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Review Article

Thermoregulated transcriptomics: the molecular basis and biological significance of temperature-dependent alternative splicing

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Temperature-dependent alternative splicing (AS) is a crucial mechanism for organisms to adapt to varying environmental temperatures. In mammals, even slight fluctuations in body temperature are sufficient to drive significant AS changes in a concerted manner. This dynamic regulation allows organisms to finely tune gene expression and protein isoform diversity in response to temperature cues, ensuring proper cellular function and physiological adaptation. Understanding the molecular mechanisms underlying temperature-dependent AS thus provides valuable insights into the intricate interplay between environmental stimuli and gene expression regulation. In this review, we provide an overview of recent advances in understanding temperature-regulated AS across various biological processes and systems. We will discuss the machinery sensing and translating temperature cues into changed AS patterns, the adaptation of the splicing regulatory machinery to extreme temperatures, the role of temperature-dependent AS in shaping the transcriptome, functional implications and the development of potential therapeutics targeting temperature-sensitive AS pathways.

The molecular basis of temperature-dependent alternative splicing

While mammalian body temperature is often perceived as constant, it is in fact subject to a myriad of influences. From circadian rhythms to physical activity, hormonal cycles and disease states, the body's temperature regulation is a finely tuned process that responds dynamically to internal and external stimuli. At the cellular level, temperature is perceived by several mechanisms, including temperature-sensitive transmembrane receptors or the expression of heat-shock proteins. These temperature-sensing mechanisms are essential for cellular function and survival at potentially noxious temperatures, but often require rather extreme temperatures to become activated (heat shock pathway) or are restricted to certain cell types such as primary sensory neurons (TRP channels). In contrast, temperature-sensitive alternative splicing (AS) serves as a temperature responsive process that is universally found across all tested mammalian cell types and responds ultrasensitive to changes in temperature [1]. Indeed, splicing changes can respond in a thermometer-like fashion to subtle changes in the physiological temperature (see an example in Figure 1A). This behavior implies the presence of a sensitive molecular thermosensor. Biochemical characterizations revealed that the activity of recombinant and pure CDC-like kinases (CLKs), a class of protein kinases that phosphorylate SR splicing factors, is highly responsive to physiological temperature changes, with higher activity in cold (Figure 1B, left). This sensitivity is conferred by the kinase activation segment, which adopts an active conformation in cold but displays higher flexibility and adapts an inactive conformation in response to increasing temperature [2]. The interplay of temperature-insensitive SR protein kinases (SRPKs) and temperature-sensitive CLKs sets the phosphorylation state of SR proteins [3], a group of RNA-binding proteins (RBPs) characterized by stretches of serine arginine (SR) repeats [4]. Importantly, the

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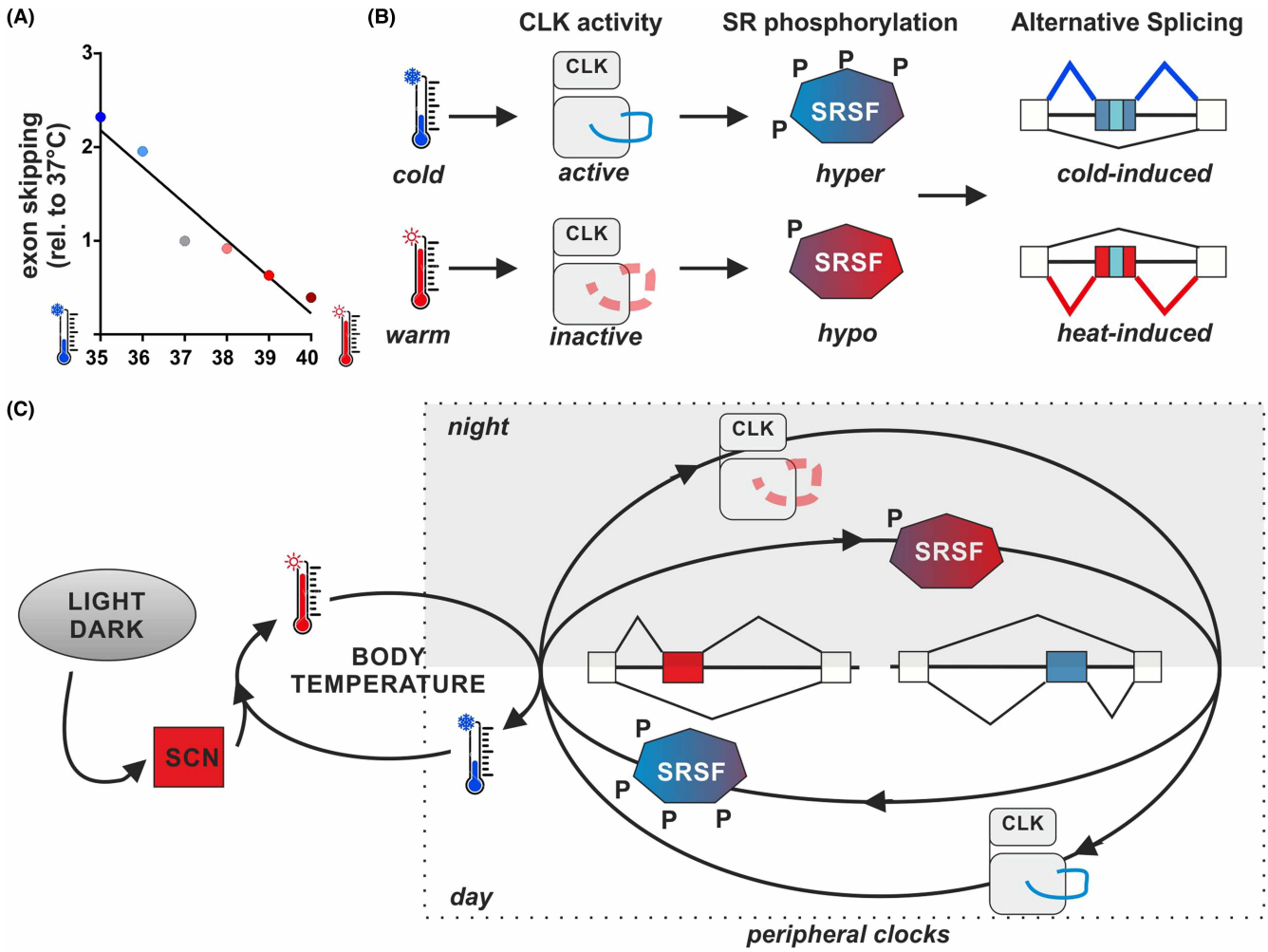

Figure 1. CDC2-like kinases (CLKs) regulate temperature-dependent alternative splicing.

(**A**) Ultrasensitive response of alternative splicing to temperature changes. Alternative splicing changes linearly and in a thermometer-like fashion in response to subtle shifts in physiological temperature. Mean exon skipping levels are shown normalized to 37°C for exclusion of U2AF26 exon 6 and 7. Line depicts linear regression fit. Data are derived from [1]. (**B**) Mechanism of temperature-controlled exon inclusion. Alternative splicing is influenced by temperature through the activity of CLKs and resulting SR protein phosphorylation state. Temperature-dependent CLK activity is mediated by the kinase activation segment which adopts an ordered, active conformation at colder temperatures. Depending on their phosphorylation state, the type of SR protein (see Figure 2C) and the binding position relative to the alternative exon, SR proteins either favor exon inclusion in cold conditions (hyperphosphorylated) or exon inclusion in warm conditions (hypophosphorylated). (**C**) Regulation of rhythmic alternative splicing by CLKs and SR proteins. Rhythmic changes in body temperature, controlled by the central mammalian pacemaker in the suprachiasmatic nucleus (SCN), induce 24-h rhythms in body temperature and consequently in alternative splicing. Daily fluctuations in body temperature drive rhythmic variation in CLK activity and downstream SR protein phosphorylation levels, which in turn modulate alternative splicing patterns.

temperature sensitivity of CLKs is sufficient to globally alter SR protein phosphorylation levels in cells and *in vivo* [1], with hyperphosphorylation in cold and hypophosphorylation in warm (Figure 1B, middle). SR-proteins are sequence-specific RBPs that are generally considered as activators of splicing, and both their subcellular localization as well as their activity are affected by their phosphorylation level (also see below for SR protein specific differences). While SR proteins generally regulate all types of AS, we are referring here to their dominant activity in controlling alternative exon inclusion/skipping. To activate splicing (in a temperature-dependent manner) *in trans*, SR proteins require a specific *cis*-regulatory binding site within or in proximity to the temperature regulated exon, and depending on the position of this element(s) and the nature of the interacting SR protein(s) alternative exons can be separated into heat and cold-induced (Figure 1B, right). SRSF2 and SRSF7 represent two SR

proteins with higher activity in the hypophosphorylated state observed in heat [1], leading, especially for SRSF2, to heat-induced exon inclusion if binding occurs within alternative exons [5]. RNA-sequencing of mammalian cells incubated at different temperatures revealed that temperature-dependent exon inclusion is a widespread process with hundreds to thousands of exons being affected [1,2,5,6]. Pharmacological inhibition specifically of CLK1/4 reduces or abolishes the temperature-response of a large fraction of these exons, suggesting that temperature-regulated SR protein phosphorylation via these two kinases is a major — but certainly not the only — driver of temperature-dependent AS [2].

Regulation by rhythmic changes in body temperature also offers a molecular explanation for the early observation of a quasi-circadian AS event in the mouse *U2af26* gene [7]. Body temperature cycles are directly controlled by the central mammalian pacemaker in the suprachiasmatic nucleus of the brain. Consequently, they can induce 24-h rhythms in AS, which can adapt to the external light-dark regime and persist in constant darkness. This finding is intriguing and aligns with the ultrasensitive nature of the splicing thermometer. The daily ∼1.5°C differences in mouse body temperature appear to be sufficient in driving rhythmic variation in CLK activity and downstream SR protein phosphorylation levels (Figure 1C).

Adaptation of the splicing regulatory machinery to extreme temperatures

In addition to the impact of slight temperature changes on alternative exon inclusion, a more extreme heat-shock induces a significant splicing repressive effect [8], leading to substantial intron retention (IR). Interestingly, a subset of genes is spliced efficiently, likely co-transcriptionally, under such conditions [9]. Both *Clk1* and *Clk4* exhibit even enhanced splicing efficiency of introns 3 and 4, surrounding the alternative heat-induced exon 4, under heat conditions (Figure 2A). Consequently, cold temperatures reduce the fraction of protein-coding *Clk1/4* mRNAs mainly by IR (unpublished, based on RNA-seq data) and retention of the transcripts in the nucleus. However, these retained introns likely undergo splicing upon recovery from cold shock. IR within *Clk1/4* involves the SR protein SRSF4 and autoregulation via active CLK1/4. Pharmacological inhibition of CLK activity promotes splicing of introns 3 and 4 [10], suggesting a repressive effect of active CLKs on their own intron splicing. In contrast with cold temperatures, heat-shock leads to an accumulation of high amounts of protein-coding Clk1/4 mRNAs [11]. Upon recovery from heat shock, CLKs are recruited to nuclear stress bodies containing the long non-coding RNA HSATIII and high levels of unphosphorylated SR proteins. This recruitment is crucial for efficient rephosphorylation of SR proteins [12].

The repression of splicing during heat-shock is closely linked to the dephosphorylation of the SR-protein SRSF10 [8], as depicted in Figure 2B. This dephosphorylation of SRSF10 upon heat is consistent with the reduced activity of CLKs mentioned earlier. Furthermore, it is facilitated by 14-3-3 proteins, which protect SRSF10 at normal temperatures but dissociate from it upon heat-shock, allowing dephosphorylation by protein phosphatase 1 (PP1) [13]. Dephosphorylated SRSF10 acts as a general splicing repressor, leading to significant IR upon heat-shock, whereas phosphorylated SRSF10 serves as a sequence-specific splicing activator [14]. SRSF10 also regulates the inclusion of its own exon 3 through a mechanism involving competition between the major and minor spliceosomes (Figure 2C, top). The presence of an exonic enhancer element in exon 3, recognized by phosphorylated SRSF10 in cold conditions, promotes inclusion of exon 3 via the minor spliceosome [15]. Inclusion of exon 3 generates a premature termination codon (PTC) in exon 2 through use of the minor spliceosome-dependent 5′ splice site, therefore resulting in nonsense-mediated decay (NMD) of this isoform [15]. Consequently, the expression level of SRSF10 mRNA inversely correlates with its activity, exhibiting high expression and low activity during the heat phase of a simulated 24-h temperature rhythm, and vice versa during the cold phase (Figure 2C, top). This expression pattern is dependent on the presence of the autoregulatory exon 3 [6]. Similarly, an antagonistic activity/abundance pattern is observed for SRSF2 (Figure 2C, bottom), consistent with the higher activity of dephosphorylated SRSF2 during heat-shock conditions (see above). SRSF2 also regulates its own expression by promoting the inclusion of a degradation-inducing exon, a process facilitated by dephosphorylated SRSF2 upon heat [16]. These findings demonstrate SR protein-specific differences in activity in response to altered phosphorylation levels and suggest that the temperature-responsive splicing machinery adapts its own expression levels to the ambient temperature. It is an intriguing hypothesis that these adaptations may play important roles not only in the adaptation to constantly varying temperatures and the return to normal temperature but also in the formation of a temperature memory, which could aid in protecting against repetitive temperature stress. A temperature memory, also called priming, is prominently

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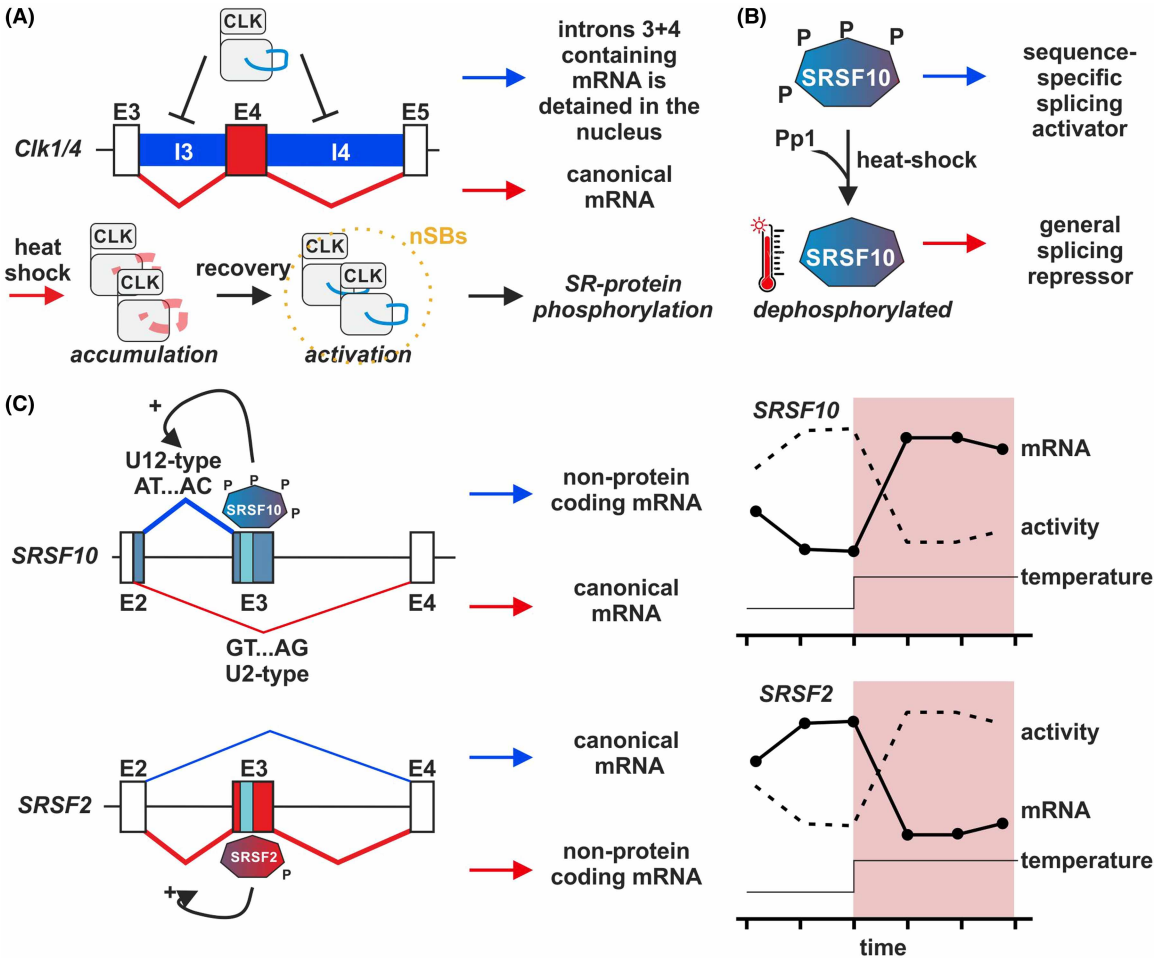

Figure 2. Autoregulation of CLK and SR proteins in response to temperature changes.

(**A**) Stress-responsive intron retention (IR) of CLK1/4 mRNA. Clk1/4 mRNA splicing is reduced at normal and even more at colder temperatures, resulting in the accumulation of intron-retaining RNA isoforms within the nucleus (based on RNA-seq data from different temperatures and [11]). Treatment with a CLK1/4 inhibitor promotes the maturation of Clk1/4 mRNA by facilitating the splicing of these retained introns, indicating a repressive effect of active CLKs on their own intron splicing [10]. Similarly, exposure to heat shock triggers the maturation of Clk1/4 mRNAs [11]. Following heat shock recovery, Clk1/4 proteins catalyze the rephosphorylation of SR proteins, particularly SRSF4 and SRSF10, within nuclear stress bodies (nSBs). Stress-induced Clk1/4 expression controlled through splicing regulation thus upholds the phosphorylation status of SR proteins during stress and recovery. (**B**) Dephosphorylated SRSF10 acts as a global splicing repressor. SRSF10 exhibits different functionalities depending on its temperature-dependent phosphorylation state. Upon CLK1/4 dependent hyperphosphorylation at colder temperatures, SRSF10 acts as sequence-specific splicing activator. In contrast, upon dephosphorylation in heat (reduced activity of CLK1/4 and accessibility towards PP1 phosphatase activity) SRSF10 serves as a general splicing repressor, leading to significant global intron retention. (**C**) Examples for temperature-specific adaptation of SR proteins. SRSF10 regulates the inclusion of its own exon 3 through competition between major (U2-type with GT/AG consensus splice sites) and minor (U12-type with AT/AC consensus splice sites) spliceosomes (top). In cold conditions, phosphorylated SRSF10 recognizes an exonic enhancer element (light blue) in exon 3, promoting its inclusion via the minor spliceosome. This inclusion generates a PTC in exon 2 through usage of the minor splice site, leading to nonsense-mediated decay of this isoform. Consequently, SRSF10 mRNA expression inversely correlates with the activity of SRSF10 as sequence-specific splicing activator, showing high mRNA expression and low protein activity during the heat phase and vice versa during the cold phase of a 24-h temperature rhythm. SRSF2 exhibits an antagonistic activity/abundance pattern, consistent with higher protein activity in warm conditions (bottom). It regulates its own expression by promoting the inclusion of a degradation-inducing exon in its dephosphorylated state upon heat. In this model, the activity of SRSF10 or SRSF2 proteins is controlled through their phosphorylation status and their potential to induce inclusion of their own autoregulatory exon.

observed and investigated in plants, where an initial priming exposure increases tolerance to subsequently occurring extreme temperatures [17]. AS has been suggested to play a role in this process in plants [18], but whether similar mechanisms exist in mammals remains an open question.

Evolutionary conservation of temperature-dependent AS

It is intriguing to note that the autoregulatory exons within SR proteins, which mediate the SR protein-specific adaptation to temperature, exhibit an exceptionally high degree of evolutionary conservation at the sequence level (see examples in Figure 3A). In fact, most genes encoding SR proteins contain ultraconserved autoregulatory exons [19,20], meaning they share identical 100–200 nucleotide-long sequences among mouse, rat and human, with high conservation observed in other vertebrate species as well. These evolutionarily conserved autoregulatory exons play a crucial role in coordinating the expression levels of the entire network of SR proteins [15,21] and are essential for viability, unlike many other ultraconserved elements [22]. Similarly to the conservation observed in SR protein-encoding genes, the alternative exon 4 of CLK1/4 and its surrounding introns display an extremely high degree of conservation across vertebrates (Figure 3B). This level of conservation within the main regulators of temperature-dependent AS, especially within mammals and birds [23], strongly links this autoregulatory network to endothermy. Consistently, the SRSF2 mRNA displayed a very similar temperature response of exon 3 splicing in cell lines from human, mouse, hamster or chicken [6].

In contrast with ultraconserved exons within SR proteins, there are also examples for functionally relevant temperature-dependent exons specific for certain species. The gene encoding the mouse TATA-box binding protein (TBP) contains two cold-induced exons within its 5′-untranslated region (5′-UTR), that do not show any conservation outside rodents (Figure 3C). Exon skipping upon warmer temperatures results in a short, unstructured 5′-UTR while inclusion of two exons (termed X and Y) results in a longer 5′-UTR that is predicted to form secondary structures. Interestingly, luciferase reporter assays revealed a higher translation efficiency for these longer, structured 5′-UTRs, suggesting temperature-controlled expression of TBP protein [1]. In consequence, in mice, low body temperature during the day results in inclusion of TBP exons X and Y and a peak of TBP protein expression at the beginning of the night, correlating with maximal expression of TBP target genes (Figure 3C). Thus, temperature-dependent AS can generate rhythms in gene expression by controlling the levels of a single transcription factor in a species-specific manner.

Global shaping of the temperature-dependent transcriptome by AS coupled to mRNA decay

In addition to controlling inclusion of cassette exons that alter parts of the coding region of their mRNAs, temperature-dependent AS can also directly influence rhythmic gene expression. As highlighted for SR proteins, temperature-dependent AS can create mRNA variants that are targets for degradation. These variants can be identified and degraded by various mRNA decay pathways, including NMD. Temperature-dependent AS events that lead to decay can be roughly divided into four categories (our focus is again on cassette exon splicing): (i) decay induced by cold through exon inclusion, (ii) decay induced by heat through exon inclusion, (iii) decay induced by cold through exon exclusion, and (iv) decay induced by heat through exon exclusion (Figure 4A, left). Inclusion-induced decay events primarily lead to degradation if PTC-containing exons are included, while exclusion-induced decay is mainly caused by the introduction of frameshifts and the generation of new PTCs in downstream exons of the mRNA (Figure 4A, right). A global analysis of the impact of AS coupled to mRNA decay on the temperature-dependent transcriptome in primary mouse hepatocytes detected over one thousand temperature-dependent splicing events influenced by the presence of cycloheximide (CHX) [6]. CHX globally halts translation, thus blocking translation-dependent decay pathways and thereby stabilizing poison variants. Many of these events align with known NMD targets, suggesting that NMD is the main decay pathway in this setting. This is further supported by the finding that a significant proportion of events sensitive to temperature and CHX are stabilized upon knockdown of the NMD-factors SMG6/SMG7 [24], emphasizing a pivotal role of NMD in degradation of temperature-controlled decay isoforms. There is a clear correlation between reduced gene expression and temperature-dependent generation of poison isoforms in normal conditions (as indicated in Figure 4A, right), while CHX treatment abolishes this correlation entirely. This strongly indicates that AS and mRNA decay are an important factor driving temperature-dependent changes in gene expression levels. Strikingly, mouse body temperature rhythms are sufficient to generate 24-h gene expression rhythms in genes containing temperature-controlled AS-NMD isoforms, thereby representing a core clock independent oscillator of

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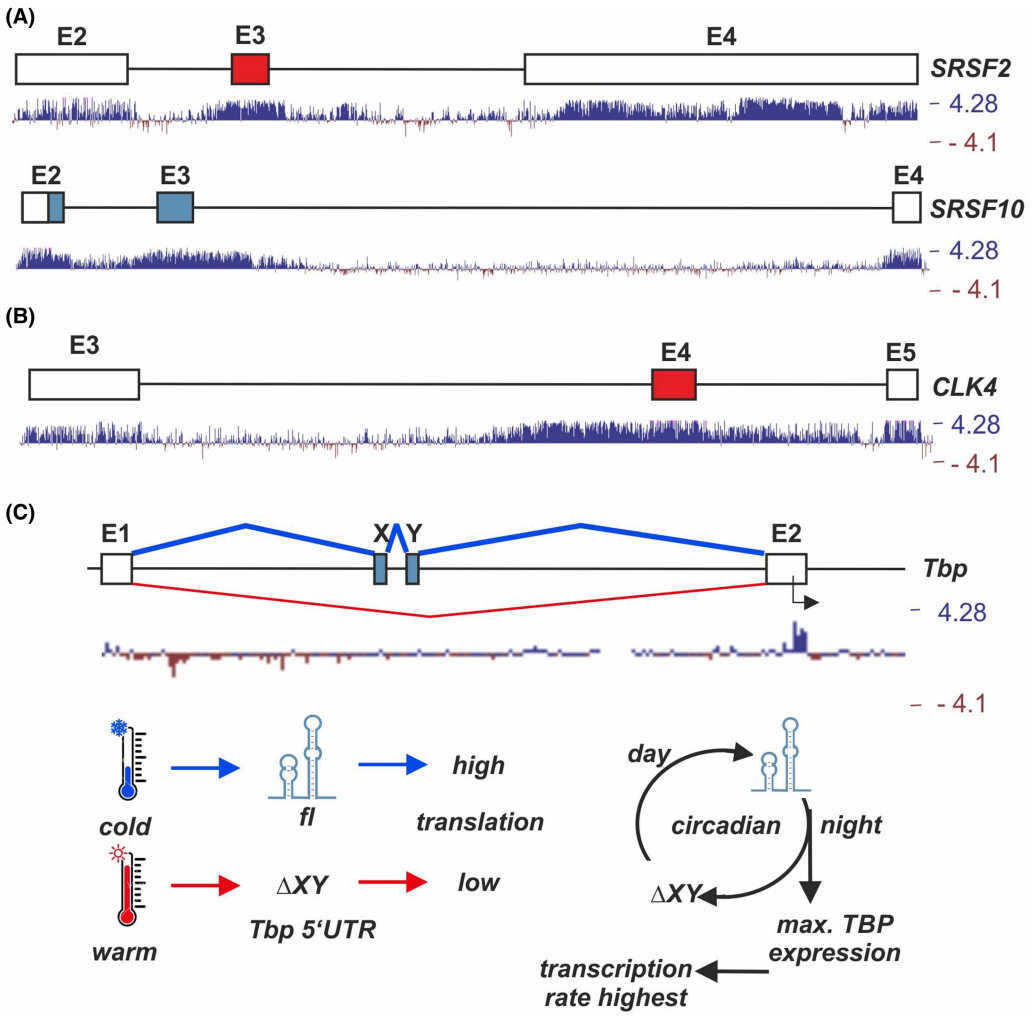

Figure 3. Temperature-dependent alternative splicing: from ultra-conserved to species-specific.

(**A**) Ultra-conserved exons regulate temperature-dependent expression of SR proteins. Shown are the autoregulatory heat-induced exon 3 of SRSF2 (red box, top) and cold-induced minor spliceosome-dependent alternative 5′-splice site of exon 2 coupled to exon 3 (blue boxes, bottom) of SRSF10 with surrounding constitutive exons. Below each exon-intron structure evolutionary conservation across 35 vertebrate species. Blue indicates high conservation values, red low conservation values. Conservation values and images are derived from the UCSC genome browser. (**B**) Evolutionarily conserved introns regulate temperature-dependent expression of CLKs. Shown is the autoregulatory heat-induced exon 4 of CLK4 (red box) with surrounding constitutive exons. Below the exon-intron structure evolutionary conservation is indicated as in A. Note the extremely high conservation of introns surrounding exon 4. (**C**) Rodent-specific exons control circadian TBP translation. Top: Shown are the rodent specific cold-induced exons X and Y (blue boxes) with surrounding constitutive exons 1 and 2. The translation start site is marked by an arrow. Below the exon-intron structure evolutionary conservation is indicated as in **A**. Note the absence of conservation in the 5′-UTR sequence. Bottom: Cold-induced exon X and Y inclusion forms a structured full-length (fl) 5′-UTR with high translation rate, while exon skipping (ΔXY) upon heat forms a shorter translationally less active 5′-UTR. Circadian body temperature cycles result in rhythmic differences within the 5′UTR of TBP, as indicated on the right. Lower body temperature during the day results in accumulation of the fl-isoform, producing maximal TBP expression and the highest transcriptional activity at the onset of the night.

gene expression [6]. This rhythm is not considered circadian as it requires the constant presence of a 24-h temperature rhythm, but is faster entrainable to a novel temperature regime than the classical circadian clock [25].

Temperature-dependent AS-coupled to mRNA decay is particularly common in RBPs, suggesting that altering the expression of RBPs is a central adaptive mechanism upon temperature changes. Altered RBP expression may

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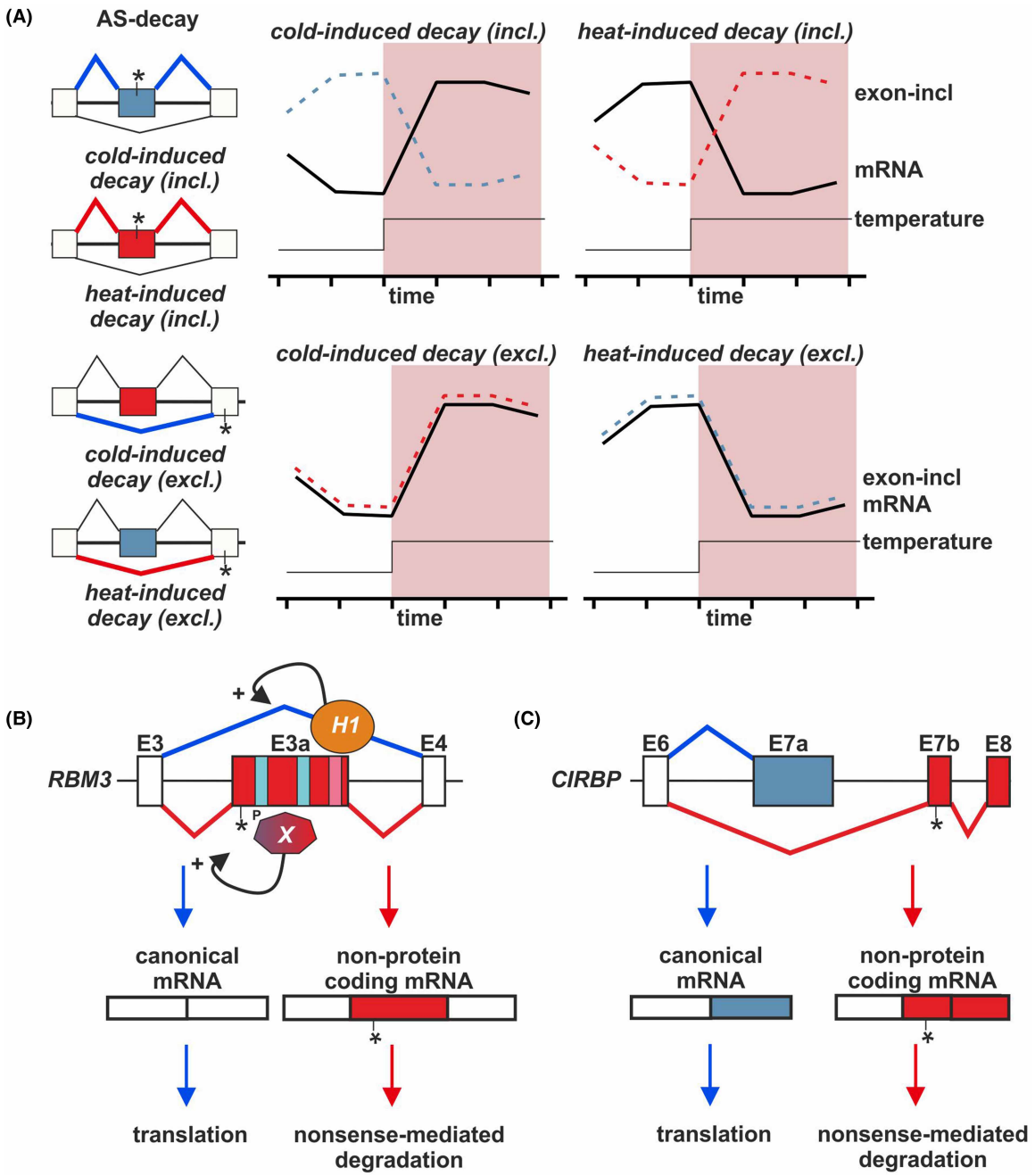

Figure 4. Global shaping of the temperature-dependent transcriptome by alternative splicing coupled to mRNA decay.
Part 1 of 2

(**A**) AS-decay generates 24-h rhythms in gene expression. Temperature-dependent alternative splicing directly impacts rhythmic gene expression by generating mRNA variants targeted for degradation. These variants undergo decay via various mRNA decay pathways, including nonsense-mediated decay (NMD). Temperature-dependent AS events leading to decay can be categorized into four types based on exon inclusion or exclusion induced by cold or heat. Inclusion-induced decay primarily occurs when exons with premature termination codons (PTCs) are included (upper two examples), while exclusion-induced decay results from frameshifts and new PTCs in downstream exons (two lower examples). Temperature-controlled AS-decay is highly prevalent in RBPs, suggesting a central role in temperature adaptation. Correlations of exon inclusion and the expected changes in gene expression are summarized on the right. (**B**) Cold-induced RBM3 expression regulated by AS-NMD. The RBM3 mRNA contains a temperature-dependent poison exon (E3a), which is included at warmer temperatures, resulting in NMD-mediated degradation of the transcript. At colder temperatures, E3a gets substantially excluded, leading to the generation of the canonical mRNA and

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Figure 4. Global shaping of the temperature-dependent transcriptome by alternative splicing coupled to mRNA decay.
Part 2 of 2

protein production. Two putative ESEs in E3a are likely bound by SR-proteins (unknown SR protein is indicated by X) at warmer temperatures (light blue boxes), promoting adjacent splice site usage and E3a inclusion. Additionally, E3a contains an ESS (light red box) which is bound by HNRNPH1 (H1) at colder temperatures promoting E3a exclusion. (**C**) Cold-induced CIRBP expression regulated by AS-NMD. In contrast with RBM3, cold-induced expression of CIRBP is regulated through alternative 3′ ends. Its warm-induced isoform utilizes an alternative exon (E7b), which is coupled to exon 8 inclusion, and contains a PTC. Omitting exons 7b and 8 at colder temperatures therefore increases CIRBP mRNA expression.

control downstream events on a global scale, thereby amplifying the impact of temperature signals. As mentioned above, ultraconserved elements within SR protein genes control their expression through poison exon splicing, and the majority of these exons are temperature sensitive. This likely results in a temperature-controlled pattern of SR protein expression, further regulated through their phosphorylation by CLKs, with transcriptome-wide consequences for (pre-)mRNA processing. Another example of a temperature-controlled RBP is the well-known cold-expressed protein RNA binding motif protein 3 (RBM3). Already mild cold-exposure (32–34°C) is sufficient to strongly induce RBM3 expression [26]. A detailed analysis of the *RBM3* gene revealed the presence of a previously not annotated exon (termed E3a) in intron 3, containing multiple PTCs thus acting as a poison exon [27]. At normothermia (37°C) E3a is highly included, while mild hypothermia promotes exclusion of E3a and production of the canonical, protein-coding mRNA. E3a inclusion results in very efficient NMD-mediated degradation of the *RBM3* mRNA (Figure 4B) making E3a a heat-induced poison exon. Mapping *cis*-regulatory elements in E3a revealed two putative, evolutionarily conserved exonic splicing enhancers (ESEs) and one exonic splicing silencer (ESS) to be involved in the temperature-dependent regulation of E3a [27,28]. While the two ESEs are likely bound by SR proteins promoting E3a inclusion at higher temperatures, the G-rich ESS is bound by heterogeneous nuclear ribonucleoprotein H1 (HNRNPH1) at lower temperatures thereby repressing E3a inclusion. Strikingly, temperature-dependent expression of RBM3, in many transcriptome- or proteome-wide analyses the strongest cold-induced mRNA and protein, is completely abolished in genome engineered cell lines lacking exon 3a. This suggests that temperature-dependent AS-NMD is solely responsible for the massive induction of RBM3 expression in cold [27].

Another example of a cold-expressed RBP is cold-induced RBP (CIRBP), a protein associated with circadian rhythm, sleep, and body temperature regulation [29,30]. In contrast with RBM3, the *CIRBP* mRNA does not contain a temperature-dependent cassette exon, but is regulated mainly through alternative 3′ ends. Instead of the canonical last exon 7a, the warm-induced isoform uses an alternative exon (E7b), which is coupled to exon 8 inclusion, making the stop codon in E7b a potential PTC (Figure 4C) [2]. Interestingly, a strong evolutionary conservation downstream of the canonical polyadenylation site including the 3′ splice site of E7b was observed in all vertebrates, suggesting an evolutionarily conserved regulatory mechanism [2]. As for the poison exon in RBM3, genetic deletion of exons 7b and 8 in HEK293 cells resulted in increased *CIRBP* mRNA expression and decreased temperature responsiveness. Furthermore, the E7b containing isoform is strongly stabilized by global translation inhibition using CHX, consistent with a function as poison isoform, and E7b inclusion correlates with decreased CIRBP expression. Additionally, another study suggests a splicing-dependent regulatory element in *CIRBP* intron 1, which may contribute to temperature regulation [31]. The authors hypothesize that temperature-sensitive RNA secondary structures could influence splice site recognition and splicing efficiency in a temperature-dependent manner. Predicted noncanonical splice acceptor and donor sites, as well as potential RNA structures within intron 1 support this hypothesis. Finally, splicing-dependent cold-induced CIRBP expression is also observed in hibernating mammals [32], a process additionally involving the splicing efficiency of intron 6 [33], which is also temperature-dependent in non-hibernating mammals. Cold-induced CIRBP expression therefore appears to be controlled at several levels, all of them using temperature-controlled AS as the mechanistic basis. These examples, from global regulation to individual cases, together underline the fundamental impact of AS-NMD in shaping the temperature-dependent transcriptome.

Functional outcomes of temperature-controlled AS

Temperature-dependent AS patterns essentially reflect one part of the 'molecular state' of a cell at a certain temperature. In turn, manipulation of AS at normothermia offers a potential means to simulate potentially beneficial molecular responses to temperature changes, without altering the temperature itself. For instance, therapeutic

hypothermia (TH) is a medical therapy where the body temperature of patients is intentionally lowered (typically to ~32–34°C). It is utilized in various clinical settings such as cardiac arrest [34–36], brain injuries, or stroke [37–40] but especially demonstrates efficacy for the treatment of hypoxic-ischemic encephalopathy (HIE) in neonates. Although TH treatment of HIE is practised worldwide with good results [41–43], its use is strongly limited due to significant side effects and countermeasures of the human body that can only be suppressed through induced coma and strong concurrent medication. Therefore, manipulating AS events that mediate the beneficial effects of hypothermia without actually changing body temperature holds huge therapeutic potential. One such case, in which a single splicing event could mimic hypothermia on the molecular level, is the poison exon in RBM3, as recent studies have shown that RBM3 alone mediates (at least parts of) the beneficial effects of TH by promoting the formation of new synaptic contacts and protecting against synaptic loss in neurodegenerative *in vivo* models [44,45]. As RBM3 is strongly induced in hypothermia, induction at normothermia represents a promising therapeutic strategy. The mechanistic understanding of the cold-induction of RBM3 via exclusion of a poison exon, discussed above, opened the possibility to manipulate this AS event in order to promote exclusion and thereby induce RBM3 expression independent of temperature. Indeed, antisense-oligonucleotides (ASOs) targeting one of the ESEs promote E3a exclusion at normothermia, resulting in elevated RBM3 protein levels *in vitro* and *in vivo* (Figure 5A). Notably, a single administration of an ASO results in long-lasting increase in RBM3 expression in mouse brains. In prion-diseased mice, this treatment leads to remarkable neuroprotection, with prevention of neuronal loss and spongiosis in the hippocampus [27]. Interestingly, although the neuroprotective role of RBM3

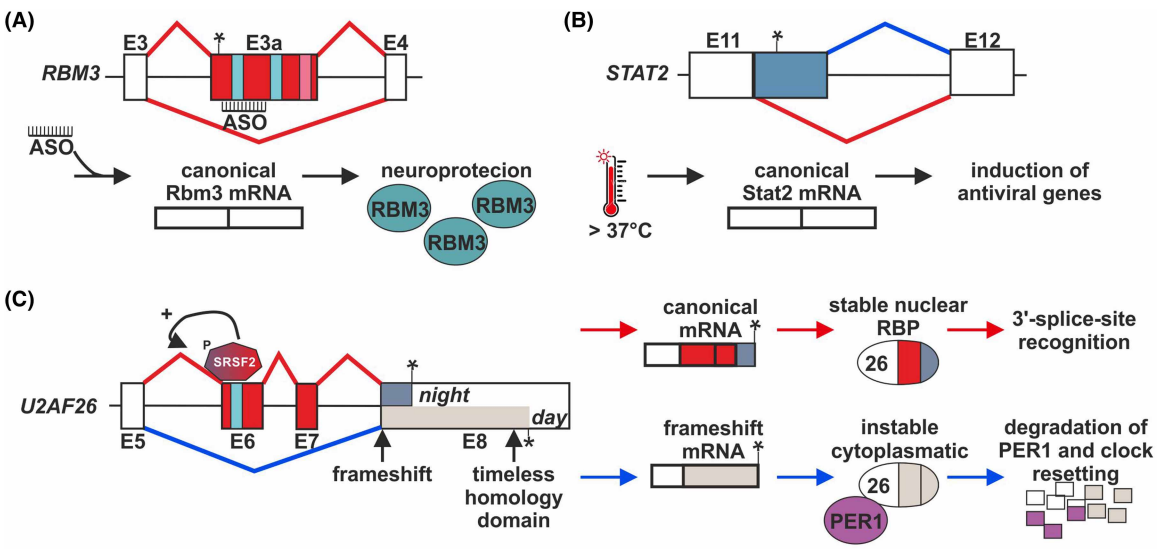

Figure 5. Functional outcomes of temperature-controlled alternative splicing.

(**A**) ASO-induced RBM3 expression protects neurons from degeneration at normothermia. RBM3 plays an important role in mediating beneficial effects of hypothermia such as promoting new synaptic connections and protecting against synaptic loss. Understanding the mechanism behind its cold-induction through exclusion of a poison exon has led to therapeutic strategies targeting this splicing event. Antisense-oligonucleotides (ASOs) targeting an ESE promote E3a exclusion and result in long-lasting increased RBM3 levels *in vivo* and neuroprotection in prion-diseased mice. (**B**) Body temperatures above 37°C induce STAT2 expression leading to activation of the innate immune system. Temperature-dependent AS coupled to nonsense-mediated decay (AS-NMD) in *Stat2* regulates its expression. At lower temperatures, a temperature-dependent alternative 5′ splice site in exon 11 is favored, leading to a premature termination codon, triggering NMD and preventing functional protein formation. Conversely, higher temperatures (>37°C) promote usage of the canonical 5′ splice site, increasing STAT2 expression. STAT2 is a key transcription factor in the antiviral response, enhanced expression upon a subtle temperature increase thus triggers antiviral gene expression in the sub-pathological temperature range. (**C**) Temperature-induced frameshift in U2AF26 regulates the circadian clock. In the mouse gene U2AF1L4, encoding U2AF26, AS of exons 6 and 7 is controlled by the body temperature of mice. At warm body temperature (night), exon 6/7 inclusion produces a full-length mRNA encoding a nuclear RNA-binding protein involved in AS regulation. Colder body temperature (day) induce exon skipping, resulting in a frameshift and translation into the supposed 3′UTR generating a new C-terminus that localizes the protein to the cytoplasm. This altered isoform interacts with the core clock protein PERIOD, leading to its degradation.

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is widely acknowledged, its molecular function is only partly understood. An interesting hypothesis is that RBM3, and potentially related cold-induced RBPs such as CIRBP, could act as RNA chaperones to prevent the aggregation of RNAs at colder temperature. Similarly, other stress conditions that reduce translation, for example through phosphorylation of eIF2alpha, increase the formation of potentially toxic RNA aggregates, which could be (partially) prevented by RBM3 and other RNA chaperones [46].

The significance of AS-mediated functional adaptations to temperature changes within the body is further demonstrated by another example of a temperature-dependent AS-NMD event in signal transducer and activator of transcription 2 (STAT2). STAT2 is a transcription factor activated upon viral infection and plays a key role in the antiviral response. The *STAT2* mRNA contains a temperature-dependent alternative 5′ splice site in exon 11. Usage of this alternative 5′ splice site is higher at lower temperature and leads to a frameshift and the inclusion of a PTC, thus preventing the formation of a functional protein and leading to NMD. Interestingly, temperatures higher than 37°C favor the usage of the canonical 5′ splice site resulting in increased *STAT2* expression, which eventually induces the type I interferon response and nitric oxide production upon a subtle temperature increase in the sub-pathological temperature range (Figure 5B). A mild temperature increase is further associated with a strong induction of antiviral gene expression in monocytes, providing a potential explanation for the individual sensitivities towards viral infections [47]. This model may also contribute to the explanation why older people with lower body temperature showed higher susceptibility to severe Sars-CoV-2 infection than children, who have on average a higher body temperature.

In the examples mentioned so far temperature regulated AS mainly serves its function by controlling gene expression levels via splicing coupled to mRNA decay. But there are also examples for temperature-controlled AS events leading to the formation of protein isoforms with altered functionality. Within the mouse gene U2AF1L4, encoding for the U2AF35 paralogue U2AF26, AS of the two penultimate exons 6 and 7 is regulated by temperature (Figure 5C; see also Figure 1A). At warm temperatures an enhancer element in exon 6 recruits SRSF2, in its hypophosphorylated state, resulting in exon 6 and 7 inclusion [1,5]. This full length mRNA encodes for a nuclear RBP with a function in AS regulation [48], that — like U2AF35 — can also control translation upon shuttling to the cytoplasm [49,50]. Upon cold exposure, phosphorylation of SRSF2 (and likely SRSF7) results in strong skipping of exons 6 and 7, a phenomenon occurring in mice exclusively during the day when body temperature is low [7]. Interestingly, skipping of exons 6 and 7 induces a frameshift that generates a novel open reading frame expanding into the supposed 3′UTR. This phenomenon, AS of frameshift-inducing penultimate exons leading to translation into the '3′UTR' and a new C-terminus of the protein, is frequently found in the mammalian transcriptome [49]. In the case of the U2AF26 gene the novel C terminus localizes the protein to the cytoplasm, and consistent with its homology to the *Drosophila melanogaster* TIMELESS protein, is involved in controlling the stability of the interacting core clock protein PERIOD. As a consequence, mice lacking U2AF26 show arrhythmic expression of PERIOD in peripheral tissues and show a faster adaptation to a new light-dark regime in jet-lag experiments, suggesting an involvement of U2AF26 and its temperature-controlled AS in stabilizing the core circadian clock against external perturbations [7].

Temperature-dependent AS in other organisms

Although this review focuses on temperature-controlled AS in mammals, noteworthy examples have been described in other species, most prominently in ectothermic organisms that experience a larger temperature fluctuation than endothermic organisms. A prominent example is the role of AS in adapting the transcriptome to temperature changes in plants. Interestingly, despite the large evolutionary distance between plants and mammals, temperature-controlled regulation of SR proteins by AS-NMD is found in both systems, pointing to a fundamental function in dynamically adjusting SR protein expression across evolution [6]. For a detailed introduction to temperature-controlled AS in plants we refer to several recent reviews covering this topic [51–54]. Another interesting example is temperature-dependent IR in *D. melanogaster* with a potential role in regulating the circadian rhythm [55]. The authors showed an alteration in the splicing pattern of *timeless* (*tim*), an essential component of the circadian clock, in response to temperature changes. In addition to the canonical transcript (*tim-L*) two cold-specific isoforms are generated upon cold (18°C) exposure: one previously described isoform, referred to as *tim-cold* [56–58] contains intron 16 and *tim-short and cold* (*tim-sc*), which contains intron 11 including a cleavage and polyadenylation site. Conversely, another isoform, termed *tim-medium* (*tim-M*), containing intron 13, becomes more abundant at higher temperatures. This isoform switching plays a crucial role in regulating TIM protein levels, resulting in reduced expression of the canonical TIM-L protein at 18°C. As overall *tim* mRNA expression was not altered at different temperatures, the observed reduction in

TIM-L at lower temperatures is rather the result of changed *tim* splicing isoform distribution and post-transcriptional regulation at the respective temperatures. Thus, temperature-dependent AS of *tim* might serve as a sensor for the circadian clock's adaptation to temperature variations.

Temperature-dependent AS may also play a role in temperature-dependent sex determination (TSD) and temperature-induced sex reversal (TISR). TSD and TISR are found in many vertebrate species, like all crocodilians, most turtles, some lizards and some fish. While in TSD the sex of the offspring is determined by the temperature at which eggs are kept during a particular time of embryonic development and not by genotypic factors, in TISR the genetically determined sex can be overridden by alterations in temperature. Interestingly, several studies observed temperature-dependent IR events occurring in genes of the *Jumonji* chromatin modifier family like *Jarid2* and *Jmjd3* [59] in TSD/TSIR species. For example, in alligators (*A. mississippiensis*) *Jarid2* and *Jmjd3* IR specifically occurs at the female producing temperature, while in a turtle species (*T. scripta*) IR variants appear at the male producing temperatures, likely controlled through temperature-dependent CLK activity [2]. The high relative abundance of the intron retained variants, which all contain PTCs, suggests that the transcripts escape NMD and may therefore result in the production of protein variants that play a role in the TSD process. However, the precise functionality of these IR isoforms and a potential impact on TSD remain to be investigated. Temperature-dependent AS has further been shown in TSD-related genes, like *doublesex and mab3-related transcription factor 1* (*Dmrt1*) [60], *Lysine demethylase 6B* (*Kdm6b*) [61] and *SRY-box9* (*Sox9*) [62]. While it has been shown that *Kdm6b* and *Dmrt1* directly regulate sex determination in *T. scripta*, sex-fate decision is a complex interplay of different pathways and it remains elusive how these temperature-dependent AS variants contribute to the process of sex determination.

Outlook

The last years have led to the realization that even subtle changes in body temperature, as observed in humans and other endothermic mammals, are sufficient to globally control AS, and, through AS-NMD, gene expression. One mechanism, based on temperature-controlled SR protein phosphorylation by CLKs, has been identified and the functionality of some individual temperature-controlled AS events has been uncovered. However, many questions remain to be addressed, which promises fascinating discoveries in the coming years. We would like to highlight four areas of active research with especially high potential:

1. While hundreds or even thousands of AS events are controlled in a temperature-dependent manner, the functionality of individual splicing events with respect to the resulting protein isoform and its impact on the cell has been investigated in very few cases. It thus remains a wide-open question how temperature-controlled AS impacts on cellular functionality. Answering this question requires tailored molecular and cell biological approaches for every individual splicing event and is difficult to perform in high throughput settings and therefore, addressing the functionality of (temperature-controlled) AS will remain an active research field for many years to come. In addition, it remains an open question whether it is the concerted splicing change in many RNAs that is required for cellular adaptation to different temperatures, or whether this adaptation is based on a few key splicing events.
2. One mechanism for temperature-controlled AS, based on CLK-mediated phosphorylation of SR proteins, has been elucidated in some detail. However, it seems very likely that other mechanisms exist. It is an intriguing possibility that the RNA itself can act as a thermosensor due to its conformational flexibility and the formation of alternative secondary structures. Especially in bacteria (but also in other organisms such as plants), RNA thermometers are widely used to control translation through altering the accessibility of the Shine-Dalgarno-Sequence, during heat shock, cold shock, or during infection and adaptation to the respective host temperature [63–65]. It seems possible or even likely that similar regulatory mechanisms have evolved in mammals to control gene expression in a temperature-dependent manner. In line with this idea, the role of RNA secondary structures in controlling AS is rapidly gaining attention [66,67], and it is well conceivable that some of these structures are temperature sensitive. RNA-based thermosensors could act independently of the trans-acting environment across different tissues, which could be advantageous if a concerted response across an entire organism is required. Related to this, it will be interesting to determine global and tissue-specific temperature-controlled splicing events and their mechanistic basis.
3. As described above, hypothermia is used in some clinical settings for its neuroprotective role. Likewise, hyperthermia is used as adjuvant therapy in some cancer treatments. In both cases, the molecular events that lead

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to the beneficial effect of altered temperature are only beginning to be understood. One example is RBM3, which mediates at least some of the neuroprotective effects of hypothermia. Future research will uncover additional beneficial regulators of thermotherapies in various tissues and disease conditions. The recent discovery that cold-induced expression of RBM3 is entirely dependent on AS-NMD opens up the possibility of ASO-based splice-modulating therapies to increase RBM3 at normothermia. This example also shows how a detailed mechanistic understanding of temperature-regulated gene expression can lead to the development of new therapeutic concepts. Given the wide impact of AS and AS-NMD on the temperature-controlled transcriptome, we consider it very likely that other beneficial mediators of thermotherapies are controlled through AS-based mechanisms as well and will therefore be amenable to ASO-based therapeutic strategies.

4. The remarkable sensitivity of both the temperature-sensitive kinase and endogenous alterations in AS underscores the rapid and precise response of this system to even minor fluctuations in the body temperature of endothermic organisms. This sensitivity aligns with the narrow body temperature range of homeothermic organisms, making even subtle temperature changes crucial signals for the body. Physiological temperature shifts of 1–2°C, generated by the circadian rhythm, delineate activity and rest phases, while more significant variations in temperature, such as those induced by fever or nutrient deprivation (+/− ∼4° C), serve as acute stress signals prompting immediate physiological adjustments to address these threats. From an evolutionary standpoint, it's intriguing to investigate how these systems have evolved from heterothermic organisms accustomed to more pronounced and prolonged fluctuations in body temperature, likely responding only to severe and enduring temperature changes as indicators of significance. Has the temperature-responsive splicing mechanism evolved from an ancient temperature-sensitive system, subsequently acquiring increased sensitivity (as evidenced by temperature-dependent kinases found in various vertebrates and even invertebrates [2])? The conservation of certain elements of this mechanism across all mammals and birds further supports a single evolutionary origin and underscores its vital role closely linked to homeothermic life. Moreover, the presence of temperature-sensitive SR protein autoregulation in plants suggests that a similar mechanism was independently acquired during evolution [6]. Investigating the evolutionary origins and adaptations to life under vastly different temperature regimes is an essential avenue for future research, especially in the light of global warming.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Florian Heyd: Supervision, Funding acquisition, Writing — original draft, Writing — review and editing. **Tom Haltenhof**: Writing — original draft, Writing — review and editing. **Marco Preussner**: Visualization, Writing — original draft, Writing — review and editing.

Abbreviations

AS, alternative splicing; ASO, antisense-oligonucleotide; CHX, cycloheximide; CIRBP, cold-induced RNA-binding protein; CLK, CDC-like kinase; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; HIE, hypoxic-ischemic encephalopathy; IR, intron retention; NMD, nonsense-mediated decay; PP1, protein phosphatase 1; PTC, premature termination codon; RBM3, RNA binding motif protein 3; RBP, RNA-binding protein; STAT2, signal transducer and activator of transcription 2; TBP, TATA-box binding protein; TH, therapeutic hypothermia; TISR, temperature-induced sex reversal; TSD, temperature-dependent sex determination.

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