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Opinion

The role of alternative splicing in adaptation and evolution

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Regulation of gene expression plays a central role in adaptive divergence and evolution. Although the role of gene regulation in microevolutionary processes is gaining wide acceptance, most studies have only investigated the evolution of transcript levels, ignoring the potentially significant role of transcript structures. We argue that variation in alternative splicing plays an important and widely unexplored role in adaptation (e.g., by increasing transcriptome and/or proteome diversity, or buffering potentially deleterious genetic variation). New studies increasingly highlight the potential for independent evolution in alternative splicing and transcript level, providing alternative paths for selection to act upon. We propose that alternative splicing and transcript levels can provide contrasting, nonredundant mechanisms of equal importance for adaptive diversification of gene function and regulation.

Highlights

Transcriptional and post-transcriptional regulation of gene expression plays an integral role in development, physiology, and adaptation.

Recent studies indicate that variation in transcription and alternative splicing have distinct genetic bases, allowing transcript levels and alternative splicing to evolve independently.

Rapid technological development has opened (nearly) all organisms to the investigation of the role of alternative splicing in adaptation.

Alternative splicing potentially provides a mechanism for reducing constraints on highly pleiotropic genes.

Transcript levels and transcript splicing may play complementary or contrasting roles in adaptive evolution.

Going beyond gene transcription

Understanding the molecular mechanisms that link genetic and environmental variation to phenotypic variation is a key aspect of evolutionary and ecological research. To date, transcriptomic studies have mostly focused on transcript level changes ('gene expression') to understand the mechanisms underlying developmental processes, phenotypic plasticity, and adaptation [1–3]. Yet, the regulation of genes goes far beyond their transcript levels. Despite our growing knowledge on the mechanisms and functions of **post-transcriptional gene regulation** (see Glossary) [4,5], little is known about the evolutionary dynamics and functional role of these mechanisms over microevolutionary timescales and in varying ecological contexts. This opens up an important question: have we been mostly ignoring an important puzzle piece in our understanding of how phenotypic diversity evolves and how species adapt to variable environments?

Alternative splicing is one of the multiple essential post-transcriptional mechanisms and enables the generation of structurally variable transcripts from a single gene [i.e., **isoforms**; e.g., through the inclusion or skipping of exons (e.g., **cassette exons**) or the retention of introns] (Figure 1 and Box 1) [6–12]. While it is still debated if most splicing events are just noise and nonfunctional [13], alternative splicing can increase transcriptome and proteome diversity and regulate transcript levels following transcription (Figure 1) [4,5,7,14,15]. It has been shown that alternative splicing is central to many organismal aspects, such as (i) development and physiology [9]; (ii) establishment of tissue identity [4,16]; (iii) that its disruption often leads to disease [5,17–20]; and (iv) most importantly for the eco-evolutionary research, that it can rapidly evolve, overcome functional constraints, and respond to environmental differences [10,21–25].

The role of alternative splicing in development, ecology, evolution, and plasticity

Since its discovery four decades ago, alternative splicing went from being seen as a curious phenomenon to a fundamental regulatory mechanism in eukaryotes [4,5,16,26]. Developmental genes are enriched for alternative splicing events (reviewed in [26]), and developmentally dynamic

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alternative splicing events can be conserved across species [16,22]. Yet, comparative studies across deeply divergent species have also suggested that alternative splicing evolves more quickly, is more species-specific than transcript level changes, and increases in complexity with organismal complexity [22,27–29]. This led to the idea that alternative splicing plays a key role in the evolution of phenotypic differences between species.

In contrast to deep evolutionary timescales, the role of alternative splicing in microevolutionary processes is only beginning to be understood. Analyses of alternative splicing on microevolutionary timescales paint a variable picture in which alternative splicing is not consistently more divergent than transcript levels [21,30,31]. For example, the magnitude of changes in alternative splicing is similar to the magnitude of changes in transcript levels in the postglacial divergences of the salmonid fish Arctic charr (*Salvelinus alpinus*) [21] and cichlid fish adaptive radiations [30]. However, because each gene can produce multiple isoforms [30], isoform diversity can potentially increase more rapidly than divergence in transcript levels. The potential for the very rapid evolution of isoform diversity (e.g., during domestication [8]) supports a role in adaptive diversification. Yet, comparative studies of alternative splicing and transcript levels over varying evolutionary timescales are scarce, leaving the relative paces of splicing and transcription level evolution largely unanswered.

In support of an adaptive role for splicing divergence, alternative splicing has been linked to adaptive divergence and sometimes discrete phenotypes in several species (Table 1) [32–35]. For example, the use of an alternative **5′ splice site** of the gene *MSX2A* in freshwater three-spined stickleback (*Gasterosteus aculeatus*) results in expression of a truncated loss-of-function transcript, leading to shorter dorsal spines [32]. The differential inclusion of noncoding exons that increase protein translation in the *Agouti* gene has been associated with lighter fur coloration in deer mice (*Peromyscus maniculatus*) inhabiting sandy environments [33]. Expression from alternative transcription start sites of the *vgll3* gene associates with male puberty timing in Atlantic salmon (*Salmo salar*), possibly through, for example, mRNA regulatory sites in the 5′ untranslated region (UTR) or by changing the N-terminal protein sequence [34]. Exon skipping in the per2 gene associates with increased body fat accumulation in cave ecotypes of the Mexican tetra *Astyanax mexicanus*; alternative splicing excludes a pparγ binding domain from the per2 protein, thus removing normal par2/per2 repression of the adiposity-controlling regulatory factor pparγ and increasing the expression of pparγ target genes [35].

Changes in isoform diversity through alternative splicing have also been shown to play key roles in phenotypic plasticity. For example, isoform expression of the *tim* gene in *Drosophila melanogaster* switches according to the ambient temperature leading to temperature-dependent *tim* function [23,36]. Cold stress induces the alternative splicing of hundreds of genes in skeletal muscle of fishes, including tens of genes showing parallel isoform expression plasticity across deeply divergent species [Atlantic killifish (*Fundulus heteroclitus)*, three-spined stickleback, and zebrafish (*Danio rerio*)], suggesting these are conserved plastic responses to cold acclimatization [37]. Alternative splicing has also been identified as an important mechanism of plastic change in response to biotic factors [10,24]. The relative contribution of changes in transcript splicing compared with transcript level for stress responses seems to differ across taxa (e.g., with alternative splicing potentially playing a stronger role in the environmental stress response of plants compared with animals [10]). However, more widespread comparative studies are needed to confirm this.

Potential for independent evolution of splicing and transcript level

The adoption of large-scale splicing-aware analyses has revealed that across development, different gene sets show changes in either transcript splicing or transcript levels, but rarely both [9]. Of all alternatively spliced genes during mammalian organ development, an average of

Glossary

Alternative splicing: a mechanism by which structurally variable transcripts are generated from a single gene through inclusion/exclusion of exons or introns, or changes in exon length.
Capacitor: a molecule that acts as a mechanism that can buffer (conceal) the effects of genetic variation on a phenotype. When disturbed by, for example, mutation, the molecule releases genetic variation from buffering that leads to the appearance of phenotypic variation. Heat shock protein 90 (Hsp90) is a well-known example of a potential capacitor.
Cassette exon: an exon in between two other exons that can either be included or excluded from a mature mRNA to form two distinct isoforms.
***Cis*-regulation:** gene regulatory mechanisms that co-segregate with the gene they influence; often described as situated on the same chromosome and closely linked such as enhancer and promoter elements. Convey allele-specific effects on gene expression.
Complex traits: complex traits are encoded by a large number of loci so that their precise genetic architecture is often unknown (e.g., human height).
Evolutionary capacitance: a framework whereby genetic variation can have either observable or concealed effects on the phenotype. In the concealed state, it allows for genetic variation to accumulate without being exposed to natural selection.
Expression quantitative trait loci (eQTLs)/splicing quantitative trait loci (sQTL): eQTL and sQTL describe the statistical association between genetic variation and quantitative variation in the expression or splicing of a gene, respectively. eQTL and sQTL can be mapped to the genome and can be due to *cis*- or *trans*-regulatory mechanisms.
Genetic architecture: a concept that describes the number of loci involved in encoding variation in a trait, the magnitude of their effects on the trait, their frequencies in a population, and their mutual relationships with each other and the environment.
Isoform: a distinct structurally variable transcript produced by a single gene through alternative splicing.
Nonsense-mediated decay (NMD): a highly regulated mechanism that degrades defective pre-mRNA

~10% changed in transcription level as well [16]. Such independence of alternative splicing and transcript levels indicates that these two processes are controlled (at least partially) by distinct mechanisms. Splicing and transcription, however, occur simultaneously within the cell nucleus (co-transcriptional splicing), and splicing is dependent on mRNA transcription for efficiency (reviewed by [5]). Due to their interconnectedness, the extent to which splicing and transcript levels can evolve independently has been unclear.

Several recent studies suggest functionally nonredundant roles of splicing and transcript levels in phenotypic diversification. Alternatively spliced genes often do not differ in transcript levels and influence different biological processes, such as in postnatal skeletal muscle development of mouse (*Mus musculus*) [38], in the parallel ecological divergence of Arctic charr [21], between plastic pea aphid (*Acyrthosiphon pisum*) morphs [24], and in response to heat stress in *D. melanogaster* [36], or between sexes in three bird species (mallard duck *Anas platyrhynchos*, turkey *Meleagris gallopavo*, and helmeted guineafowl *Numida meleagris*) [25]. Both mechanisms contribute independently to **complex trait** associations in humans [17,39]. However, variation to this tendency exists, and the two mechanisms were observed acting together in association with jaw morphology between cichlid fish species [30] or in plastic changes in response to cold acclimation in fishes (Atlantic killifish, three-spined stickleback, and zebrafish) [37]. We suggest that the independent evolution of alternative splicing and differential gene expression, affecting different gene sets with nonredundant functions (contrasting scenario in Figure 2A), is a common and potentially important phenomenon in plasticity and over microevolutionary timescales. However, further intra- and interspecific comparisons are needed to test under which circumstances splicing and transcript levels coevolve to complement each other (complementary scenario in Figure 2A), or if this pattern is highly idiosyncratic and largely dependent on, for example, the species, phenotype, **genetic architecture**, and/or evolutionary history.

Genetic basis of variation in splicing and transcript level

Genetic mapping of splicing and transcript level variation in humans has shown that loci underlying variation in splicing [called **splicing quantitative trait loci (sQTL)**] are largely distinct from those influencing transcript level variation [**expression (e)QTLs**] [39]. Splicing variation in humans largely maps to ***cis*-regulatory** variation (Figure 2B) and is constrained to coding sequences [39], especially to post-transcriptionally spliced introns [40]. A similar tendency is seen in *Arabidopsis thaliana* [41,42]. While it has been observed that *cis*-regulatory divergence plays an important role in adaptive divergence of transcript levels [43–46], it remains unknown to which extend *cis*-regulatory evolution affects alternative splicing patterns on microevolutionary timescales.

Splicing is also influenced by ***trans*-regulatory** variation in **RNA-binding proteins (RBPs)** and **splicing factors** that influence the splicing machinery [47] (Figure 2B), which has been shown to be predominant for splicing evolution in sunflower domestication compared with *cis*-regulatory divergence [8]. Such *trans*-effects have the potential to influence splicing in a large set of genes through gene regulatory networks (e.g., [48,49]). For transcript levels, *trans*-effects can be due to almost any gene expressed in a cell [50,51], which has been suggested to translate into a polygenic architecture of traits influenced by transcript level variation [52]. Whether splicing shows a similarly complex *trans*-architecture, and how this might influence trait evolution mediated by splicing, remains largely unknown.

Splicing variation can also be due to epigenetic mechanisms, enabling rapid responses to environmental change and potential routes for the decoupling of splicing and transcript level evolution. For example, (i) **nucleosome** positioning demarcates exons and is independent from mRNA levels [53]; (ii) post-translational modifications of histones interact with RNA-binding factors

molecules (e.g., pre-mRNA containing premature stop codons).
Nucleosome: a basic unit of eukaryotic chromatin that represents DNA that is wrapped around one core histone complex.
Pleiotropy: a concept that describes the effects of variation in a single locus (e.g., gene) on multiple distinct traits; non-pleiotropic variation influences a single trait, pleiotropic variation influences multiple traits.
Post-transcriptional gene regulation: an overarching term to describe mechanisms that regulate transcript levels and transcript structure following the transcription of a gene (e.g., through alternative splicing, miRNAs, RNA editing, and RNA modifications).
RNA-binding protein (RBP): RBPs form ribonucleoprotein complexes with single-stranded or double-stranded RNA to regulate post-transcriptional processes such as splicing, RNA localization, and stability.
5′ splice site: sequence at the 5′ end of an intron that is recognized by the splicing machinery. Also referred to as splice donor.
Splicing factor: RBPs that are directly involved in regulating or mediating the splicing of introns from pre-mRNA.
***Trans*-regulation:** gene regulatory mechanisms that are unlinked to the gene they influence; often described as diffusible molecules such as transcription factors. Convey non-allele-specific effects on gene expression.

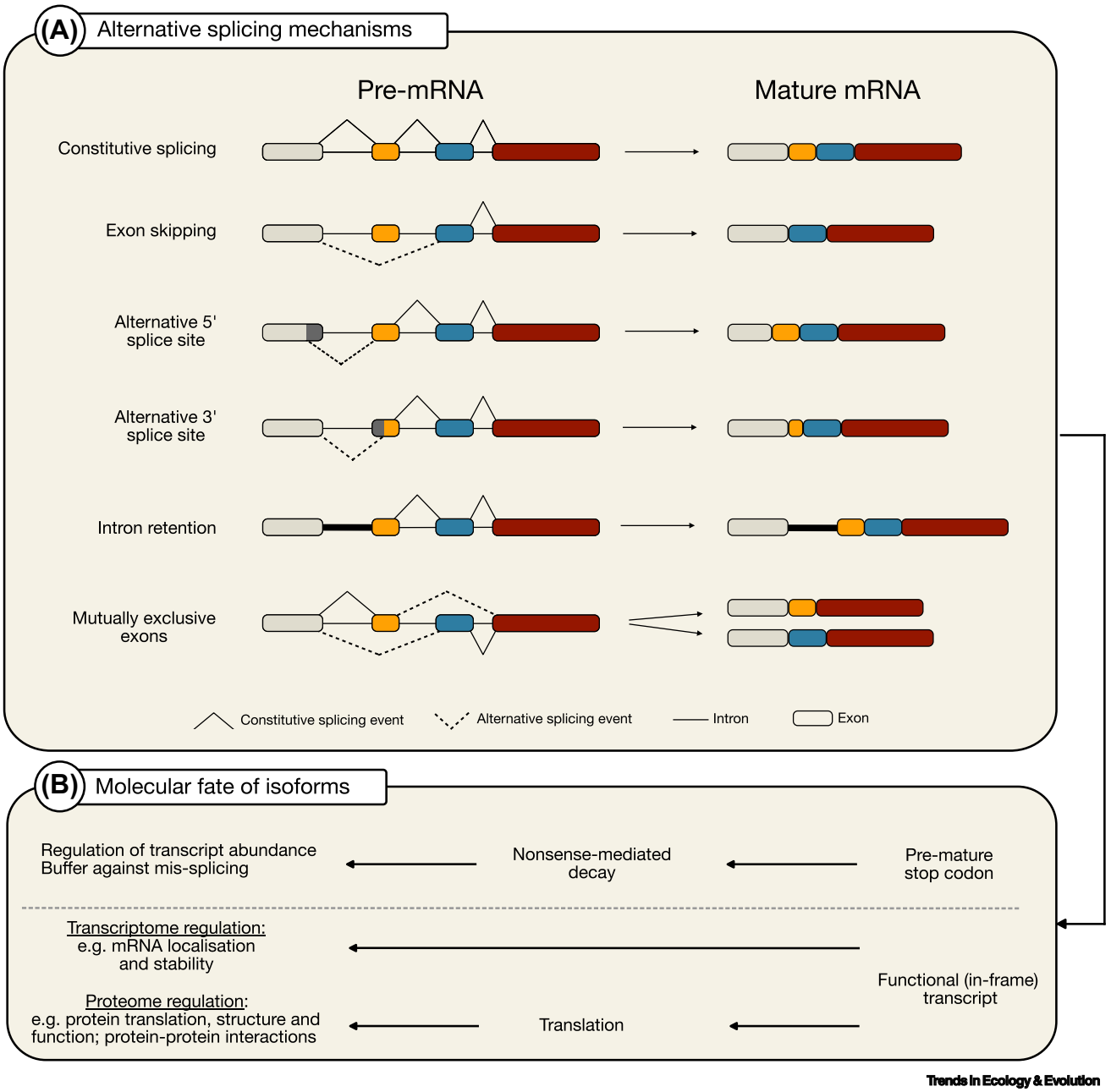


Figure 1. Alternative splicing mechanisms and the molecular fates of isoforms. (A) Alternative splicing can be achieved through multiple distinct mechanisms that include exon skipping, alternative 3′ and 5′ splice sites, intron retention, or a combination thereof. (B) Alternatively spliced transcripts can have two general molecular fates; transcripts containing premature stop codons are degraded through nonsense-mediated decay; in-frame alternative splicing alters the function of the transcripts at the post-transcriptional (mRNA localization and stability) and/or post-translational (protein sequence and modifications) level.

[54]; and (iii) RNA methylation modulates accessibility of splicing factors to splice sites (e.g., [5,55]). Furthermore, DNA methylation can alter alternative splicing patterns [5] and has been linked to splicing differences between honey bee (*Apis mellifera*) castes [56]. Altogether, a more precise determination of the genetic and epigenetic architectures of splicing and transcript level variation is required to fully understand the interdependence in the evolution of these traits.

Box 1. Alternative mechanisms of isoform formation and their role in adaptation

Alternative splicing is not the sole mechanism creating variation in transcript structures, rather, the majority of human isoform diversity between tissues involves alternative start and termination sites [73]. Alternative polyadenylation can lead to transcripts with different 3′ untranslated regions (UTRs). Over half of human genes show alternative polyadenylation (reviewed in [74]), and the mechanism can mediate association with complex traits [75]. Alternative polyadenylation from intronic adenylation sites creates truncated proteins that have been associated with the development of cancer [76], and the diversification of DNA- and RNA-binding proteins and protein–protein interactions [77].

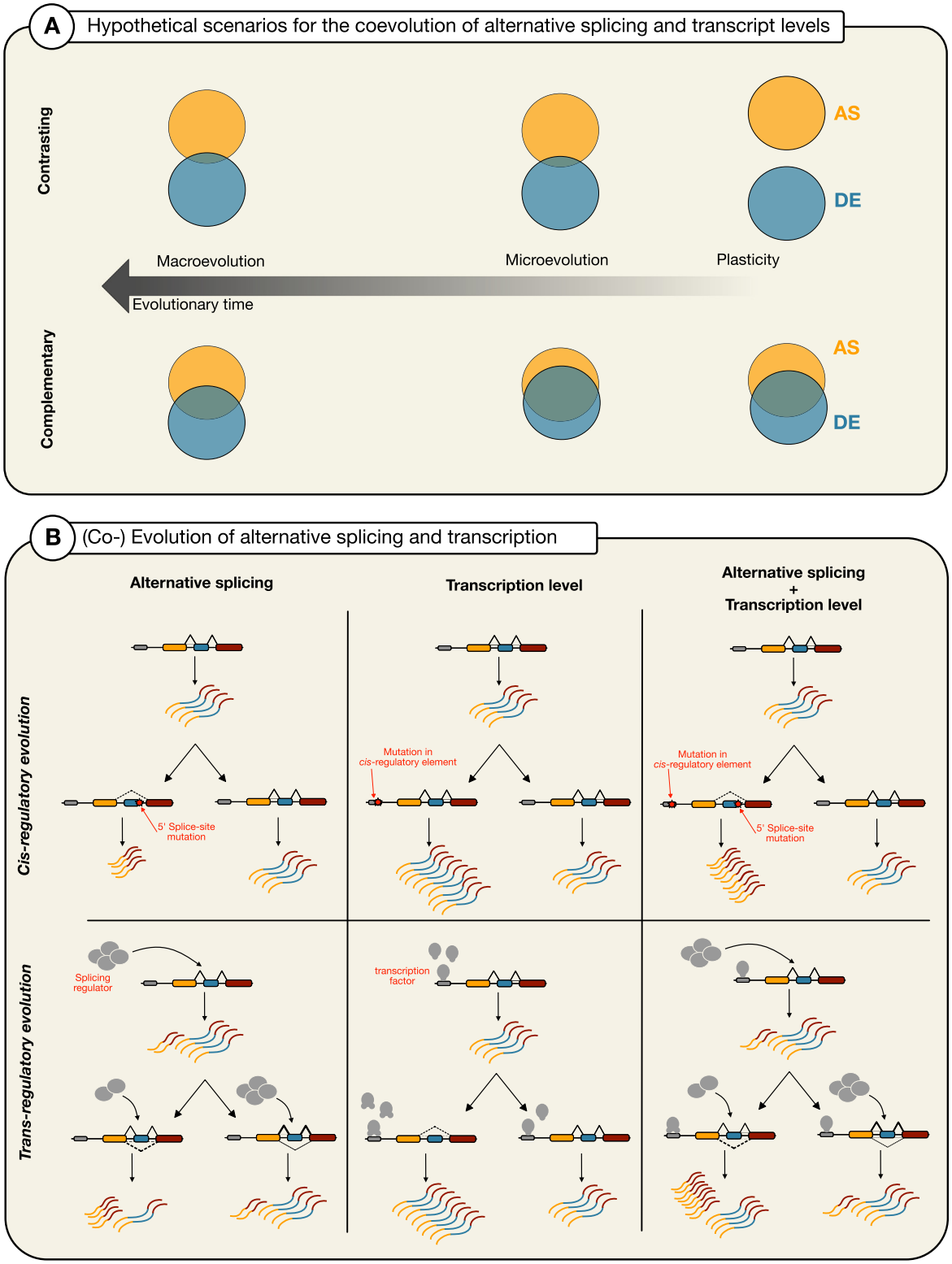
Alternative transcription start sites can create alternative 5′ UTRs and/or alter the coding sequence by inclusion or exclusion of first exons. As a general rule, variation in 3′ and 5′ UTRs is created on the transcriptional level, but their effects are mainly post-transcriptional. This is the case of the *Agouti* gene in deer mouse, for example, where a noncoding exon transcribed from an alternative transcription start site increases protein translation efficiency, leading to lighter fur coloration in sandy environments [33]. Both 3′ and 5′ UTRs are also dense in regulatory sites for RNA-binding proteins and regulatory RNAs, and thus influence, for example, mRNA stability and translation. Alternative polyadenylation and transcription start site usage can also create alternative polypeptide sequences with different characteristics as seen in the examples described in the main text.

The adaptive potential of variation in splicing and transcript level is further influenced by their **pleiotropy**. On the one hand, alternative splicing can be a mechanism of increasing transcriptional diversity in genes that have pleiotropic effects, such as developmental or sexually selected genes. For example, splicing might offer a resolution of sexual conflict through the generation of sex-specific isoforms in pleiotropically constraint genes [25]. On the other hand, genetic differences in the expression of alternative isoforms are relatively stable across tissues [39] (i.e., having strong pleiotropic effects, compared with variation in transcript level). It remains

Table 1. A nonexhaustive list of cases in different species where alternative splicing has been associated with phenotypic plasticity or adaptive differences

Species	Focus[a]	Splicing type[a,b]	Adaptation	Tissue	Refs
Arctic charr (*Salvelinus alpinus*)	GW	Various (NS)	Benthic–limnetic divergence	Skeletal muscle	[21]
Atlantic salmon (*Salmo salar*)	*vgll3*	Alternative 5′ UTR/exon	Age at maturity	Testes	[34]
Cichlids (*Cichlidae*)	GW	Various (NS)	Trophic divergence	Pharyngeal jaw	[30]
Killifish (*Fundulus heteroclitus*)	GW	Various (NS)	Cold acclimation	Skeletal muscle	[37]
Mexican tetra (*Astyanax mexicanus*)	*per2*	ES	Surface-cave adaptation	Liver	[35]
Stickleback (*Gasterosteus aculeatus*)	*MSX2A*	A5′SS	Dorsal spine length	Multiple tissues	[32]
Stickleback (*G. aculeatus*)	GW	Various (NS)	Cold acclimation	Skeletal muscle	[37]
Zebrafish (*Danio rerio*)	GW	Various (NS)	Cold acclimation	Skeletal muscle	[37]
Deer mouse (*Peromyscus maniculatus*)	*Agouti*	Alternative 5′ UTR/exon	Coloration	Dorsal and ventral skin tissue	[33]
Duck (*Anas platyrhynchos*)	GW	Mostly ES/MXE	Sex differences	Spleen, gonads	[25]
Helmeted guineafowl (*Numida meleagris*)	GW	Mostly ES/MXE	Sex differences	Spleen, gonads	[25]
Turkey (*Meleagris gallopavo*)	GW	Mostly ES/MXE	Sex differences	Spleen, gonads	[25]
Drosophila melanogaster	GW	Mostly ES	Temperature response	Whole body	[36]
Human lice (*Pediculus humanus*)	GW	Various (mainly ES)	Head versus body lice	Whole bodies (pooled)	[70]
Pea aphids (*Acyrthosiphon pisum*)	GW	Various (Mainly ES and MXE)	Wing polyphenism; reproduction	Whole body	[24]
Arabidopsis thaliana	GW	Mostly *cis*-regulatory	Association with environment	Leaves	[42]
A. thaliana	*P5CS1*	ES	Proline accumulation (drought adaptation)	Seedlings	[71]
Sunflowers	GW	Mostly IR	Domestication	Above-ground tissue	[8,72]

[a]Abbreviations: A5′SS, alternative 5′ splice site; GW, genome-wide; ES, exon skipping; IR, intron retention; MXE, mutually exclusive exons; Various (NS), splice type not specified.
[b]See Figure 1 in the main text for types of alternative splicing.



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unclear how the pleiotropy of alternatively spliced gene functions and splicing variation impacts evolutionary trajectories.

The fate and functional roles of alternative isoforms

A major challenge remains in understanding how the functions of different isoforms and polypeptide variants differ. Efforts have been made to predict isoform functions [57], but the task will remain challenging, particularly in non-model species for which isoform function cannot be easily tested. Protein-coding isoforms might have functional roles as distinct from each other as independent genes [15,26]. Documented adaptive mechanisms through alternative splicing include loss-of-function [32], increased translation [33], and changes in protein interactions [35]. Recent work has shown that polypeptide variants are expressed in proportion to their mRNA isoforms [7,14]. Similarly, mRNA level variation translates into protein level variation, albeit as a complex function involving post-transcriptional buffering [17,58–61]. Regulatory variation in splicing and transcript level therefore potentiates not only qualitative changes in the proteome (such as, e.g., different enzymatic or regulatory functions, protein localization, or protein–protein interactions), but also evolutionary fine-tuning of isoform levels.

An important characteristic of alternative splicing is that most genes express a single dominant isoform and multiple alternative isoforms at much lower levels. This has been interpreted as evidence for nonfunctionality of isoforms (e.g., [13]). However, we should not categorically exclude lowly expressed isoforms as having feeble evolutionary significance. For example, while most genes show consistent patterns of splicing between *Drosophila* genotypes in benign environments, environmental stress greatly exacerbates splicing differences, thus revealing hidden variation in splicing for selection to act upon [23,36]. Hence, to determine the functionality of isoforms, screening isoform diversity across environments might provide additional evidence. Furthermore, alternative isoforms that are lowly expressed are predicted to evolve in a nearly neutral fashion, free from negative selection on the major isoform function, thus allowing for exploring low-fitness areas of the genotype–phenotype map that can eventually become adaptive [62]. This resembles protein function diversification following gene duplication [63], whereby redundancy in function allows for accumulation of nearly neutral variation that potentiates further functional novelty [64,65]. Functional diversification of isoforms through such nearly neutral processes tends to act on microevolutionary timescales [62].

All eukaryotes seem to have a common mechanism that protects organisms from the deleterious effects of erroneous splicing, **nonsense-mediated decay (NMD)** (reviewed in [66,67]) (Figure 1). It has been proposed that NMD can act as a buffer allowing cells to safely experiment on alternative splicing, which could ultimately lead to the emergence of new functional proteins [5]. We bring forth the idea that this is akin to the theory of **evolutionary capacitance**, whereby genetic variation can accumulate if its potentially harmful effects on the phenotype are buffered by a molecular mechanism. There are multiple ways transcripts can evade NMD which varies between cells, individuals, and environments [68], providing potential routes for buffered variation in splicing. We hypothesize that NMD could serve as a **capacitor** that enables the hidden

Figure 2. Mechanisms of expression evolution through alternative splicing (AS) and transcript levels (DE). (A) Two hypothetical scenarios for the contrasting or complementary coevolution of AS and DE at different stages of adaptation and/or evolution. For example, AS and DE can potentially influence different gene sets leading to two general hypotheses on how gene regulation coevolves over microevolutionary timescales: splicing and DE can influence distinct gene sets so that their evolution shows contrasting patterns, or shared gene sets, whereby they would play complementary roles in adaptation. Which of these two scenarios predominate is an outstanding question. The extent of overlap between gene sets can differ between adaptive scenarios (plastic response versus evolutionary change) and along evolutionary timescales (macro- versus microevolution). Note that these are not all of the possible scenarios. Circles denote hypothetical gene sets. (B) The ways in which AS and DE evolve can involve both *cis*- and *trans*-acting mechanisms. If splicing and DE play complementary roles in adaptive processes, the mechanisms are predicted to co-evolve to optimize the abundances of specific transcript isoforms. Note that, for simplicity, not all combinations of *cis/trans*/splicing/expression are drawn.

accumulation of genetic variation in alternative splicing and potentiates future adaptation. This process is distinct from the near neutrality of lowly expressed isoforms discussed previously, as NMD potentiates the accumulation of hidden splicing variation that may be highly expressed or otherwise have stronger deleterious effects. Therefore, selection on isoform expression level is not necessary to gain adaptive benefit. Much work is needed to evaluate this hypothesis and to identify the individual NMD factors that could function as capacitors.

Concluding remarks

The focus of most eco-evolutionary transcriptomic studies still lies on transcript levels; we argue that alternative splicing plays an important but widely ignored role in adaptation and phenotypic change. We highlight that isoform ratios and transcript levels have the potential to vary independently from one another through their distinct (epi)genetic basis. The evolutionary implications for the decoupling of splicing from transcription regulation are simple: evolution can act on transcript structures and transcript levels separately, thus harnessing independent mechanisms for evolutionary change and modulating different biological processes through distinct mechanisms.

We further argue for the need for a framework to test the selective function of alternative splicing in a similar way to transcript level. Possible approaches to this end could lever on parallel evolution [21,43] or comparative analyses in taxa where isoform usage can be analyzed on both short and long timescales. The coevolution of these processes can possibly be approached through studying regulatory networks as a compound phenotype of total and isoform expression [69], rather than distinct networks. However, a lack of studies that assess the evolution of alternative splicing and transcript levels across evolutionary timescales and on the same datasets limits our understanding of the coevolution of splicing and transcription in eco-evolutionary contexts (see Outstanding questions). Overall, we suggest that transcriptomic studies should more regularly incorporate analyses of alternative splicing, ideally using long-read sequencing and/or specific analytical approaches (Box 2), to gain a more complete understanding of the mechanisms and genes involved in phenotypic evolution and environmental response.

Box 2. Analytical and technological approaches to study alternative splicing in non-model systems

Investigating alternative splicing is not a trivial undertaking as it is challenging to accurately reconstruct isoforms from short-read sequencing data [78]. However, many different methods for identifying alternative splicing events from short-read sequencing data have been developed. In general, these methods can be divided into isoform-based approaches, which rely on the reconstruction and quantification of isoforms, and count-based methods, which quantify differences in read counts between the studied units (e.g., exons or junctions) and are more powerful and accurate than the former [79]. Count-based methods can further be divided into exon-based methods, which quantify the differential expression of individual exons between groups, or events-based methods, which quantify distinct splicing events (e.g., intron retention or exon skipping [79,80]). Compared with exon-based methods, events-based methods have the advantage that they can provide information on the type of splicing event [79].

Recent development of long-read cDNA and RNA sequencing has the potential to revolutionize the study of alternative splicing, as full transcripts/isoforms can be completely sequenced, removing the need for complicated isoform reconstruction (e.g., [78,81–83]). Similar to short-read sequencing data, multiple different approaches for studying alternative splicing using long-read data have been developed over the last few years (e.g., [78,81–84]). While long-read sequencing has more power and a higher accuracy, it remains more expensive and therefore out of reach for many research projects. However, as mentioned previously, short-read RNA-seq data can still be effectively used to investigate alternative splicing in a wide range of species (e.g., [16,21,24,30]) and provide a good first step to understand its role in ecology and evolution.

Similar to transcript level, advanced technological approaches also enable the investigation of alternative splicing on the single-cell level (e.g., [85,86]), particularly using long-read single-cell sequencing, but this is more challenging and inaccurate than expression level analyses due to high rates of read dropouts and potential mistakes in isoform quantification [87]. However, understanding the changes in alternative splicing on the single-cell level will provide a deeper understanding of how splicing changes on the tissue level are related to, for example, changes in tissue composition rather than alternative splicing in individual cells, particularly in very heterogeneous tissues [16].

Outstanding questions

Is alternative splicing evolving faster than transcript levels, and how does this differ across evolutionary timescales, adaptive scenarios, and taxa?

What is the role of coevolution of transcript splicing and level in adaptive evolution? Does adaptation lead to the optimization of the two mechanisms with respect to one another?

Through which mechanisms is alternative splicing predominantly contributing to adaptive phenotypic differences: proteome diversification, post-transcriptional regulation of transcript level, or some other?

What is the evolutionary potential in alternative splicing, what are the mechanisms that potentiate the evolution of alternative transcripts (e.g., *cis*- versus *trans*-regulation and NMD), and how important are these mechanisms in evolution?

Does alternative splicing play a role in resolving evolutionary constraints in pleiotropic genes? How does pleiotropy in splicing variation impact the adaptive role of alternative splicing?

Can NMD mediate evolutionary capacitance? If so, which parts of the NMD pathway function as potential capacitors?

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Author contributions

A.J. initially conceived this opinion piece, and J-P.V. and A.J. contributed equally to the ideas presented and writing of the manuscript.

Declaration of interests

No interests are declared.

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