Sp7*, *Spp1*, *Sirt1*, and Nrp2, which are implicated in the altered differentiation potential and increased senescence of these cells. Significantly, the alternative transcripts that are generated in *Ybx1* knockouts mimic the functional decline of BMSCs during aging; that is, they encode less potent activators of osteogenesis and inhibitors of adipogenesis than the endogenous transcripts [25].

In mouse oocytes, *Cdk9* undergoes age-associated alternative splicing; using morpholino-substituted antisense oligonucleotides, Li et al. were able to induce age-associated exon skipping in *Cdk9* transcripts in oocytes isolated from young mice. Embryos that developed from these oocytes had increased DNA damage and a higher load of apoptotic cells, mimicking the phenotype of the embryos of older mice [24]. This is similar to the observation that Troponin T, which modulates the interaction between myosin and actin, is subject to alternative splicing with age; in older individuals, the β spliceoform is predominantly expressed, and this spliceoform is known to drive weaker muscle contraction, suggesting that mis-splicing of *TNNT3* contribute to a loss of muscle strength during aging [82, 83]. Thus, not only does altered splicing have a generally deleterious effect on the aging process, but specific age-associated splicing events directly contribute to aging phenotypes.

Elevated cryptic transcription drives aging phenotypes in fibroblasts and stem cells

Chromatin state dysregulation drives increased cryptic transcription during aging

As described above (Figure 1), cryptic transcription is normally repressed downstream of H3K36me3. However, the mechanisms by which cryptic transcription is repressed break down with age as the chromatin becomes more open. This is primarily due to a decline in intragenic H3K36me3 [9, 22, 67, 68]; the loss of this scaffold results in reduced Kdm5b and Dnmt3b recruitment, which renders cryptic promoters become more accessible and drives the elevation of cryptic transcription. H3K4me3 accumulates at cryptic promoters during aging, likely as the result of reduced Kdm5b, rendering them more accessible to Pol II [9, 10, 51]. Likewise, lessened Dnmt3b recruitment within gene bodies contributes to a reduction in CpG methylation, further opening cryptic promoters to allow transcription initiation from these sites [9, 10, 44]. Thus, chromatin state changes that are driven by reduced H3K36me3 promote cryptic transcription initiation during aging (Figure 1).

Age-associated cryptic transcription increases transcript diversity during mammalian aging

Cryptic transcription is a phenomenon that has mostly been studied in budding yeast, in which the simple gene structure makes identifying such transcripts relatively easy [46]. Cryptic transcription has long been known to be deleterious in this organism; it increases with age and severely limits its lifespan [84]. Until recently, the impact of cryptic transcription on mammalian aging has not been studied. However, several recent papers have identified elevated levels of cryptic transcription in a variety of tissues during aging and senescence in humans and mice. These include skin, liver, and brain; senescent fibroblasts; and several types of adult stem cells, including mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), neural stem cells (NSCs) [9, 10].

The genes subject to increased cryptic transcription during aging and senescence in human MSCs, fibroblasts and the mouse liver have a limited overlap with differentially expressed genes in these tissues. Likewise, there is tissue specificity of age-associated cryptic transcription, as when comparing genes that specifically generate cryptic transcripts in the aged/senescent state in mouse liver and human fibroblasts, Sen et al. found that while there is a significant overlap in orthologous genes, the majority of genes are unique to each tissue [10]. Finally, cryptic transcription occurs in genes relevant to tissue function and, particularly, aging. In MSCs, genes with elevated cryptic transcription are involved in telomere maintenance, translation, and protein folding [9], while in both senescent fibroblasts and aged mouse liver, genes with elevated cryptic

transcription are broadly related to signal transduction and ECM, which are known to be disrupted during aging [1].

Perturbations that increase cryptic transcription mimic aging phenotypes

Several studies implicate increased cryptic transcription in driving aging phenotypes (Figure 2). Conditional knock out of *Setd2* in hematopoietic stem cells causes a myeloid bias during differentiation [11], similar to what is seen during aging [85]. Likewise, loss of *Setd2* from bone marrow stem cells biases them towards adipogenic differentiation [12], while in MSCs, *SETD2* knockdown reduces proliferation, both of which occur during aging [86]. Additionally, oocyte-specific knockout of *Setd2* compromises oocyte quality and causes sterility [13]; reduced oocyte quality causes the age-associated fertility decline in females [87]. Reduced DNMT3B function also mimics several aspects of aging. In HSCs, double knock out of *Dnmt3a* and *Dnmt3b* promotes self-renewal vs. differentiation [88, 89], which phenocopies the expansion of the progenitor pool in aged mice [86]. Specific knockdown of *Dnmt3b* in the hippocampus of adult mice impairs recognition memory [14], which is also impaired during aging [90]. Together, these studies suggest that elevated cryptic transcription may contribute to numerous aging phenotypes.

Concluding remarks

Altered splicing patterns and elevated cryptic transcription both increase the structural diversity of transcripts in a variety of tissues during aging, though relatively few studies have examined the impact of transcripts variants on the aging process. As splicing dysregulation and cryptic transcription largely affect genes that are not differentially expressed with age [9, 15–18, 23, 24, 60, 69, 71, 74, 75], the effects of this increased transcript diversity are not considered in most aging studies, although it is essential to understand how these novel transcripts affect the aging process. As discussed above, proteins generated from structurally novel transcripts may have altered functions, and thus, are likely to have a negative effect the processes in which they are involved. As these include aging and tissue function-related processes, cryptic and differentially spliced transcripts likely have a negative effect on both tissue function and longevity.

Recent work has shown that altered splicing and cryptic transcription are pervasive during aging and occur in genes that are relevant to tissue function or involved in processes that are disrupted during this process, consistent with early reports from leukocytes and the brain [9, 10, 15, 17, 18, 23, 24, 60, 70, 71, 75]. Interestingly, in related MSCs and BMSCs, there is a divergence of genes subject to increased alternative splicing and those with elevated cryptic transcription during aging. While alternatively spliced transcripts in BMSCs appear to affect the differentiation potential of these cells, cryptic transcripts in MSCs arise from genes involved more generally in aging [9, 25]. This may point to a divergence in the mechanisms by which cryptic transcription and alternative splicing regulate aging. Critically, perturbations that dysregulate splicing and increase cryptic transcription phenocopy occur in certain aspects of aging in many tissues [11–14, 24, 25, 34, 35, 37, 88, 89], suggesting that these novel transcripts directly impact aging.

Detecting altered splicing and cryptic transcripts is considerably more technically difficult than assessing overall gene expression. The technologies developed to globally assess the transcriptome, microarrays and next generation sequencing, have a limited ability to robustly detect qualitative, as opposed to quantitative, changes to transcripts. The fact that cryptic and mis-spliced transcripts are relatively low abundance in the pool of poly-adenylated RNA [9, 21, 44] further complicates their experimental detection. Thus, relatively little is known about how splicing changes and cryptic transcription contribute to the aging process. The recent proliferation of long read sequencing, particularly Nanopore sequencing, shows promise in overcoming these limitations. Indeed, long read sequencing shows considerable improvement over Illumina sequencing in quantification of transcript spliceoforms [91]. As this technology is applied to the question of aging, we anticipate that the increased transcript diversity in aged tissues will be more broadly appreciated and that future work will uncover how novel transcripts drive organismal aging.

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