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# Regulation of cold-inducible RNA-binding protein (CIRBP) in response to cellular stresses

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## Highlights

- CIRBP is a conserved, stress-responsive RNA-binding protein.
- CIRBP acts as a regulator of key pathways such as cell cycle and inflammation.
- CIRBP regulations range from transcription to protein modification and localization.
- Alternative splicing mechanisms modulate CIRBP expression and function.

## Abstract

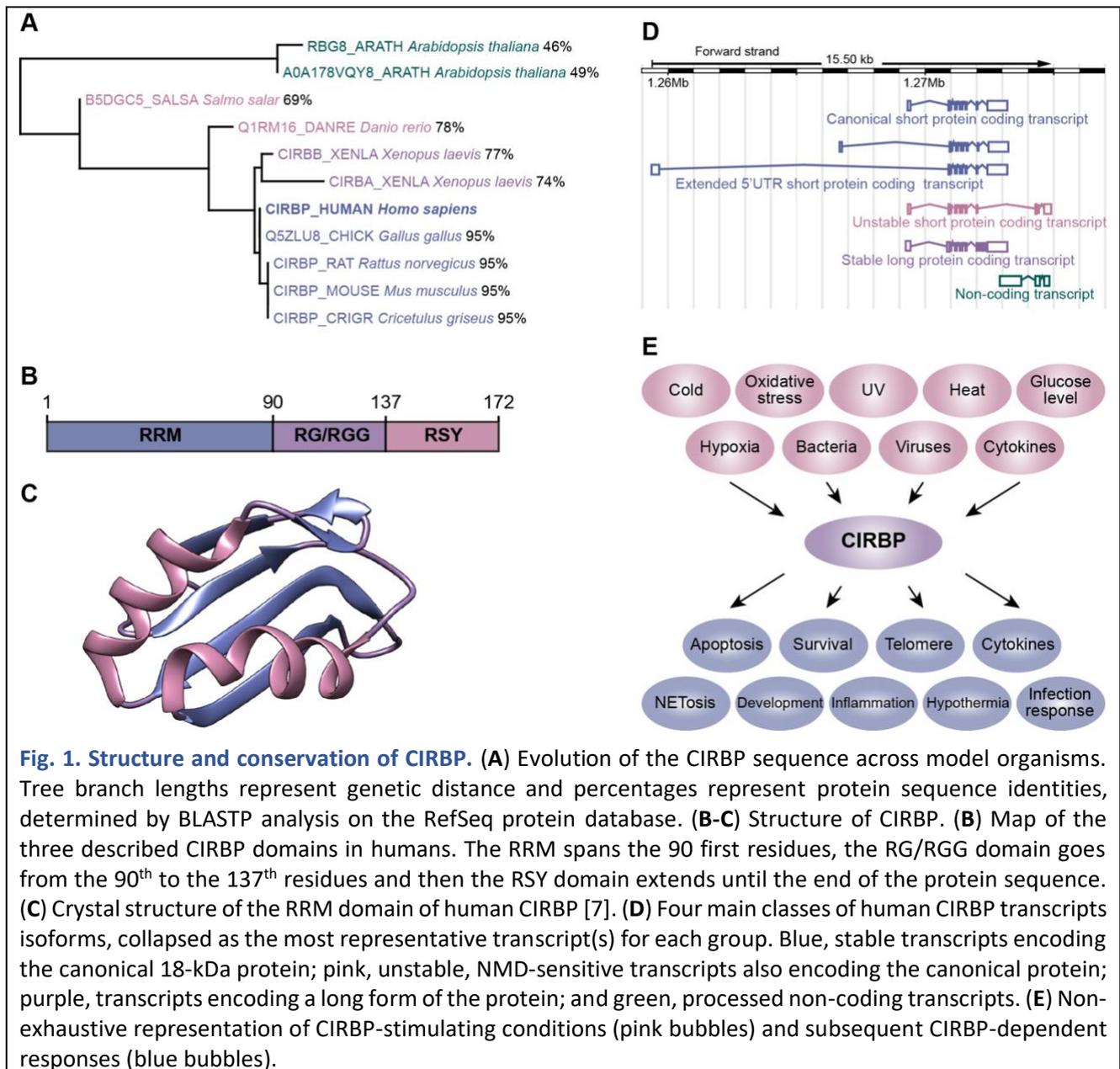
Cold-inducible RNA-Binding protein (CIRBP) is a general stress-response factor in vertebrates harboring two domains: an RNA-recognition motif and a regulatory domain rich in RG/RGG motifs. CIRBP has been described to bind mRNAs upon various stress conditions (cold, infections, UV, hypoxia...) and regulate their stability and translation. The proteins encoded by its targets are involved in key stress-responsive cellular pathways including apoptosis, inflammation, cell proliferation or translation, thus allowing their coordination. Due to its role in regulating central cellular functions, the expression of CIRBP is tightly controlled. We review here current understanding of the multiple mechanistic layers affecting CIRBP expression and function. Beyond transcriptional regulation by cold-responsive elements and the use of alternative promoters and transcription start sites, CIRBP undergoes various alternative splicing (AS) events which, depending on conditions, modulate the stability of CIRBP transcripts and/or impact the sequence of the encoded polypeptide. Typically, whilst CIRBP expression is induced in the context of hypothermia or viral infection, AS events preferentially address alternative isoforms towards mRNA degradation pathways in response to heat stress or to bacterial-secreted pore forming toxins. Post-translational modifications of CIRBP, mostly in its RGG domain, also condition CIRBP subcellular localization and access to its targets, thereby promoting or inhibiting their expression. For instance, phosphorylation and methylation events gate CIRBP nuclear to cytoplasmic translocation and control its recruitment to stress granules. Considering the therapeutic potential of modulating the expression and function of this central player in stress responses, a fine understanding of CIRBP regulation mechanisms deserves further attention.

## Keywords

CIRBP, alternative splicing, stress, post-translational modification, subcellular localization, translation.

## 1. Introduction

The cold-inducible RNA-binding protein (CIRBP, also referred to as CIRP or hnRNP A18) is part of the family of glycine-rich RNA-binding proteins (GRP) carrying an RNA recognition motif (RRM) [1]. CIRBP orthologs are extremely conserved across vertebrate species, and also share strong homologies with regulators of the same family in plants (Fig. 1A) [2]. In vertebrates, CIRBP is expressed in a wide variety of tissues [3] and usually encodes several protein isoforms, the most representative one being usually a 18-21 kDa polypeptide. It was first identified as a DNA-Damage-induced transcript [1,4] and hypothermia-induced protein [5] and later described as a key response factor to a broad panel of both extracellular and intracellular stresses [2], which is also involved in the development of various diseases [6].



CIRBP preferentially binds the 3'-untranslated regions (UTRs) of its target mRNA transcripts and regulates their polyadenylation site selection [8], stability and translation [9], thus impacting key cellular pathways, such as apoptosis, cell cycle or inflammation [2]. The polyvalence of CIRBP response to different contexts could be linked to the multiple layers of regulation of the gene itself.

For instance, while the structure of the protein appears quite simple, several post-translational modifications have been described [10,11], which tune the role of CIRBP essentially by affecting its sub-cellular localization. Additionally, regulations of the transcription and alternative splicing (AS) of CIRBP impact its transcript stability and translation levels, thereby affecting downstream CIRBP regulatory functions.

CIRBP regulation is thus multileveled and multifactorial. This versatility allows its regulatory role to be co-opted for coordinating responses to various types of stressors across a broad range of tissues and species, although a thorough landscape of CIRBP relevance in vital cellular functions is still missing. In this review, we aim at summarizing CIRBP known regulation mechanisms, from transcription to protein relocalization, in the context of intracellular and extracellular stresses or physiological variations.

## 2. CIRBP is a conserved RNA-binding stress response regulator

### 2.1. CIRBP sequence conservation and functional domains

The CIRBP locus is located on chromosome 19 in humans and on chromosome 10 in mice, and encodes a 18.5 kDa protein (for the main isoform in these species). The protein amino-acid sequence is very conserved across species; human CIRBP shares 95% identity with murine, rat and chicken CIRBP, 74 to 78% with *Xenopus* xCIRBP or with its homolog in zebrafish, and under 50% with members of the GRP family in *Arabidopsis thaliana* (GRP7 and GRP8. [12], arguing of an early origin of this family of regulators in Eukaryotes (Fig. 1A). The CIRBP protein comprises three main domains (Fig. 1B). The RNA-binding domain (RBD), also called RNA-recognition motif (RRM), is located in the N-terminal region of the protein and carries two consensus and highly conserved ribonucleoprotein sequences (RNP1 and RNP2) [1]. It adopts the classical fold of this domain consisting of four antiparallel beta-sheets stacked between two alpha helices (Fig. 1C), which creates an interaction platform with its targets. It has been shown to bind specific mRNA targets, preferentially in their 3'-UTRs [13]. A putative 51-nucleotide consensus binding motif containing 6 invariant nucleotides in positions 5, 13, 14, 19, 29 and 51 has been proposed, based on the comparative analysis of the sequence of CIRBP mRNA targets identified by an immunotube-based strategy [10]. However, to date, the high number of putative CIRBP targets identified in RNA immunoprecipitation- or cross-linking and immunoprecipitation assays followed by sequencing argues for a rather low specificity in their recognition [10,14–16]. In the C-terminal region, the unstructured arginine-glycine (RG)-rich domain is characterized by its decoration with several RG/RGG motifs. This type of domain, common to CIRBP and other stress-induced proteins, is generally involved in mRNA processing and transport [13]. As a target of various post-translational modifications, the RGG domain also serves as protein regulation platform [17]. Some authors consider that this domain is subdivided into an N-terminal RGG-rich portion containing three RGG repeats [18] and a C-terminal arginine, serine, tyrosine (RSY)-rich domain [19], both serving adjacent roles for protein cellular relocalization.

If the structure of the main form of the protein is rather simple, the transcription of the CIRBP loci and further maturation of its pre-mRNA can result in multiple mRNA transcripts. So far, 46 transcript variants have been identified in humans, among which 22 stable isoforms and 17 unstable isoforms (targeted for degradation by the nonsense-mediated decay —NMD—, see below) are coding, all in all resulting in at least 17 distinct CIRBP-encoded polypeptides differing in their amino-acid sequence. AS of CIRBP RNA isoforms thus represents both a quantitative regulatory layer and a potential reservoir of diversification for CIRBP protein sequence, structure and function around a common core.

## 2.2. CIRBP expression landscape across tissues

CIRBP displays a broad range of constitutive expression patterns across tissues as assessed in mice [3], with stronger levels in the brain, testis, lungs and heart and milder expression in the liver, thymus, spleen, stomach, intestine, colon kidney and uterus. Upon mild hypothermia, for instance during hibernation or circadian variations of body temperatures, CIRBP expression is amplified *via* the regulation of AS of its pre-mRNAs towards the shortest protein-coding isoform in the brain of mice [3,20], in the heart of Syrian hamsters [21] and in *Xenopus* oocytes [22]. CIRBP expression is also largely impacted in cancer development, where it can act as an oncogene or as a tumor suppressor [6], depending on the tissue.

CIRBP localization is predominantly nuclear except in *Xenopus* oocytes [18], but the protein can be translocated to the cytoplasm and even released into the extracellular space upon specific stimulations, such as mild hypothermia, inflammation or exposure to ultraviolet (UV) light [2]. The spatiotemporal regulation of CIRBP functions is thus dictated by its shuttling across cell membranes. Although ubiquitously expressed, CIRBP protein-coding isoforms, localization and functions can thus differ depending on the tissue, or in specific pathophysiological contexts.

## 2.3. Functions of CIRBP in stress responses and inflammation

Despite its name, CIRBP responds to a plethora of stresses beyond hypothermia. It is also positively regulated by UV exposure [1] and mild hypoxia [23], and negatively regulated by inflammatory cytokines [24], heat shock [25], bacterial infection [16], low circulating glucose [26] and oxidative stress [27] (Fig. 1D). In response to these stressors, CIRBP acts itself as a gene expression regulator.

In rodents, mild hypothermia induces a change in CIRBP transcript isoform proportions by AS, which in turn favors CIRBP protein production [3,20,21,28]. CIRBP binding, preferentially on a 51-base pair motif located in the 3'-UTRs of mRNAs, has been shown to stabilize its targets, inhibit their deadenylation and increase their translation by interacting with eIF4G1 [10]. Conversely, the recruitment of CIRBP to membraneless organelles named stress granules (SGs) upon certain stresses has been shown to repress translation [11].

CIRBP targets are mostly involved in stress responses, but also in development, translation and circadian clock. CIRBP activation thus affects a large panel of cellular pathways, such as cell survival, cell death, proliferation, differentiation, tumorigenesis, telomere maintenance, etc. [2] (Fig. 1D). Recent evidence also points CIRBP as a player in responses to viral and bacterial infections, with a rather pro-infectious role [16,29].

Independently of its RNA-binding characteristics, extracellular CIRBP can also act as damage-associated molecular pattern (DAMP) upon its release, and then as a sensor of sepsis or hemorrhagic shock, thereby playing an important role as a mediator of inflammatory responses in macrophages, neutrophils, lymphocytes and dendritic cells [30].

The large panel of stressors to which CIRBP responds and the multiple pathways it impacts require a fine-tuning of its expression and function depending on conditions (Fig. 2).

## 3. Quantitative and qualitative regulations of CIRBP transcripts

A number of signaling pathways impact CIRBP expression. It is inhibited by TGF- $\beta$  and TNF signaling in human and murine neurons [24], while the GSK3- $\beta$  kinase not only phosphorylates the CIRBP protein, but also increases CIRBP mRNA levels during the development of Alzheimer's disease [31]. CIRBP expression is also inhibited by intracellular calcium influx in U-2 OS human cells [32]. In contrast, upon hypothermia, CIRBP expression is induced *via* the transient receptor potential vanilloid 4 TRPV4 receptor, although in a mechanism independent of this receptor ion channel activity [32]. Similar results were recapitulated with receptors TRPV3 and 8 of the same

family [33]. CIRBP mRNA levels were also found upregulated in response to porcine reproductive and respiratory syndrome virus (PRRSV) [34], and by avian erythroblastosis virus E26 oncogene homolog-1 ETS-1 in Thp1 cells [35] as driver of inflammation.

### 3.1. Regulation by CIRBP transcription

The first evidence of CIRBP regulation at the transcription level were brought by the observation that CIRBP could be transcribed from two alternative transcription start sites (TSS) upon cold shock in murine neuronal cells [36]. The isoform with the longest 5'-UTR also harbored an internal ribosome entry site (IRES), possibly promoting CIRBP cap-independent translation in a context where overall cellular translation is diminished. Further studies showed that these isoforms were under the control of two promoters, bound by a different set of transcription factors in response to cold [37]. Another study detected the presence of a temperature sensitive element within the first intron of the canonical isoform, which allowed response to circadian temperature oscillations [28]. This study also showed that neither CIRBP pre-mRNA levels, nor RNA polymerase II occupancy on the CIRBP locus were affected by cold. This suggests a splicing-dependent regulation of CIRBP levels upon temperature variations. In HEK293T cells, another temperature-responsive element was identified in the 5' region upstream of the promoter, called mild-cold responsive element (MCRE) [38]. This GC-rich octa-nucleotide, found within 1,000 bp of the transcription start site, acts as a transcriptional enhancer responsive to Sp1 binding. While the interaction in normothermia and mild hypothermia conditions is low, it is enhanced upon cold stress, when Sp1 accumulates in the nucleus, thereby increasing CIRBP transcription.

Altogether, these results suggest diverse mechanisms of regulation of CIRBP transcription, through alternative promoters, transcription start sites, enhancers or other regulatory motifs (Fig. 2). Yet, since most studies were performed in the context of hypothermia, transcriptional regulation of CIRBP in the context of stressors that repress CIRBP expression, like heat shock, would deserve further investigation.

### 3.2. Alternative splicing in favor of the canonical CIRBP transcript increases its stability and protein production

Beyond usage of alternative TSSs as described above, alternative splicing events have also been shown to finely tune the representation of CIRBP mRNA isoforms and subsequent protein production (Fig. 2). Two CIRBP mRNA isoforms with different expression pattern were initially described in the context of circadian cycles [3] and hibernation [39]. In the hearts of hibernating animals, long isoforms of CIRBP that harbor a retained intron and a premature stop codon are predominantly expressed in euthermia, but are spliced in favor of a shorter, protein-coding isoform in hibernation condition. This splicing results in a rapid increase in CIRBP protein expression as part of the response to cold. Splicing is also involved in CIRBP expression regulation upon daily temperature oscillations, as addition of the splicing inhibiting drug spliceostatin A abolished CIRBP accumulation upon mild hypothermia [28].

Broadened use of high-depth RNA-sequencing resulted in more detailed insight into CIRBP splicing patterns in different conditions. In the context of viral infection of human Huh7 cells, m<sup>6</sup>A-RNA-immunoprecipitation followed by sequencing has revealed another splicing-dependent regulation of CIRBP [29], where an isoform encoding a 32-kDa version of CIRBP was spliced in favor of the shorter, canonical protein coding transcript. A methylation mark, placed in the coding sequence near the last exon-exon junction of the canonical isoform, was lost upon infection, resulting in reduced intron retention. This resulted in sustained expression of the short, canonical form of CIRBP despite general translation inhibition upon infection. A similar loss in methylation and effect on CIRBP AS was also observed upon Thapsigargin-induced endoplasmic reticulum (ER) stress [29].

### 3.3. Alternative splicing towards non-canonical CIRBP transcripts decreases their stability and protein expression

In the context of heat shock, where CIRBP overall expression is decreased [28], an opposite mechanism of regulation by AS has been recently described. High-resolution RNA-sequencing data has revealed that, upon hyperthermia (38°C and over), reduced CIRBP expression was most likely due to a preferential splicing of its pre-mRNAs into an unstable mRNA isoform instead of the canonical one [25]. The two isoforms encode almost identical proteins, but they diverge in their 3'-UTRs, in which the unstable isoform harbors an exon-exon junction, rendering it sensitive to degradation by NMD [25,40]. The reduced stability of this transcript results in decreased overall CIRBP mRNA and protein levels. This regulation of AS has been linked to the stress responsive kinase CLK1. Upon heat shock, inhibition of the activity of CLK1 leads to the dephosphorylation of serine-arginine-rich splicing factors (SRSFs). In turn, modulation of the activity of SRSFs leads to a switch in the splicing of many genes, including CIRBP.

A very similar regulation of CIRBP has recently been identified in the context of bacterial infection of human epithelial cells [16]. The invasive bacterium *Listeria monocytogenes* secretes a pore-forming toxin, listeriolysin O (LLO), the cytolytic activity of which inhibits CLK1 and SRSF phosphorylation, then leading to CIRBP isoform switch towards the above-mentioned NMD-sensitive transcript and to a subsequent decrease in overall CIRBP expression. The switch impacts the cell response to bacterial infection, as expression of the canonical isoform favors infection, while the unstable one appears to be anti-bacterial, although the limited difference in the sequence of the two protein products (4 C-terminal amino-acids are missing in the product of translation from the unstable isoform) does not in principle give much latitude for extensive functional divergence between the two protein isoforms. Coincidentally, a shift in CIRBP-bound targets from housekeeping genes to stress-response targets was observed upon *Listeria* infection, in line with CIRBP contributing to cellular responses to infection-induced stress.

In cervical and uterine cancer cells, AS of CIRBP affecting its polyadenylation site in favor of less stable isoforms with shorter 3'-UTRs, and subsequent decrease in protein expression, negatively impact the prognosis of patients [41].

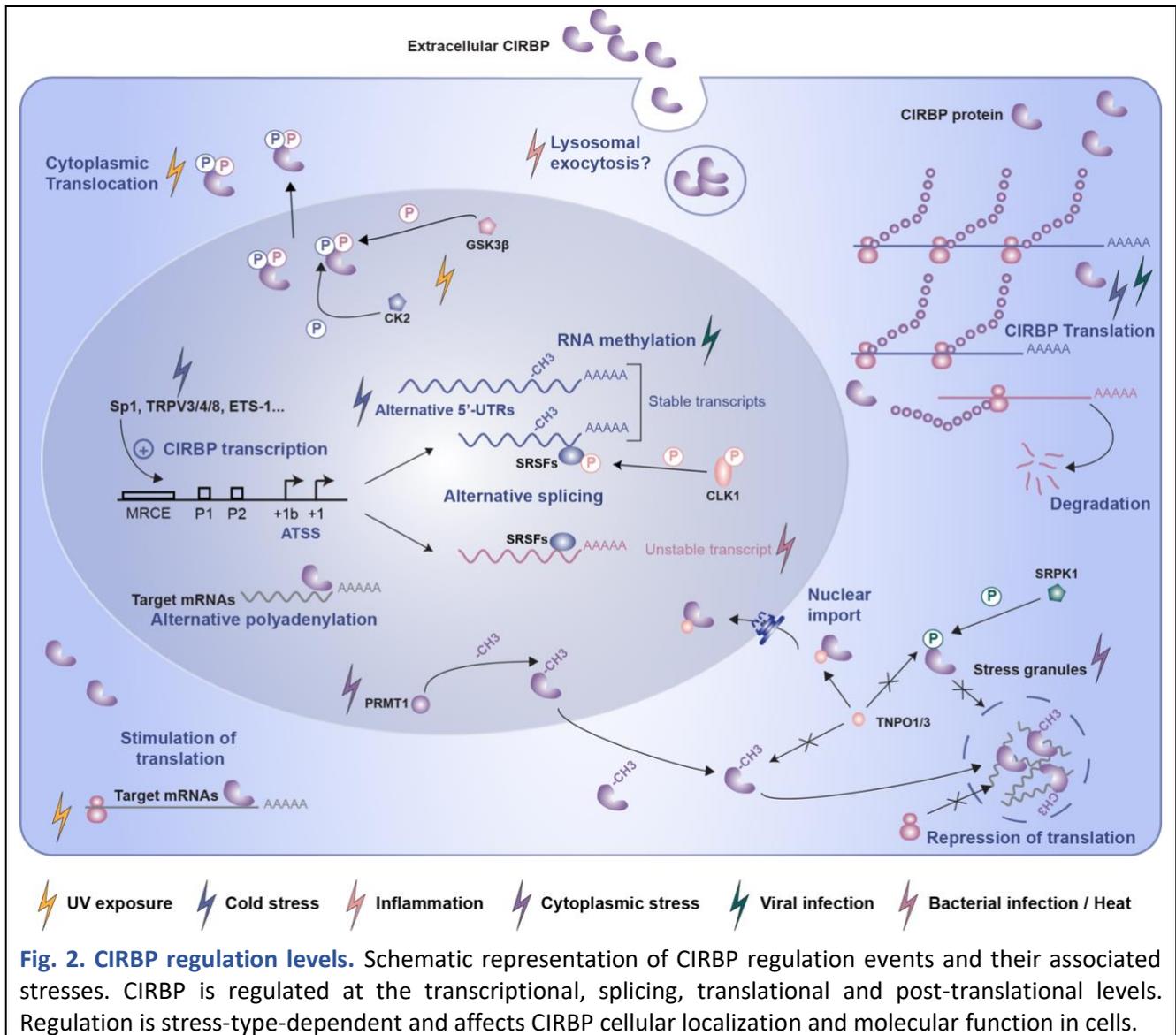
Alternative splicing of CIRBP thus allows a fine tuning of its expression, mostly centered around the intertwining of the production of the canonical isoform with that of other isoforms of different stability and/or coding potential (Fig. 2). In response to opposite types of stresses (cold *versus* heat, or high *versus* low circulating glucose levels [26]), fine-tuned modulation of CIRBP splicing and expression can craft an appropriate response for each condition.

## 4. Subcellular localization of CIRBP governs its functions

### 4.1. Intracellular CIRBP is decorated by post-translational modifications

The canonical CIRBP protein has been shown to be the target of several post-translational modifications, mostly affecting its cellular localization and subsequent function (Fig. 2). The homolog of CIRBP in *Xenopus*, xCIRBP, can interact *in vitro* with the arginine methyltransferase xPRMT1 [18]. Further experiments have shown that, in HeLa cells overexpressing xPRMT1 and transfected with xCIRBP, the latter was methylated in its RGG domain by xPRMT1, which induced its translocation from the nucleus to the cytoplasm. The methylation-dependent shuttling of CIRBP towards the cytoplasm has since been observed in other cells lines (Simian COS, human HeLa and murine NIH3T3) upon heat shock, oxidative, osmotic or ER stress [11], and is enhanced upon PRMT1 overexpression [18]. In these stress conditions, following relocalization of methylated CIRBP to the cytoplasm, it is targeted to SGs, which act as mRNA-sequestering platform upon certain stresses. This relocalization requires an intact RRM, suggesting that it is dependent on CIRBP RNA-binding properties [11]. When recruited to SGs, CIRBP acts as a translational repressor *via* the interaction of

its unstructured RGG region, but not its RRM, with sequestered mRNAs. As mentioned above, this repressive function inside SGs contrasts with the previously-described role of CIRBP as facilitating the translation of its target mRNAs in the cytoplasm [9], emphasizing the strong impact of CIRBP localization on its function.



Detailed investigation of CIRBP nucleo-cytoplasmic shuttling has revealed that its nuclear localization is dictated by the interaction between its RGG and RSY domains with the transportins TNPO1 and TNPO3 respectively [19]. Through the disruption of CIRBP liquid-liquid phase separation (LLPS) in presence of RNA, the transportins outcompete the recruitment of CIRBP to SGs. When CIRBP is methylated in its RGG domain, in contrast, the transportins, and especially TNPO1, dissociate, which opposes nuclear import, LLPS and recruitment to SGs. A following study showed that ATP binding to CIRBP was also able to disrupt LLPS and recruitment to SGs, probably by competing with RNA binding to the RGG [42]. Recently, the SRPK1 kinase was shown to phosphorylate CIRBP on the RGG domain, thereby impeding CIRBP LLPS *in vitro* and its recruitment to SGs in HeLa cells, maybe by competing with PRMT1 for CIRBP availability [43]. Moreover, CIRBP phosphorylation by SRPK1 also impedes its recruitment by TNPO1.

The control of CIRBP localization by phosphorylation is substantiated by other studies. Indeed, in human RKO cells exposed to UV light, CIRBP is phosphorylated in its RGG, first by the CK2 kinase

and then, on multiple sites by the GSK3 $\beta$  kinase [10]. These modifications induce its translocation from the nucleus to the cytoplasm, where it interacts with many target mRNAs, including that of the genotoxic stress associated kinase ATR, and increases its expression. Phosphorylation of CIRBP by GSK3 $\beta$  upon UV exposure additionally impacts its binding activity to targets such as thioredoxin (TRX) [9]. Interestingly, although SGs are formed upon UV exposure, CIRBP is not recruited to these compartments in this context [13]. Neither is it translocated to the cytoplasm in response to mild hypothermia [11], emphasizing yet again the versatility of CIRBP regulation mechanisms as a means to adapt its function to various stresses. Various redundant or complementary mechanisms are thus deployed, in an intricate network of regulations, to adjust the sub-cellular localization of CIRBP and thereby tightly control its function within the cells (Fig. 2).

#### 4.2. Extracellular CIRBP

In addition to its intracellular role, CIRBP has been characterized as a DAMP that is translocated to the extracellular space in patients and murine models undergoing hemorrhagic shock or sepsis, and in macrophages under hypoxic stress [44] (Fig. 2). How exactly CIRBP is translocated from the cytoplasm to the extracellular space remains unclear, but as CIRBP lacks a signal peptide, its translocation through the canonical ER-Golgi route has been ruled out. Cell fractionation experiments detected CIRBP enriched in the lysosomal fraction, indicating that CIRBP could be released through lysosomal exocytosis. Another hypothesis suggests that cell necrosis releases CIRBP in the circulation where it can bind as a DAMP to the TLR4 receptors of neighbor cell and induce the release of inflammatory cytokines through activation of the NF- $\kappa$ B pathway, of the inflammasome through NLRP3, or through NETosis [30]. After its release, extracellular CIRBP is mostly involved in organizing inflammatory responses.

#### 5. Conclusions and future challenges

CIRBP regulation is multileveled, tissue-, cell type- and species-dependent, with sometimes opposed roles liable to a specific context. Although large parts of its regulatory pathways have been deciphered in various stress conditions, the precise molecular mechanism that govern CIRBP regulation at the transcript, protein or cellular localization level in a given context are still incomplete. The rise in the use of long-read sequencing technologies should help to clarify the diverse AS events affecting CIRBP expression and to pinpoint their consequences on transcript stability and protein expression. They could also shed light on apparent contradictory functions of CIRBP in distinct contexts, in case they depend on the expression of alternative isoforms.

Another argument in favor of systematically reviewing CIRBP expression patterns in any given condition also lies in its therapeutic potential. Indeed, it is already thoroughly described as a key factor in the development of many cancers [2], inflammatory diseases [6], degenerative pathologies [31], in responding to viral [29] and bacterial [16] infections, and more. Its role in the development of cancers is dual; while CIRBP is mostly described as a strong oncogene relevant in aggressive prognosis for melanoma, breast, colon and prostate cancers as well as in the induction of metastasis [2], it can also act as a tumor suppressor in other pathological conditions, such as gynecological and nasopharyngeal cancers [45,46]. Enhancing CIRBP expression through so-called therapeutic hypothermia has launched promising anti-apoptotic effects resulting in protection against organ injury in animal models of ischemia and cardiac failure [47,48]. Some strategies, like CIRBP neutralizing antibodies [49], the use of the small CIRBP-derived peptide C23 [50], or using CIRBP as a vaccination platform [51] already yield promising results in treating hepatic ischemia, sepsis or tumoral targeting. Given the utmost significance of this factor in disease outcome, research on its regulation represents a tempting track of development relevant to public health.

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## Declaration of competing interest

The authors declare that they have no conflict of interest.

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