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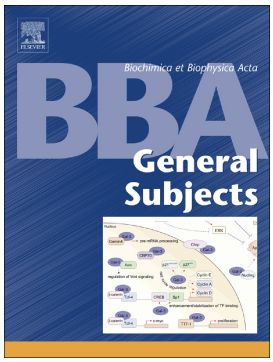

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What's new about CNBP? Divergent functions and activities for a conserved nucleic acid binding protein.

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ABSTRACT

Background: Cellular nucleic acid binding protein (CNBP) is a conserved single-stranded nucleic acid binding protein present in most eukaryotes, but not in plants. Expansions in the *CNBP* gene cause myotonic dystrophy type 2. Initially reported as a transcriptional regulator, CNBP was then also identified acting as a translational regulator.

Scope of review: The focus of this review was to link the CNBP structural features and newly reported biochemical activities with the recently described biological functions, in the context of its pathological significance.

Major conclusions: Several post-translational modifications affect CNBP subcellular localization and activity. CNBP participates in the transcriptional and translational regulation of a wide range of genes by remodeling single-stranded nucleic acid secondary structures and/or by modulating the activity of trans-

actin n cell proliferation control. Besides, CNBP has been linked with neurodegenerative, inflammatory, and congenital diseases, as well as with tumor processes.

General significance: This review provides an insight into the growing functions of CNBP in cell biology. A unique and robust mechanistic or biochemical connection among these roles has yet not been elucidated. However, the ability of CNBP to dynamically integrate signaling pathways and to act as nucleic acid chaperone may explain most of the roles and functions identified so far.

KEYWORDS: G-QUADRUPLEX, NUCLEIC ACID CHAPERONE, VIRAL INFECTION, INFLAMMATORY RESPONSE, CELL PROLIFERATION, CRANIOFACIAL DEVELOPMENT.

ABBREVIATIONS: AD, Alzheimer disease; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; APP, amyloid precursor protein, *Bvht*, *Braveheart*; CCHC, Cys-Cys-His-Cys; Ccl, chemokine (C-C motif) ligand 3; CK1, casein kinase I; CNBP, cellular nucleic acid binding protein; DM2, myotonic dystrophy type 2; eEF1A, eukaryotic elongation factor 1A; eEF2, eukaryotic elongation factor 2; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; G4, G-quadruplex; Gis2p, gluconeogenic growth suppressor 2 protein; HMGCR, hydroxymethylglutaryl-coenzyme A reductase; *HuR,* human antigen R (also named *ELAV like RNA binding protein 1 or ELAVL1*); IFN, interferon; Il, interleukin; IRES, internal ribosomal entry site; JNK, c-Jun N-terminal kinase ; LAST, lncRNA Assisted Transcription Stabilization; lncRNA, long non-coding RNA; LPS, lipopolysaccharide; MBNL, muscle blind-like; *mmp*, matrix metalloproteinases; mRNP, messenger RNP; NC, neural crest; NCp, nucleocapsid protein; NHE III_1, nuclease hypersensitive element III_1; NM23-H2, Nucleoside diphosphate kinase B; ODC, ornithine decarboxylase; PABP1, Poly(A)-binding protein 1; PDAC, pancreatic ductal adenocarcinoma; P-bodies, processing bodies; PEST, Pro, Glu, Ser or Thr-enriched; *PGM1*, Phosphoglucomutase 1 gene; PKB (also named AKT), protein kinase B; PKC,

prote ctive oxidative species; SG, stress granules, SHH, sonic hedgehog; sIBM, sporadic inclusion body myositis; SLE, systemic lupus erythematosus; ssDNA, single-stranded DNA; Sufu, suppressor of fused; TAK1, tat-associated kinase 1; TCS, Treacher Collins Syndrome; TLR, toll-like receptor; TOP, terminal oligopyrimidine tract; ZNF9, zinc-finger protein 9.

1. INTRODUCTION

Cellular nucleic acid-binding protein (CNBP, also named ZNF9) was originally identified as a small human single-stranded nucleic acid-binding protein in an expression library screening with a sterol regulatory element from the hydroxymethylglutaryl-coenzyme A reductase (HMGCR) promoter [1]. More than thirty years after its discovery, numerous studies have shown that CNBP is exclusively present in eukaryotes. Apart from metazoans, CNBP orthologues have been identified in fungi [2–4] and in protozoans [5], but no CNBP orthologues have been described in plants [6].

CNBP is a strikingly conserved protein mainly formed by zinc knuckles (tiny zinc fingers) of the CCHC (Cys-Cys-His-Cys)-type. CNBP binds to both single-stranded DNA (ssDNA) and RNA molecules; therefore, it has been classified as a DNA- and RNA-binding protein [7]. Although much progress has been made in understanding the structure, biochemical activity, and functions of CNBP, many aspects remain yet to be elucidated. A review about the main CNBP structural features, biochemical activities, and biological functions was published in 2010 [8]. For this reason, here we mostly review the knowledge generated over the last decade. We focus our efforts on linking the newly CNBP described functions with the reported structural features and biochemical activities. We also discuss the biological and pathological significance of the CNBP roles.

2. *CNBP structure and isoforms*

and fungi was found [9]. Regardless of the protein taxonomic origin, all CNBP orthologous contain six to seven tandem CCHC-type 14-amino acids zinc knuckle motifs (Figure 1A) [6,10]. The CCHC zinc knuckles present in CNBP are remarkably similar to those ones found in the human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein (NCp), being most of them capable of replacing structurally and functionally the HIV1-NCp CCHC domains [11]. In the linker joining the first and second CCHC zinc knuckles, vertebrate CNBP contains an Arg/Gly (RG)-rich motif (Figure 1A) required for proper CNBP nucleic acid binding and chaperone activities [12]. RG-rich motifs have been proposed as a Novel Interesting Quadruplex Interaction motif useful for predicting G-quadruplex (G4) binding proteins [13]. This finding, along with experimental biochemical evidence described below (see Sections 5 to 7), allowed to classify CNBP as a G4 binding protein [14].

Analyses of cDNA sequences revealed the presence of two alternatively spliced products, *CNBPa* and *CNBPb*, in mammals [15] and *X. laevis* [16,17]. The *X. laevis* "b" isoform is slightly different from the mammalian "b" isoform due to distinctive alternative splicing events. A unique isoform similar to mammalian "b" isoform was found in *Gallus gallus* [18], and a single shorter and slightly different isoform in fish [9,19]. The pre-mRNA coding for human CNBP contains alternative splice sites in close proximity (2–12 nucleotides distance), also called 'tandem alternative splice sites' (TASS) [20]. The presence of TASS might explain the existence of transcript variants, either a long or short isoform depending on which splice site is used, as well as protein isoforms with subtle differences of just few amino acids [20], but potentially displaying structural and functional differences [9]. The expression of six different isoforms was reported in HEK293 human cells. CNBP isoform 3, which lacks the third CCHC motif, and isoform 6, which contains deletions in the third CCHC motif and the C-terminus of the RG-rich motif, are the most abundant in this cell line [21]. Eight described isoforms and one potential isoform computationally mapped can be retrieved from the Uniprot database (https://www.uniprot.org/uniprot/P62633#P62633-1, accessed on 12 July 2021). So far, neither structural nor functional differences have been reported among CNBP isoforms

prod along with the RG-rich motif, probably generated by the proteolysis of the full-length "a" isoform (see below and Figure 1A), shows differential biochemical and biological behavior. A deletion mutant mimicking the shorter-proteolytic isoform fails to bind *in vitro* to RNA and, although it binds *in vitro* to ssDNAs with lower affinity, is unable to act as nucleic acid chaperone [12]. Noteworthy, CNBP deletion mutants lacking either the RG-rich motif or the N-terminal CCHC motif plus the RG-rich motif act as dominant negatives in *Xenopus* developing embryos, abrogating normal neural crest development [12]. Besides, the total or partial lack of the RG-rich motif in human CNBP abolishes sustained transcriptional activation of inflammatory genes [22].

Most of the knowledge on the biochemical and biophysical properties of CNBP come from experiments performed with recombinant CNBP fused to different protein tags. The use of tags is certainly advantageous for purifying recombinant proteins; however, the presence of such tags might affect their structure and/or function [23]. To avoid this, a simple method for CNBP purification that yields good amounts of homogeneously pure, fully active, and tag-free CNBP was settled [24]. Size-exclusion high-performance liquid chromatography revealed that tag-free CNBP forms homodimers independently of nucleic acid binding, which coexist with monomers as non-interconvertible forms or in slow equilibrium. According to circular dichroism spectroscopy, CNBP has a secondary structure dominated by random-coil and β-sheet coincident with the sequence-predicted repetitive zinc knuckles motifs. CNBP structural stability increases in the presence of single-stranded nucleic acid targets, in a similar fashion as other nucleic acid chaperones. Based on this data, CNBP was suggested as an intrinsically disordered and flexible protein with interspersed structured zinc knuckles that acquires a more rigid structure upon the nucleic acid binding [24].

3. *CNBP post-translational modifications*

The ; are summarized in Figure 1B and briefly described below.

3.1. Methylation: Boisvert et al. [25] firstly identified CNBP as a putative symmetric dimethylarginine containing protein in an immuno-purification study using a specific antibody for methylarginine protein complexes. Lately, methylated CNBP was found in a large-scale identification of protein Arg methylation screening carried out in T cells [26]. Differentially methylated CNBP can be selectively recognized by antibodies against spliceosome Sm proteins (anti-Sm autoantibodies) specific to the autoimmune disease systemic lupus erythematosus (SLE). CNBP thus was suggested as a novel autoantigen recognized by the Sm-positive SLE patients [27]. In HeLa cells, CNBP is specifically methylated in the RG-rich motif (Figure 1A) by protein Arg methyltransferase 1. Inhibition of methylation does not affect the CNBP nuclear localization; however, it obstructs the binding to RNA targets [28]. This finding raises interesting questions regarding the methylation effect on location and substrate availability (Figure 1B, Methylation branch).

3.2. Proteolysis: CNBP contains a putative PEST (Pro, Glu, Ser or Thr-enriched) proteolysis region adjacent to the RG-rich motif (Figure 1A and 1B, Proteolysis branch). Both, the removal of the RG-rich motif and the PEST region [12], and the partial or total deletion of the RG-rich motif [22], yield a shorter protein lacking most of the biochemical features. The proteolysis product is usually detected in Western blot assays; however, its existence *in vivo* and biological relevance have not been completely addressed yet.

3.3. Oxidation: Cys is one of the rarest amino acids present in proteins and is involved in a plethora of chemical reactions and complex mechanisms [29]. CNBP contains 10-times more Cys residues than the average polypeptide submitted to SWISS-PROT Data Bank. The aromatic/basic microenvironment surrounding the groups of three Cys in each zinc knuckle could raise their sensitivity to reactive oxidative species (ROS). CNBP emerged among the more oxidized proteins in a redox proteomic assay that identified proteins whose level of oxidation is different in two cancer cell lines [30]. Remarkably, Cnbp depletion in developing zebrafish causes a significant increase of ROS [31]. Zebrafish embryos incubated in the presence of H_2O_2 show lower amounts of Cnbp, which is degraded through the proteasome pathway [31].

These during embryonic development (Figure 1B, Oxidation branch) and, perhaps, also in carcinogenesis.

3.4. Ubiquitination: A putative ubiquitination site is located at the conserved Lys 77 of human CNBP (Figures 1A and 1B, Ubiquitination branch). CNBP ubiquitination leads to degradation, which is abrogated by the presence of the proteasome inhibitor MG132 [31,32]. CNBP ubiquitination and proteasome degradation is prevented by binding to Sufu (suppressor of fused), a component of the sonic hedgehog (SHH)/patched signaling pathway [32]. As described immediately below, CNBP phosphorylation boosts CNBP-Sufu interaction.

3.5. Phosphorylation: The C-terminal of the seventh CCHC zinc knuckle holds a conserved Ser/Thr phosphorylation site (Figure 1A). During zebrafish embryonic development, Cnbp is differentially phosphorylated by protein kinase A. While CNBP phosphorylation does not change nucleic acid binding capability, it promotes *in vitro* the CNBP nucleic acid chaperone activity [33]. In medulloblastoma cells, CNBP is phosphorylated by AMP-activated protein kinase (AMPK) in response to SHH stimulation. Phosphorylation increases association with the protein Sufu and abrogates proteasome-mediated CNBP degradation [32]. CNBP stimulates the synthesis of ornithine decarboxylase (ODC) by direct binding to the internal ribosome entry site sequence (IRES) within the 5' UTR of ODC mRNA [34,35]. Therefore CNBP phosphorylation eventually enhances ODC translation, with the consequent increase of polyamines synthesis [32] (Figure 1B, Phosphorylation/Ubiquitination branches). On the other hand, after lipopolysaccharide (LPS) stimulation in macrophages, CNBP phosphorylation by casein kinase I (CK1), tat-associated kinase 1 (TAK1), and protein kinase C (PKC) promotes CNBP nuclear translocation and dimerization [22], which subsequently induces immune response activation (see below, Sections 5, 6, and 8). CNBP phosphorylation by TAK1, induced by RNA virus infections, promotes CNBP nuclear translocation [36]. In murine lung epithelial carcinoma cells, the activation of PKC, extracellular signal-regulated kinase

(ERK) actor (EGF) stimulation leads to CNBP phosphorylation and nuclear translocation [37].

4. *CNBP subcellular localization*

Data discussed below support the notion that CNBP is located in both the nucleus and the cytoplasm, wherein it may also be part of large RNA-protein granules.

4.1. *CNBP localizes both in the nucleus and the cytoplasm.*

CNBP was identified 30 years ago as a nuclear protein involved in transcription regulation by binding to ssDNA in the nuclei of human liver hepatocellular carcinoma (HepG2) cells [1]. The significant amount of evidence suggesting that CNBP participates in transcriptional control [1,14,15,22,31,36,38–51] reinforces the notion that CNBP is a nuclear protein. The nuclear localization was indeed detected by Western blot performed on nuclear extracts from mouse macrophages, splenocytes and lung epithelial carcinoma cells [22,36,37,45], as well as by immunolocalization in human [1,27,28,40,42,44,48] and mouse [22,45,49] cultured cells, fish gonads [19,52], and mice [38], fish [9,53], and amphibian embryonic cells [54].

Besides, a role of CNBP in translational regulation has been well-documented [2,4,21,28,32,34,35,55–65], which suggests a cytoplasmic CNBP localization. Actually, CNBP was detected by Western blot in the cytoplasm of human HEK293 [21] and HeLa cells [14,28], as well as by immunolocalization in mouse macrophages, splenocytes and lung epithelial carcinoma cells [22,37,45], amphibian and fish somatic and germ cells [9,19,52,54], and fish and amphibian embryonic cells [9,53,54]. Particularly during amphibian and fish early embryogenesis, CNBP is maternally inherited and stored in blastomeres mainly as a cytoplasmic protein; at later developmental stages, CNBP is also detected in the nucleus of embryonic cells [9,19,53,54]. The CNBP mutant isoform lacking the first CCHC and the RG-rich motif also shows dual subcellular localization [53], thus suggesting that the motif responsible for subcellular translocation is located downstream the RG-rich motif. A nuclear localization sequence has been identified *in silico* [9]

(Figu ation after phosphorylation [22]. Therefore, CNBP translocation from the cytosol to the nucleus, at least in murine cells, appears to be mainly the consequence of regulated phosphorylation induced by LPS [22] and EGF [37] stimuli, or by RNA viral infection [36].

4.2. *CNBP is a component of stress granules*.

Processing bodies (P-bodies) and stress granules (SG) are ribonucleoprotein (RNP) structures involved in the storage of particular mRNAs during periods of stress and the maintenance of protein homeostasis [66]. CNBP localizes in SG upon arsenite treatment of HeLa cells, but depletion of CNBP does not affect SG formation. The gluconeogenic growth suppressor 2 protein (Gis2p, the yeast CNBP orthologue) was localized both in P-bodies and SG, which contain messenger RNPs (mRNPs) stalled at a step prior to 60S subunit joining [67,68]. In view of this, CNBP was suggested interacting with mRNAs during translation initiation and contributing to the translational repression of some mRNAs during stress [69].

5. *CNBP consensus binding sites*

The CNBP preferential DNA binding site was firstly defined based on the octanucleotide sequence GTG(G/C)GGTG present in the promoter region of the genes coding for the low density lipoprotein receptor and the HMGCR proteins [1]. Later, a systematic *in vitro* binding analysis of oligonucleotide representing sequences found in reported CNBP targets showed that the preferred binding motifs are single-stranded nucleic acids containing G-tracts constrained by an organized sequence environment [70]. In agreement, a yeast one-hybrid assay using mouse and zebrafish genomic DNA libraries revealed that Cnbp preferentially binds to G-rich DNA sequences displaying the GAGGGGGAGGGGGG 14-nucleotide consensus [39]. The knowledge of this consensus sequence drove the prediction of several novel CNBP

putat [14]

and *in vivo* in developing zebrafish [14,39,41].

Similar to that formerly reported [39], pull-down assays using mouse genomic DNA followed by computational analyses yielded a 26-nucleotide G/A rich consensus sequence [22]. Pull-down assays also allowed to identify two additional consensus binding sequences (AAATGAGA and CTGAAAAT) into promoter regions of a subset of immune-related genes [22]. CNBP was then found binding to the CTGAAAAT consensus sequence present in the promoters of some mouse tumor-related genes [37] and to the AAATGAGA consensus sequence located into the promoter of the human antigen R gene (*HuR,* also named *ELAV like RNA binding protein 1 or ELAVL1*) [47].

Apart from binding to ssDNA, CNBP was also described as an RNA binding protein participating in the translational control by binding to mRNAs [2,57,58] into their 5' UTRs [9,28,55,56,61–64,70], IRES [2,32,34,35,59,61], coding regions [21], and 3' UTRs [4,21], as well as binding to rRNAs [71], long non-coding RNAs [60,72,73], circular RNAs [47], and positive-sense (+gRNA) and negative-sense (-gRNA) RNA strands of SARS-CoV-2 genome [74,75]. The first CNBP RNA-consensus binding sequence was informed for the yeast orthologue Gis2p as a sequence composed of 14 GAN trinucleotide repeats, wherein N refers to any of the four nucleotides [57]. Later, by using different approaches, both the UGGAGAAC [21] and GGAGGAG [76] sequences were defined as the major human CNBP consensus RNA-binding sequences.

Despite the different approaches and the variations in the lengths of the identified CNBP consensus sequences, data indicate that most of the CNBP binding sequences share the characteristic of containing G-enriched motifs. Figure 2 summarizes the CNBP-binding ssDNA and RNA consensus sequences reported so far.

6. *CNBP regulates the expression of a wide range of genes*

Many have been reported and extensively revised previously [8]. Therefore, here we mainly describe the CNBP target genes found in the last decade, as well as the novel data achieved on previously reported targets.

6.1. *CNBP targets for transcriptional regulation*

CNBP was reported as a negative and positive transcriptional regulator of several genes (Table 1) displaying divergent and apparently unrelated functions [8]. By using a CNBP consensus binding ssDNA sequence [39], novel targets of transcriptional regulation were identified, and a role of CNBP in transcriptional regulation was demonstrated both *in vitro* and *in vivo* in zebrafish embryonic development. Cnbp up-regulates *tbx2b* and *smarca5*, *tcf7l2* (formerly *tcf4*), *ptk7*, and *cdk14* (formerly *pftk1*) [39][39,41], while it down-regulates *wnt5b* [39]. *Wnt5b, tcf7l2*, *ptk7*, and *cdk14* are involved in Wnt signaling pathways, which is crucial in multiple steps of cranial neural crest formation and craniofacial cartilage development [77]. Furthermore, CNBP represses the transcription of human *NOG* and zebrafish *nog3* orthologous genes [14], which are required for proper craniofacial development [78,79]. Therefore, it was suggested that CNBP plays a role in modulating the expression of genes involved in the neural crest formation and craniofacial development, with possible consequences on the 'fine tuning' of rostral head development [14,41].

On the other hand, CNBP was reported participating in the transcriptional activation of murine immune-related genes. CNBP induces the transcription of sustained inflammatory cytokines including *interleukin* (Il)-*1b*, *Il-6*, *Il-12b*, and *Il-15*, as well *as chemokine (C-C motif) ligand 3* (*Ccl3*), *Ccl4*, *Ccl5*, *Ccl7*, *Ccl9*, and *Ccl22* [22]. Besides, CNBP activates its own expression in response to LPS stimulus by persistently binding to its own promoter, producing a positive feedback mechanism of autoregulation. Hence, robust levels of CNBP induce maximal transcription of interleukin during a prolonged LPS stimulation [22]. Since CNBP also induces *Il-12b* transcription in response to diverse microbial pathogens through interaction with c-REL transcription factor [45], CNBP has emerged as a key transcriptional regulator required for activating and maintaining the innate immune response [22,45]. In the same direction, CNBP activates the transcription

of ty[illegible]n the promoter regions and with the IFN regulatory factor (IRF) 3 (and probably IRF7) in a synergistic response to RNA viruses infections [36].

CNBP participates in the transcriptional regulation of genes involved in different oncogenic and tumorigenic processes as well. CNBP enhances the transcription of the proto-oncogene *c-MYC* through binding to the G-rich strand of the nuclease hypersensitive element III_1 (NHE III_1) located upstream the P1 promoter [14,38,40,46,48,51]. It was suggested that CNBP binds to Nucleoside diphosphate kinase B (NM23-H2) and boosts *c-MYC* transcription by interacting with the transcriptional silencing G4 formed within NHE III_1 [40]. *c-MYC* transcriptional regulation involves the interaction of the NHE III_1 with other proteins, such as SP1, Nucleolin and hnRNP K, which, along with CNBP and NM23-H2, make up a complex regulatory network [14,40,46]. In addition to this, CNBP was reported activating the transcription of *KRAS* oncogene in HeLa cells [14]. Conversely, CNBP down-regulates the transcription of both the *hnRNP K* gene in fibrosarcoma cells [42,43] and the Phosphoglucomutase 1 gene (*PGM1*) in the context of hepatocellular carcinoma [44].

As mentioned above, CNBP was reported participating in the complex transcriptional regulation of the *HuR* gene, involved in gastric cancer progression. CNBP activates *HuR* transcription by binding to the promoter region, but this activation is prevented by the binding of circular RNA *circ-HuR* to CNBP. In gastric cancer tissues and cells, *circ-HuR* levels are reduced, thus enabling transcriptional activation of *HuR* mediated by CNBP [47]. Finally, CNBP activates the transcription of other genes associated with tumorigenesis, such as the murine matrix metalloproteinases (*Mmp*)*-2*, *Mmp-14*, and transcription factor *E2f2* genes after EGF stimulation [37].

In summary, CNBP is involved in the transcriptional regulation of genes linked to the cranial neural crest development, mouse inflammatory innate immune response, and oncogenic processes.

CNBP was initially described as a translational regulator of mRNAs containing 5' terminal oligopyrimidine tracts (TOP-mRNAs) encoding ribosomal proteins and translational elongation factors [55,56,62–64,69,80]. IRES mediated translational activation of ODC by CNBP has also been reported [2,32,34,35,61]. In *Drosophila melanogaster*, dCNBP activates the translation of both *dMyc* through an IRES-dependent mechanism [59] and *dOdc* by binding to its coding region [61]. CNBP was also suggested as a translational regulator of RAS protein by binding to human *HRAS* mRNA [58]. CNBP was found promoting the binding of the long non-coding RNA (lncRNA) Assisted Transcription Stabilization (LAST) to the 5' UTR of LAST-target mRNAs, thus leading to an increase of the stability and translation of such transcripts [60]. Finally, a photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation experimental approach identified more than 4000 mRNAs containing CNBP binding sites [21]. Functional analyses, including RNA sequencing, ribosome profiling, and quantitative mass spectrometry, revealed that CNBP binding does not influence the abundance of the target mRNAs, but instead increases the translational efficiency, likely by resolving stable structures folded on target mRNAs [4,21].

Collectively, acting as an RNA binding protein, CNBP is capable of modulating the synthesis of different proteins, probably through increasing mRNA stability and/or translational efficiency by resolving RNA stable structures. A summary of the translational targets of CNBP described so far is compiled in Table 1.

7. *CNBP biochemical activities*

As described above, CNBP binds specific ssDNA and RNA sequences and acts as a transcriptional and/or translational regulator (Table 1). The following paragraphs deal with how CNBP fulfils these roles over its many targets.

7.1. *CNBP functions as a nucleic acid chaperone*

The f CNBP to the 5' UTR of TOP-mRNAs leads to the stabilization of a translational-repressive closed structure [64,81]. More recently, CNBP was reported acting as nucleic acid chaperone by promoting the rearrangements of nucleic acid secondary structure in an ATP-independent manner. Through this activity, CNBP may control transcription and/or translation by chromatin or RNA structural remodeling, eventually modulating the action of specific trans factors [12,70].

7.2. *CNBP remodels G-quadruplex structures*

Single-stranded sequences recognized by CNBP, either ssDNA or RNA, can adopt secondary structures, including non-canonical structures such as the G4 [39,70]. G4s are tetrahelical nucleic acid secondary structures formed in G-rich single stranded sequences. The core structural element is the stacking of at least two G-tetrads formed by the association of four guanines through Hoogsteen hydrogen bonding. Intramolecular G4s have been described affecting gene expression at both the transcriptional and post-transcriptional level [82].

Acting on G4 DNA, a few works support that CNBP favors the G4 folding in some promoters [40,42,43]. However, siRNA CNBP-depleted cells display a higher number of nuclear G4s *foci*, suggesting that CNBP is a global G4 unfolder potentially able to affect multiple G4-dependent nuclear processes [14]. Reinforcing this notion, CNBP was recently found as one of the G4-DNA binding sensitizers in an unbiased genome-wide study to systematically identify human genes promoting cell death (G4-sensitizers genes) when silenced by shRNA in the presence of small molecules that stabilize G4s [83]. Additionally, CNBP was found as one of the proteins that binds parallel and antiparallel G4s in a pull-down approach to identify cellular proteins that bind constrained DNA G4s with single well-defined topologies [84]. In connection with this, CNBP unfolds, *in vitro* and in an ATP-independent fashion, a tetramolecular G4 and the G4s present in the proximal promoter region (PPR) of several human oncogenes [14]. This activity may explain the transcriptional activation of *c-MYC* and *KRAS* by the unfolding of the repressive G4 located into the

respe ional activation. Indeed, *in vivo* studies using zebrafish embryos and larvae suggest that Cnbp plays a role in craniofacial development by repressing the transcription of *NOG/nog3* through its G4-unfolding activity [14].

All the evidence gathered so far agrees that CNBP unfolds G4 RNA. In the case of mRNAs, CNBP binds and resolves G4s that function as roadblocks for the ribosomes, thus enhancing translation [21]. Besides, CNBP binds and promotes the unfolding of G4s formed by both strands of SARS-CoV-2 RNA genome, probably regulating the translation of viral proteins and/or the transcription and replication processes catalyzed by the viral RNA dependent RNA polymerase [75]. CNBP also interacts with *Braveheart* (*Bvht*), a lncRNA required for proper cardiovascular lineage commitment, by binding to a 5' asymmetric G-rich internal loop able to form a G4 structure [72]. *Bvht* lncRNA 3D flexible structure is remodeled upon CNBP interaction, probably regulating its function over target chromatin [72]. In addition, CNBP binds to expansion segments of large subunit rRNA that forms G4 structures, but no functions were described for this interaction so far [71].

CNBP G4-unfolding activity is driven by (i) a higher binding affinity to unfolded G-rich sequences than to folded G4, and (ii) a direct unwinding activity on G4 core [14]. Custom DNA microarrays used to examine the binding specificities of proteins across ~15000 potential G4 structures showed that CNBP, together with the well-known G4 helicase PIF1, binds G4-DNA. However, the abrogation of G4 formation did not inhibit the binding of these proteins to the same sequences. These data suggest that the binding affinity for G-rich sequences of multiple conformations is a putative mechanism for binding genomic regions undergoing transitions in DNA conformation [85].

In summary, there is strong evidence showing that CNBP functions as a nucleic acid chaperone capable of unfolding G4 structures. Likely, through this activity, CNBP regulates different cellular processes, including transcription and translation (Figure 3). In addition, CNBP can interact with other proteins, thus defining a complex interactome (Figure 4) that potentially impacts gene expression at different regulatory levels.

8. *CNBP and human diseases*

The varied range of target genes and cellular and biological functions attributed to CNBP would be questioned if this protein had not been related to pathologies derived from the alteration of such functions. In Table 2 and the following paragraphs we present an update of the latest publications that linked CNBP to some human diseases.

8.1. *Neuromuscular degenerative diseases*

The *CNBP* gene has been largely associated with the myotonic dystrophy type 2 (DM2) condition. An expansion of CCTG repeats in intron 1 of the human *CNBP* gene causes DM2 [86]. Even though this association was reported almost twenty years ago, little is known about the molecular mechanisms of muscle atrophy in DM2. A current hypothesis states that transcribed CCUG-repeats sequester alternative splicing proteins among which are muscle blind-like (MBNL) proteins [87]. A decrease in MBNL1 levels results in splicing defects of more than 100 pre-mRNAs, leading to the disease muscular symptoms, which include myotonia, muscle weakness, muscle/joint pain/stiffness, but not mental retardation [88]. In addition, the CCUG-repeats seem to interact with proteins of the translational apparatus, thus leading to a decrease in the global rate of protein synthesis [56]. The CCUG-repeats may also titrate and reduce the CNBP levels, thus partially contributing to the DM2 phenotype [56,89]. In agreement, heterozygous *Cnbp* knock-out (KO) mice ($Cnbp^{-/+}$) show multi-organ abnormalities resembling DM2. The crossing of these *Cnbp* KO mice with transgenic mice overexpressing *Cnbp* rescues the phenotype [90]. These data position CNBP as a reasonable candidate player in the development of DM2. However, other evidence suggests alternative pathological mechanisms underlying DM2 [91]. The finding that proteasome activity is affected in DM2 muscle cells [92] points out that an imbalance between protein synthesis and degradation leads to DM2 rather than a decrease in CNBP level by itself. Remarkably, the alteration of the levels of several proteins controlling global translation and muscle contraction has been observed in surviving *Cnbp* KO

mice core component of the multimeric dystrophin glycoprotein complex that regulates membrane stability. In muscle fibers of DM2 patients, CNBP becomes predominantly a membrane protein due to an increase in the CNBP-α-dystroglycan interaction [93]. Muscle biopsies from DM2 patients also show a reduction of the CNBP-ODC-polyamine axis [61]. In connection with this, depletion of CNBP in muscles of a *Drosophila melanogaster* model for DM2 causes age-dependent locomotor defects, which correlate with impaired polyamine metabolism. This finding also links the symptoms of DM2 with CNBP, given its role in controlling the translation of ODC mRNA and, consequently, in the synthesis of polyamines [61]. Overall, data suggest that CNBP titration affects the global cellular translation and/or the transcription of essential genes to guarantee general cell physiology. Nonetheless, further research is needed to fully understand the pathogenesis of DM2 and the role of CNBP in the development of this human disease.

Sporadic inclusion body myositis (sIBM) is the most common age-related degenerative muscle disease associated with the accumulation of β-amyloid [94]. Artificial over-expression of the amyloid precursor protein (APP) in murine tissue induces pathological changes resembling degenerative sIBM pathology. APP overexpression reduces CNBP expression [94]; nevertheless, the molecular mechanisms responsible for this downregulation have not been completely elucidated yet. Askanas and Engel [95] noted the remarkable similarities between sIBM and Alzheimer disease (AD). Indeed, muscle fibers and brain tissue from sIBM and AD patients share abnormal β-amyloid, phosphorylated tau, ubiquitin, apolypoprotein E, and presenilin-1 accumulation. Furthermore, both AD and sIBM are age-related degenerative diseases associated with abnormal intracellular protein folding and oxidative stress. Until now, there are no reports connecting *CNBP* miss-regulation and AD but, considering the above-mentioned facts, a deeper examination would be worthwhile.

8.2. *Inflammatory and autoimmune diseases*

In ha erent immune challenges, suggesting its involvement in the immune response during both bacteria and viruses' infection [96]. As stated above, CNBP-binding motifs have been identified in the promoter region of mice genes coding sustained inflammatory cytokines [22]. In mice, LPS induces *Cnbp* expression through an NF-κB dependent manner and a positive auto-regulatory mechanism, which in turn enables prolonged *Il-6* gene expression. Besides, CNBP up-regulates pro-inflammatory cytokine gene expression, although in a more restricted gene signature [45]. *Cnbp*-KO mice fail to mount protective IL-12 and IFN-γ responses *in vivo*, resulting in a reduced Th1 cell immune response and an inability to control parasite replication [45]. Consistent with this role, human CNBP has been recently suggested to function as an antiviral regulator that activates the expression of pro-inflammatory cytokines in response to foreign RNA sensing. CNBP-KO mice are more susceptible to influenza virus infection than wild-type mice, and show reduced type I IFN production. In addition, macrophages and dendritic cells from CNBP-KO mice show reduced type I IFN production upon infection with a panel of RNA viruses. Mechanistically, in response to RNA virus infection, CNBP is phosphorylated and translocated to the nucleus, wherein directly binds to the promoter of the *Ifnb* gene and then recruits IRF3/7 for the maximal induction of *Ifnb* gene expression [36]. In addition, CNBP was found as the major human protein interacting with the SARS-CoV-2 RNA genome in infected cells. CNBP-KO cell lines infected with SARS-CoV-2 reach higher viral RNA levels and are sensitized to viral-induced cell death [74].

In rats, *Cnbp* expression is low in the intestine compared to other tissues [1]. Intestinal epithelial cells are key players in setting the immunosuppressive tone of the gut mucosa to inhibit overreaction against innocuous luminal antigens. These immune mechanisms heavily depend on the toll-like receptor (TLR)-signaling and the consequent secretion of pro-inflammatory cytokines and chemokines by the intestinal epithelium [97]. A dysfunctional TLR-signaling response, such as shown by patients with inflammatory bowel disease (IBD), causes a chronic inflammatory state that may lead to tissue injury and tumorigenesis [98]. In view of this, and bearing in mind that excessive *Cnbp* expression leads to increased release of a

range ce of IBD [22,45]. Autoimmune reactivity to antigens associated with nucleic acids is a hallmark of systemic autoimmune diseases [99]. Although CNBP suits as an autoimmune disease antigen [27], its role in the pathogenesis of autoimmune disease has not yet been studied in depth [22,45].

8.3. *Cell proliferation and tumor development*

Numerous studies suggested a role of CNBP in tumorigenesis and tumor progression. In the Human Protein Atlas CNBP has been classified as a gene related with cancer [100] and was found as an unfavorable prognostic marker in pancreatic ductal adenocarcinoma (PDAC) [101]. Bearing in mind that mutations in *KRAS* occur in 75–90% of PDAC [102] and that CNBP may activate *KRAS* transcription by unfolding G4 structure in *KRAS* promoter [14], it is tempting to speculate about a role of CNBP in PDAC development through G4-mediated transcriptional activation of *KRAS*. In a similar fashion, CNBP was reported enhancing the transcription of *c-MYC* proto-oncogene [48], likely through the unfolding of a repressive G4 formed in *c-MYC* promoter [14]. *c-MYC* codes for a transcription factor that activates and/or represses the expression of a large array of genes essential for multiple cell functions. Miss-regulation of *c-MYC* occurs in >70% of human cancers, and is related to increased tendency to metastasis and poor prognosis, turning this oncogene as a central driver in multiple cancers [103,104]. Collectively, data leads to suggest that CNBP favors cell proliferation and survival, and perhaps cancer development, through a general mechanism of action over certain oncogenes whose transcription is repressed by the folding of G4s in their promoters.

The aberrant activation of the SHH pathway in cerebellar granule progenitors, which causes mitotic expansion, is a leading basis of medulloblastoma [105], the most frequent brain malignancy of childhood. The activation of a non-canonical Hedgehog-dependent pathway causes AMPK-mediated CNBP phosphorylation and Sufu binding, which prevents CNBP proteasome degradation with the consequent increase of ODC translation, polyamine metabolism and proliferation of medulloblastoma cells. The

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levels in cell proliferation [32,106].

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults. CNBP overexpression boosts HCC progression by interacting with FOXJ2 and, in turn, repressing the transcription of the metabolic tumour suppressor *PGM1* gene. The enzyme PGM1 regulates the bidirectional interconversion of glucose 1-phosphate and glucose 6-phosphate. The lowering of PGM1 due to CNBP overexpression decreases glycogen content and enhances glucose consumption and lactate production, greatly accelerating the proliferation of tumor cells [44].

EGF receptor is highly expressed in cancer cells [107]. Under EGF stimulus, CNBP phosphorylation and nuclear import lead to transcriptional up-regulation of the *Mmp-2* and *Mmp-14* genes, which encode matrix metalloproteinases representing the most prominent family of proteinases associated with tumorigenesis and metastasis. Besides, EGF stimulus also promotes CNBP-mediated transcription of the *E2f2* gene, which encodes a transcription factor also associated with tumor cell migration and invasion [37].

Altogether, data reported so far position CNBP as a regulator of oncogenes expression, as well as a potential cancer diagnosis marker and therapeutic target, not only for medulloblastoma, HCC, and gastric cancer (see *HuR* regulation above, Section 6.1), but also for other tumors where evidence is still suggestive [108].

8.4. *Congenital rostral head anomalies*

CNBP is required for proper cephalic embryonic development [51,109–111], playing a role in skeletogenic neural crest development [111]. In agreement, CNBP is involved in the neurocristopathy called Treacher Collins Syndrome (TCS) [31,112,113]. TCS is a rare congenital disease (1:50 000 live births) characterized by craniofacial defects, which is caused in nearly 90% of the cases by mutations in the *TCOF1* gene, which encodes the nucleolar protein Treacle. Interestingly, a positive correlation between *CNBP* and *TCOF1*

expre been modelled in zebrafish, where embryonic Treacle depletion displays phenotypic features that recapitulate the spectrum of craniofacial abnormalities observed in TCS patients. TCS-like zebrafish embryos and larvae show an increase of ROS along with the overexpression of redox-responsive genes, and treatment with antioxidants ameliorates the phenotypic defects of craniofacial anomalies. TCS-like zebrafish embryos also show lower Cnbp abundance, while transgenic overexpression of *cnbp* rescues the TCS phenotype in a dose-dependent manner by a ROS-cytoprotective action that prevents the redox-responsive genes up-regulation but does not normalize the synthesis of rRNAs [31]. In agreement with works showing that CNBP is degraded through the proteasomal pathway [31,32], the presence of proteasome inhibitors during TCS-like zebrafish embryonic development ameliorates cranial skeleton malformations, and attenuates critical TCS manifestations, such as neuroepithelial cell death and cell redox imbalance [114]. Altogether, these findings suggest CNBP as a druggable target in mandibulofacial dysostosis.

For many of the human diseases and biological processes where CNBP has been involved, different animal models (mice, chick, zebrafish and flies) with altered CNBP expression (overexpression, knock-down and KO) have been generated. Table 3 presents a summary of the available animal models that have been reported up to date for assessing CNBP biological functions.

9. CONCLUSIONS

After accumulating a large amount of information about the CNBP biochemical activities and cellular functions, as well as the biological processes in which CNBP has been directly or indirectly involved, many new aspects emerged that have not yet been fully understood. Currently, we know that CNBP binds to G-enriched single-stranded nucleic acid sequences and unfolds G4 RNA and ssDNA secondary structures by means of its nucleic acid chaperone activity. Besides, the relatively high number of Cys residues gives the molecule the biochemical capability to act as a redox switch (Figures 1 and 5). On the other hand, experimental evidence gathered so far indicates that CNBP participates in both transcriptional and

and protein-nucleic acid associations (Figure 4) by the ability of remodeling single-stranded nucleic acid secondary structures (Figure 3) and/or by contributing to modulate the redox state of both the transcriptional and translational machineries. Furthermore, CNBP is the target of several post-translational modifications (Figure 1) that allow not only the modulation of its activities, but also the dynamic regulation of CNBP subcellular localization, opening the possibility of a role for CNBP as an integrator of different cell signaling routes.

Various studies have reported a link between CNBP and several biological and pathological processes (Table 2), many of which made use of different animal models (Table 3). Indeed, CNBP was found involved in the inflammatory response, participating in cell proliferation and tumor processes, and required for proper neural crest and heart development (Figure 5). However, it is yet difficult to establish a unique and robust mechanistic or biochemical connection among these phenomena, leading us to wonder about the actual CNBP way(s) of action. Therefore, while a lot of knowledge has been generated about CNBP in the last decade, there are still many unanswered questions. Are there other CNBP targets for gene expression regulation or cellular partners (other proteins or nucleic acids) still not described? Is it possible that CNBP displays other biochemical activities not yet identified? Could different activities be modulated by the cellular (probably redox) context? How could a normal or a pathological context affect the function of CNBP? Is CNBP dysfunction the unknown cause of any human pathology? Is it possible to advance in the knowledge of the biological function of CNBP with the available animal models? Or is it needed to develop a novel model for this purpose? Some of these issues may be addressed by using high-throughput biochemical technologies (DNA or RNA chromatin immunoprecipitation followed by high throughput sequencing, co-immunoprecipitation coupled to mass spectrometry) and establishing novel and comprehensive cellular and organismal models for studying CNBP biology.

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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure 1. CNBP primary structure and post-translational modifications. (A) Schematic representation of CNBP primary structure pointing out the main structural motifs and the identified post-translational modification sites. **(B)** Diagram showing the various post-translational modifications (solid line boxes, including the references where they were informed) that were described for CNBP with varied functional implications in normal and/or disease conditions. The impact on CNBP dimerization state, cell localization, stability or target binding preference are indicated together with the biological process (dashed line boxes) where the modifications were described.

Figure 2. Reported CNBP-binding ssDNA and RNA consensus sequences. ssDNA (above) and RNA (below) motifs informed as CNBP targets represented in the form of WebLogos and consensus sequences.

Figure 3. CNBP regulates transcription and translation through G4 unfolding activity. By unfolding DNA-G4 structures (above) CNBP represses (*Nog/nog3*) or activates (*KRAS* and *c-MYC*) transcription. By unfolding RNA-G4 structures (below) CNBP enhances translation.

Figur with CNBP.

Figure 5. CNBP activities and functions. CNBP participates in many biological processes including neural crest and heart development, cell proliferation, tumorigenesis and inflammatory response. The biochemical activities so far described for CNBP as nucleic acid chaperone and binding factor allows CNBP work as a transcriptional and translational regulating factor. Finally, its putative role as a redox switch may consent the adaptation of its activities (and cellular roles) to the physiological context.

TABLE 1. CNBP targets of transcriptional and translational regulation. Gene target name, effect on expression, type of experimental evidences and linked references.

CNBP targets of gene expression control				
Regulated process	**Gene name (species)**	**Activation/ Repression**	**Experimental evidences**	**References**
Transcription	*HMGCR* (*Homo sapiens*)	Repression	- Expression library screening	[1]
	JC virus early promoter-enhancer (JC virus)	Repression	- Southwestern blots	[50]
	β-MHC (*Homo sapiens*)	Repression	- Expression library screening	[15]
	Csf1 (*Mus musculus*)	Activation	- Expression library screening	[49]
	c-MYC (*Homo sapiens*)	Activation	- DNA-affinity column purification and identification - Electrophoretic Mobility Shift Assays (EMSAs) - Chromatin Immunoprecipitation (ChIP) in cultured cells and in zebrafish embryos - Loss-of-function in cultured cells and expression analysis with endogenous and reporter genes	[14,38,40,46,48,51]
	tbx2b (*Danio rerio*)	Activation	- Yeast one-hybrid assay - EMSA - ChIP in zebrafish embryos - *In vivo* loss-of-function in zebrafish embryos and expression analysis	[39]
	smarca5 (*Danio rerio*)	Activation		
	wnt5b (*Danio rerio*)	Repression		
	tcf7l2 or tcf4 (*Danio rerio*)	Activation	- Bioinformatic analyses - ChIP in zebrafish embryos - *In vivo* loss-of-function in zebrafish embryos and expression analysis	[41]
	ptk7 (*Danio rerio*)	Activation		
	cdk14 or *pftk1* (*Danio rerio*)	Activation		
	HNRNPK (*Homo sapiens*)	Repression	- Bioinformatic analyses - EMSA - ChIP in cultured cells - Overexpression in cultured cells and expression analysis of endogenous and reporter genes	[42,43]

	(*Mus musculus*)	Activation		
	Ccl3, *Ccl4*, *Ccl5*, *Ccl7*, *Ccl9*, and *Ccl22* (*Mus musculus*)	Activation	- DNA-protein complex pull-down - ChIP in cultured cells	[22]
	Cnbp (*Mus musculus*)	Activation		
	Il12b (*Mus musculus*)	Activation	- ChIP in cultured cells - Loss-of-function in cultured cells and expression analysis of endogenous and reporter genes	[45]
	PGM1 (*Homo sapiens*)	Repression	- EMSA - ChIP in cultured cells - Overexpression and loss-of-function in cultured cells and expression analysis of endogenous and reporter genes	[44]
	Mmp2, Mmp14, E2f2 (*Mus musculus*)	Activation	- Bioinformatic analyses - EMSA - ChIP in cultured cells	[37]
	E2f2 (*Mus musculus*)	Activation		
	KRAS (*Homo sapiens*)	Activation	- EMSA - ChIP in cultured cells - Loss-of-function in cultured cells and expression analysis with endogenous genes	[14]
	NOG / *nog3* (*Homo sapiens* / *Danio rerio*)	Repression	- EMSA - ChIP in cultured cells and in zebrafish embryos - Loss-of function in cultured cells and expression analysis of endogenous genes - *In vivo* gain-of-function in zebrafish embryos and expression analysis	[14]
	ELAVL1 or *HuR* (*Homo sapiens*)	Activation	- Bioinformatic analyses - ChIP in cultured cells - Overexpression and loss-of-function in cultured cells and expression analysis of endogenous and reporter genes	[47]
	Ifnb / Ifna4? (*Mus musculus*)	Activation	- ChIP in cultured cells - Overexpression and loss-of-function in cultured cells and expression analysis of endogenous and reporter genes	[36]
Translation	*rpl4* (*Xenopus laevis*)	Repression / Activation	- UV cross-linking and PAGE - *in vitro* translation assay	[63,64]
	RPS17, *PABP*, *EEF1A*, *EEF2*, and *RPS6* (*Homo sapiens*)	Activation	- UV cross-linking - loss-of-function in cultured cells and expression analysis of endogenous genes - RNA immunoprecipitation (RIP)	[56,62]
	ODC (*Homo sapiens*)	Activation	- RNA affinity capture and mass spectrometry (MS) - EMSA - Overexpression and loss-of-function in cultured cells and expression analysis of reporter genes	[2,32,34,35,61]
	genes required for ribosome biogenesis and assembly (*Saccharomyces cerevisiae*)	Repression	- RNA immunoaffinity isolation and analysis with yeast DNA oligo arrays - RNA pull-down - Overexpression and loss-of-function in yeasts and expression analysis of endogenous genes	[57]

Myosins (Saccharomyces cerevisiae)	Activation	analysis with yeast DNA oligo arrays - Overexpression and loss-of-function in yeasts and expression analysis of endogenous genes	[57]
HRAS (*Homo sapiens*)	Not determined	- *In vitro* transcribed RNA probed on human protein microarray. - *In vitro* and *in cellulo* RNA pull-down	[58]
dMyc (*Drosophila melanogaster*)	Activation	- Loss-of-function larvae and flies and expression analysis of endogenous genes - Loss-of-function and overexpression in cultured cells and expression analysis of reporter genes	[59]
4178 mRNAs (*Homo sapiens*)	Activation	- Photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) - EMSA and filter binding assays - loss-of-function in cultured cells, ribosome profiling and expression analysis of reporter genes	[21]
RPL2 (*Cryptococcus neoformans*)	Activation	- RNA pull-down and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) - EMSA and UV-cross-linked EMSA - loss-of-function cells and RNA stability analysis by Northern blot and ribosome profiling	[4]
CCND1, *SOX9*, *NFE2L1*, and *PDF* (*Homo sapiens*) cooperating with *LAST* lncRNA	Activation	- RNA pull-down - RIP - loss-of-function in cultured cells and expression analysis of endogenous genes	[60]
dOdc (*Drosophila melanogaster*)	Activation	- loss-of-function cells and flies and expression analysis of endogenous and reporter genes - RIP - Ribosome profiling	[61]

TABLE 2. Diseases connected to CNBP. Main characteristics, OMIM (Online Mendelian Inheritance in Man) reference number and the recent publications that link CNBP to the disease are provided.

Disorder classification	DISEASE/ CONDITION	HIGHLIGTHS/CHARACTERISTICS	REFERENCES CONNECTING TO CNBP
Degenerative diseases	**DYSTROPHIA MYOTONICA 2 (DM2)**	OMIM **#**602668 Caused by a (CCTG)n repeat expansion in the zinc finger protein 9 gene (*ZNF9*, *CNBP*; 116955)	[56,61,86,88,89,90,91,92,93]
	SPORADIC INCLUSION BODY MYOSITIS (sIBM)	OMIM #147421 The most common age-related muscle disease in the elderly that results in severe disability. Striking similarities to Alzheimer disease (AD; 104300).	[94]
Congenital disorders	**TREACHER COLLINS SYNDROME 1 (TCS1)**	OMIM **#**154500 Caused by heterozygous mutation in the 'treacle' gene (*TCOF1*; 606847) on chromosome 5q32. The features include antimongoloid slant of the eyes, coloboma of the lid, micrognathia, microtia and other deformity of the ears, hypoplastic zygomatic arches,	[31,113,114]

		palate are often present.	
	MEDULLO BLASTOMA	OMIM #155255 Caused by germline mutations in the *SUFU* gene (607035), the *BRCA2* gene (600185) and sporadically *PTCH2* (603673) and *CTNNB1* (116806) genes. Medulloblastoma is the most common brain tumor in children.	[32,106]
Cell proliferation and tumor development (increased risk/promotion of tumors)	**HEPATOCELLULAR CARCINOMA**	**OMIM #** 114550 Somatic mutations in a number of different genes have been identified in hepatocellular carcinoma. These include *TP53* (191170), *MET* (164860), *CTNNB1* (116806), *PIK3CA* (171834), *AXIN1* (603816), and *APC* (611731). Hepatocellular carcinoma is the major histologic type of malignant primary liver neoplasm. It is the fifth most common cancer and the third most common cause of death from cancer worldwide.	[44]
	GASTRIC CANCER	OMIM **#** 613659 Major cause of cancer death worldwide. Genetically heterogeneous disorder.	[47,108]
Inflammatory and autoimmune diseases	**SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)**	OMIM **#**152700 SLE is a complex autoimmune disease characterized by production of autoantibodies against nuclear, cytoplasmic, and cell surface molecules that transcend organ-specific boundaries. Multiple genes are involved in the causation of systemic lupus erythematosus.	[27]
Proposed connections to other diseasesroposed connections to	***Alzheimer disease (AD)***	**OMIM #** 104300 (AD1) Genetically heterogeneous disorder	[94]
	Inflammatory bowel disease (IBD)	**OMIM #** 266600 (CROHN DISEASE) 1; IBD1	[22,45]
	Other autoimmune disease?		[22,27,36,45]

TABLE 3: Available animal models with altered CNBP expression. Information regarding the developed animal model, the experimental strategy employed and main characteristics and findings along with the original reference.

SPECIES	MODEL/EXP. STRATEGY	EXTRA INFORMATION/FINDINGS REGARDING CNBP	REFERENCES
Mus musculus	***Cnbp*-knock-out mouse on mixed background**	ES cell lines with a single-copy proviral genome were injected into BALB/c or C57BL/6J blastocysts. The *Cnbp* transgenic line was obtained by breeding a male chimera with a female C57BL/6Jmouse. The *Cnbp* mutation was repeatedly backcrossed (>12 generations) onto the C57BL/6J inbred genetic background to improve phenotypic consistency. The mutants exhibited severe forebrain truncation in homozygous mouse embryos and various craniofacial defects in heterozygotes. A reduction in cell proliferation was observed in the anterior regions of *Cnbp*$^{-/-}$ embryos at gastrulation and neural-fold stages. In these regions, Myc expression was absent. *Cnbp*$^{+/-}$ mice, in which the expression of *cnbp* was significantly decreased, displayed many of the features of myotonic dystrophy, including muscle histological morphology, distinctive ocular cataracts and myotonic discharges and cardiac abnormalities.	[51,90]

	Cnbp-knock-out mouse on a pure C57BL background	_Cnbp_ KO mice were generated using a gene trap approach. The ES clone, containing the gene trap insertion in the first intron of the _Cnbp_ gene, was used to generate the _Cnbp_ KO model in C57BL/6 mice in the facility. Some homozygous _Cnbp_ knockout mice survived until 16 to 20 months of age. _Cnbp_-knockout mice were small at birth and remained smaller during their life span compared with wild types. Some _Cnbp_-knockout mice were weak and died during the first month after birth. Skeletal muscle of _Cnbp_-knockout mice contained small and thin fibers, with some containing centralized nuclei. Loss of Cnbp also affected sarcomeric structure. Histologic analysis demonstrated that _Cnbp_$^{-/-}$ mice had muscle atrophy at a young age, whereas heterozygous mice exhibited severe muscle loss only at advanced age. _Cnbp_-knockout skeletal muscle showed alterations in proteins encoded by TOP-containing mRNAs and proteins regulating muscle contraction. (From OMIM)	[93]
	Cnbp$^{-/-}$ mice generated using embryonic stem (ES) cells.	ES cells (_Cnbptm1a(KOMP)Wtsi_) were generated by replacing the _Cnbp_ genomic locus with a neomycin cassette under the control of a _Pgk1_ promoter. ES cells were injected into blastocysts to generate chimeric mice. _Cnbp_ heterozygous mice were obtained by gamete line transmission from mating the chimeric mice with WT C57BL/6 mice. _Cnbp_ heterozygous mice were intercrossed to generate WT and KO alleles for experiments. While _Cnbp_-deficient mice are viable, homozygous KO animals were born at a lower-than-expected Mendelian frequency. _Cnbp_-deficient mice were more susceptible to acute toxoplasmosis associated with reduced production of IL-12β, as well as a reduced T helper type 1 (Th1) cell IFN-γ response essential to controlling parasite replication. CNBP deficient mice were more susceptible to influenza virus (H1N1 PR8) infection than were wild-type mice. _Cnbp_$^{-/-}$ mice lost significantly more body weight after infection with PR8 and had significantly increased mortality rates compared to WT mice. Under viral infection, _Cnbp_$^{-/-}$ BMDMs showed significantly impaired IFN-β, IL12p40 and IL6 production compared to WT BMDMs, while viral replication is increased.	[36,45]
	Cnbp conditional KO mice	_Cnbp_ -floxed mice were generated and crossed to Vavi-Cre, Lysm-Cre, or CD11c-Cre mice to delete CNBP in hematopoietic cells, myeloid cells, or dendritic cells only, respectively. All three conditional knockout mice showed decreased survival rates after viral infection (IAV-PR8), increased viral replication and decreased type I IFN production.	[36]
Danio rerio	**Transient _cnbp_-knock-down zebrafish embryos microinjected with translation blocking Morpholino**	Cnbp depletion by translation blocking Morpholino (5¨-ATCCAAAACACTCACTGGTACTCAT-3´) caused forebrain truncation while trunk development appeared normal. Substantial reduction in cell proliferation and an increase in cell death were observed in the anterior regions of _cnbp_ morphant embryos. Cnbp depletion did not affect central neural system patterning while it caused depletion of neural crest derivatives.	[110]
	Transient _cnbp_-knock-down zebrafish embryos microinjected with splicing morpholino	The use of a Morpholino that affected the splicing of _cnbp_ pre-mRNA (5´-TATCTCTTCTTAGCTTACCCTTTCC-3´) resulted in a reduction of full-length mRNA levels along with the generation of a novel transcript coding for an isoform that may act as dominant negative proteins. The use of this Morpholino resulted in more severe phenotypes than those described for translation-blocking Morpholino. The previous splice blocking Morpholino and a new one (5'-GTTAGGAAAAACATGGCTTACCTGT-3' injected in embryos resulted in decreased levels of _il-6 mRNA_ and failure to recruit macrophages after LPS stimulation. Also increased susceptibility to Shigella infection with defective neutrophil infiltration was demonstrated. Decreased survival was informed for CNBP depleted embryos after LPS or Shigella infection.	[22,41,112]
	Stable zebrafish transgenic line overexpressing a zCnbp-eGFP	1-4 cell embryos injected with Transposase mRNA and donor plasmid (Tol system, pEF1α/zCnbp^{wt}-EGFP construct cloned in pT2AL). Fluorescent embryos were selected, bred and crossed with wild type until stable fluorescence. No strong phenotype detected. Mild alterations in the expression territory	[31,53]

		Cnbp overexpressing embryos showed milder phenotypes when injected with translation blocking Morpholino against *tcof1* (*nolc1*, Treacher Collins model in zebrafish)	
	Transient zCnbp-eGFP-overexpressing zebrafish embryos.	The mRNA coding for the zCNBP–eGFP chimera was synthesized *in vitro* and microinjected into embryos at one/two-cell stage. This treatment led to an increase in *cnbp*-mRNA of approximately 50% at 24-hours post-fertilization (hpf) stage and 20% at 54 hpf-staged larvae, causing detectable changes in the transcriptional expression of target developmental genes, although it did not generate detectable phenotypic changes in embryonic development	[14,41]
Gallus gallus	**RNAi-silencing and Retrovirus-misexpression approaches by injection and electroporation into the prospective forebrain area in chick embryos .**	The silencing of *CNBP* expression resulted in forebrain truncation and the absence of *BF-1*, *SIX3* and *HESX1* expression, but not *OTX2*. Misexpression of CNBP induced the expression of *BF-1*, *SIX3* and *HESX1* in the hindbrain, but not the expression of *OTX2*. Essential role of CNBP in forebrain formation during chick embryo organogenesis.	[109]
Drosophila melanogaster	**Tissue specific RNAi-*nubGAL4*-mediated expression of *UAS-dCNBP*^{RNAi}**	Crossing of a *nubGAL4* driver line, expressing GAL4 in the wing pouch, with dCNBP RNAi lines carrying either 1 or 2 RNAi constructs (*2×dCNBP*^{RNAi}). Knockdown of *dCNBP* in the wing territory causes a general reduction of wing size, in keeping with the reported role of dMyc in this region. Depletion of dCNBP in muscles causes age-dependent locomotor defects that are correlated with impaired polyamine metabolism due to reduced *dODC* translation	[59,61]

HIGHLIGHTS

- CNBP binds G-rich single-stranded nucleic acids and promotes G-quadruplex unfolding.
- CNBP modulates the expression of multiple genes, both at the transcriptional and translational level.
- Post-translational modifications modulate CNBP partners and subcellular localization.
- CNBP participates in cranial development, immune response and cell proliferation.
- CNBP is related to neurocristopathies, degenerative diseases and tumorigenesis.

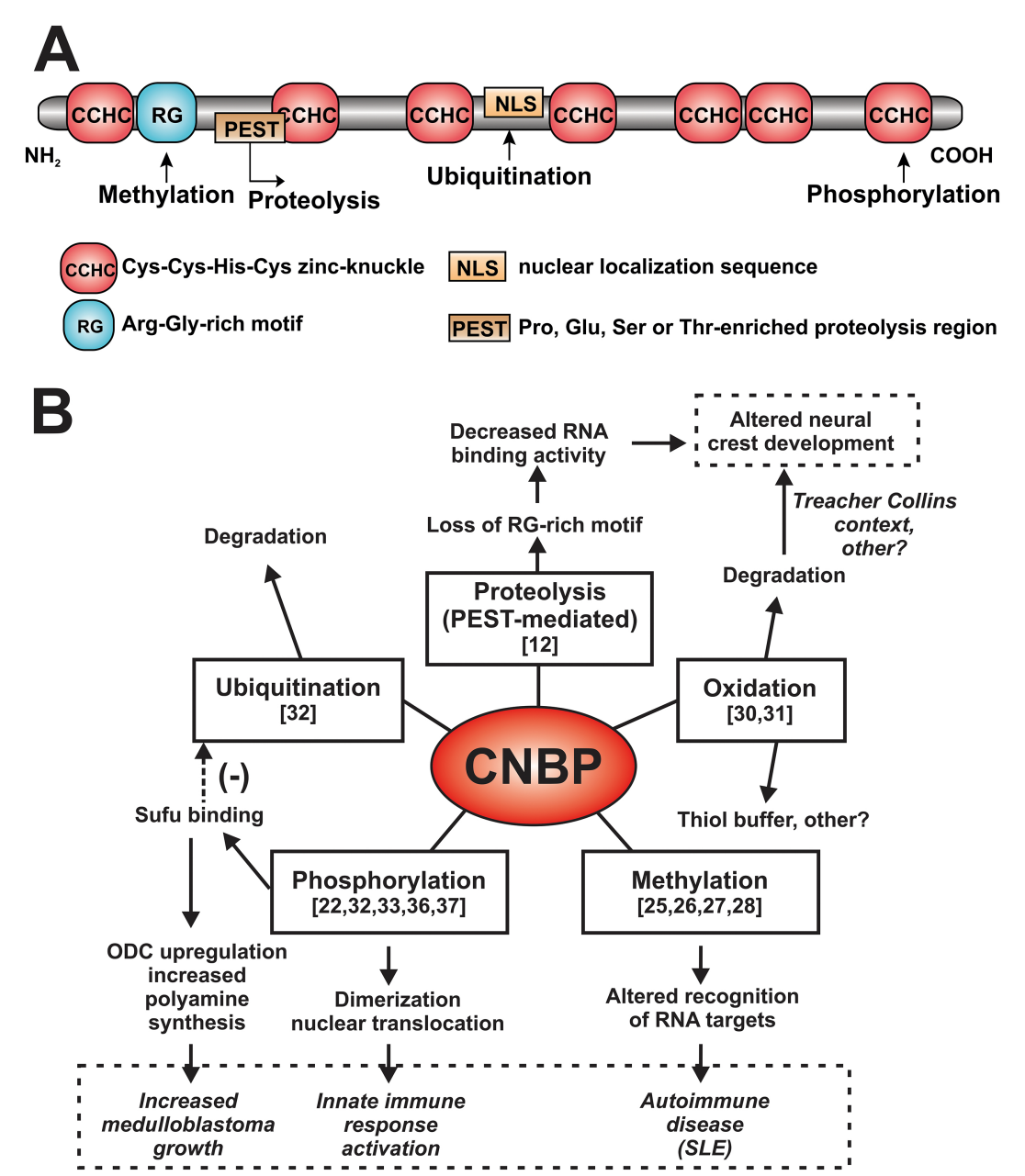
A
CCHC
RG
PEST
CCHC
CCHC
NLS
CCHC
CCHC
CCHC
CCHC
NH2
COOH
Methylation
Proteolysis
Ubiquitination
Phosphorylation
CCHC Cys-Cys-His-Cys zinc-knuckle
NLS nuclear localization sequence
RG Arg-Gly-rich motif
PEST Pro, Glu, Ser or Thr-enriched proteolysis region
B
Decreased RNA binding activity
Altered neural crest development
Loss of RG-rich motif
Treacher Collins context, other?
Degradation
Degradation
Proteolysis (PEST-mediated) [12]
Ubiquitination [32]
Oxidation [30,31]
CNBP
(-)
Sufu binding
Thiol buffer, other?
Phosphorylation [22,32,33,36,37]
Methylation [25,26,27,28]
ODC upregulation increased polyamine synthesis
Dimerization nuclear translocation
Altered recognition of RNA targets
Increased medulloblastoma growth
Innate immune response activation
Autoimmune disease (SLE)

Figure 1

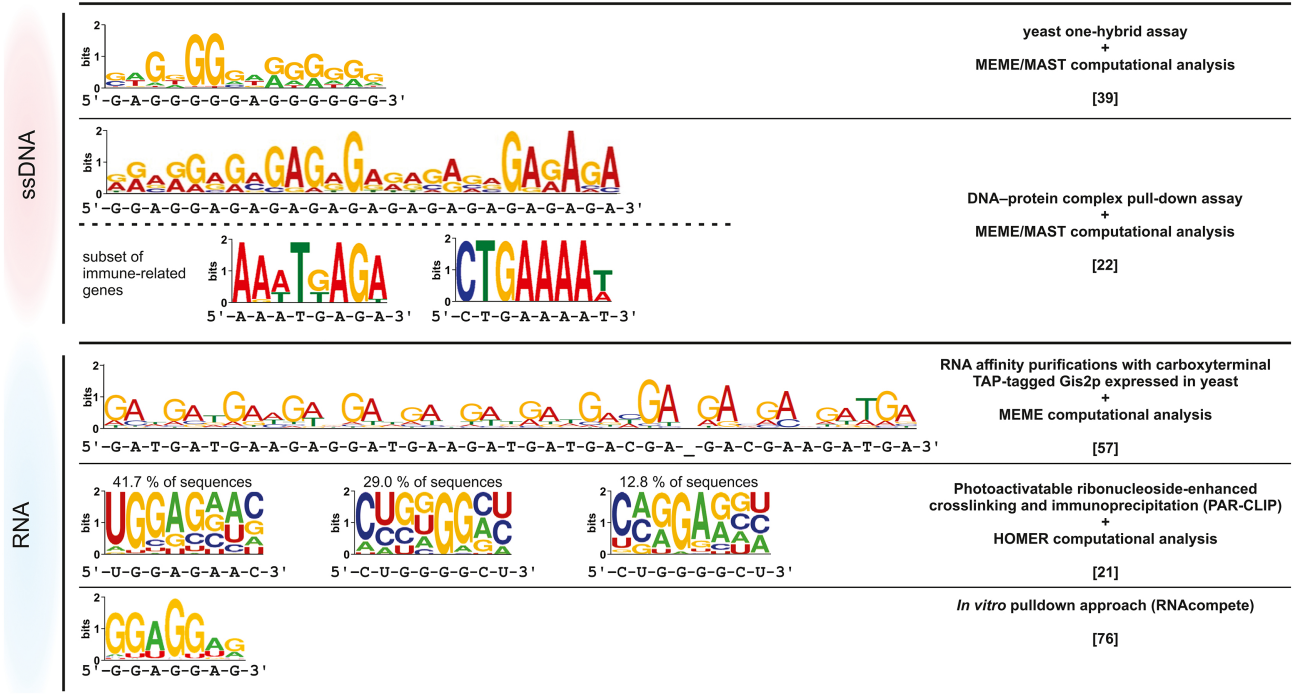
WebLogo / Consensus sequence
Experimental approach (reference)
ssDNA
RNA
bits
5'-G-A-G-G-G-G-G-A-G-G-G-G-G-G-3'
yeast one-hybrid assay
+
MEME/MAST computational analysis
[39]
5'-G-G-A-G-G-A-G-A-G-A-G-A-G-A-G-A-G-A-G-A-G-A-G-A-3'
DNA–protein complex pull-down assay
+
MEME/MAST computational analysis
[22]
subset of immune-related genes
5'-A-A-A-T-G-A-G-A-3'
5'-C-T-G-A-A-A-A-T-3'
5'-G-A-T-G-A-T-G-A-A-G-A-G-G-G-A-T-G-A-A-G-A-T-G-A-T-G-A-C-G-A-_-G-A-C-G-A-A-G-A-T-G-A-3'
RNA affinity purifications with carboxyterminal TAP-tagged Gis2p expressed in yeast
+
MEME computational analysis
[57]
41.7 % of sequences
29.0 % of sequences
12.8 % of sequences
5'-U-G-G-A-G-A-A-C-3'
5'-C-U-G-G-G-G-C-U-3'
5'-C-U-G-G-G-G-C-U-3'
Photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP)
+
HOMER computational analysis
[21]
5'-G-G-A-G-G-A-G-3'
In vitro pulldown approach (RNAcompete)
[76]

Figure 2

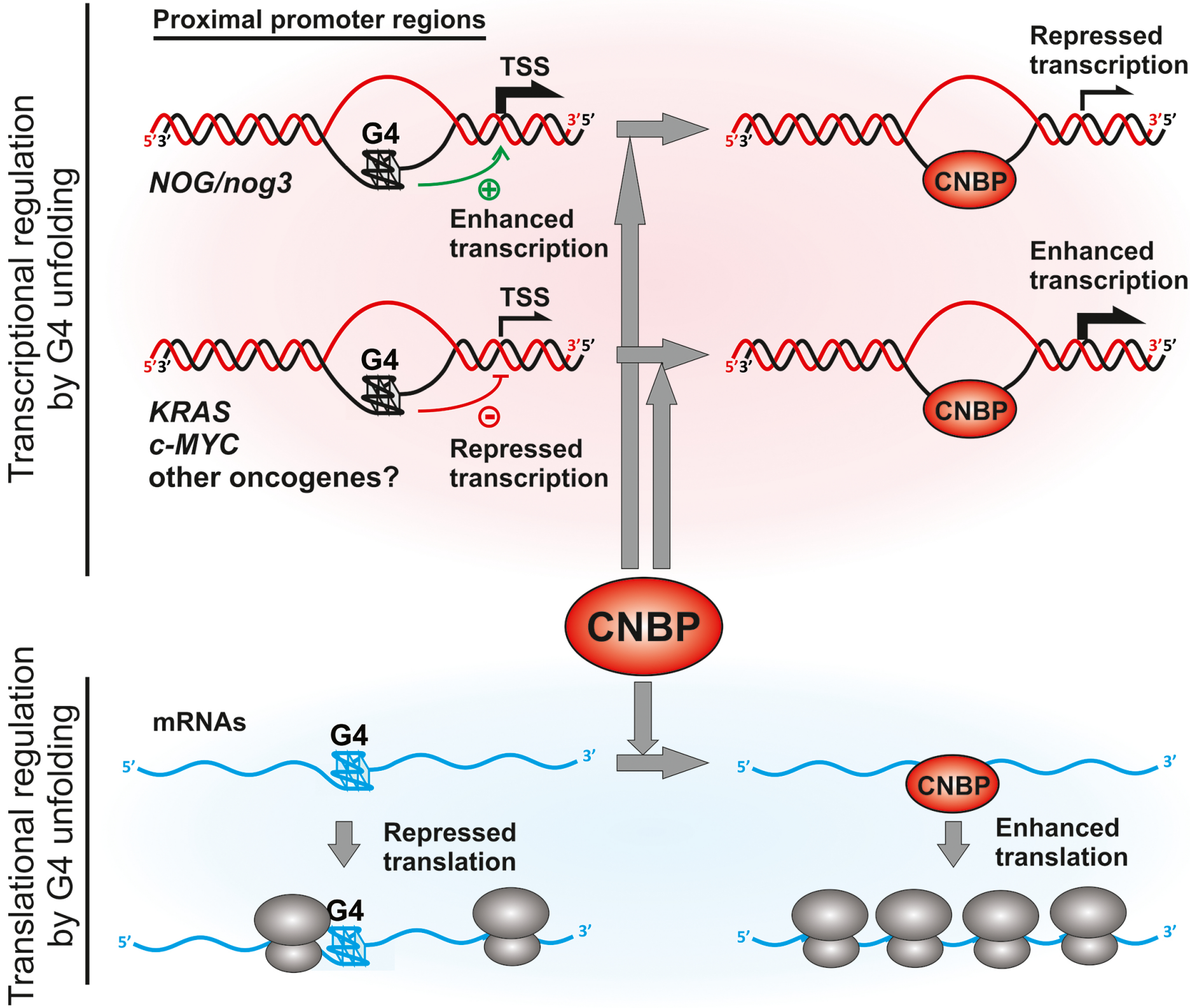
Transcriptional regulation by G4 unfolding
Proximal promoter regions
TSS
G4
NOG/nog3
Enhanced transcription
Repressed transcription
CNBP
KRAS
c-MYC
other oncogenes?
Repressed transcription
Enhanced transcription
CNBP
Translational regulation by G4 unfolding
mRNAs
G4
Repressed translation
Enhanced translation
CNBP

Figure 3

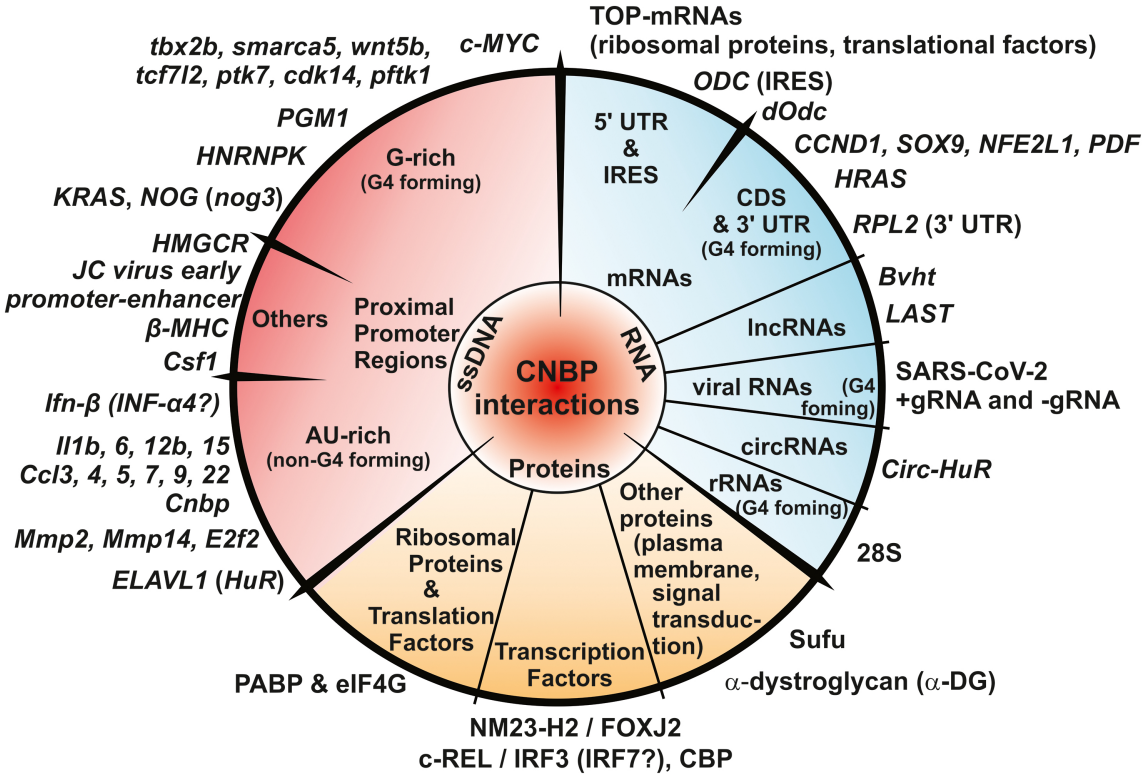
CNBP interactions
ssDNA
RNA
Proteins
G-rich (G4 forming)
Proximal Promoter Regions
Others
AU-rich (non-G4 forming)
5' UTR & IRES
CDS & 3' UTR (G4 forming)
mRNAs
lncRNAs
viral RNAs (G4 foming)
circRNAs
rRNAs (G4 foming)
Ribosomal Proteins & Translation Factors
Transcription Factors
Other proteins (plasma membrane, signal transduc-tion)
TOP-mRNAs (ribosomal proteins, translational factors)
ODC (IRES)
dOdc
CCND1, SOX9, NFE2L1, PDF
HRAS
RPL2 (3' UTR)
Bvht
LAST
SARS-CoV-2 +gRNA and -gRNA
Circ-HuR
28S
Sufu
α-dystroglycan (α-DG)
NM23-H2 / FOXJ2
c-REL / IRF3 (IRF7?), CBP
PABP & eIF4G
ELAVL1 (HuR)
Mmp2, Mmp14, E2f2
Cnbp
Il1b, 6, 12b, 15
Ccl3, 4, 5, 7, 9, 22
Ifn-β (INF-α4?)
Csf1
β-MHC
JC virus early promoter-enhancer
HMGCR
KRAS, NOG (nog3)
HNRNPK
PGM1
tbx2b, smarca5, wnt5b, tcf7l2, ptk7, cdk14, pftk1
c-MYC

Figure 4

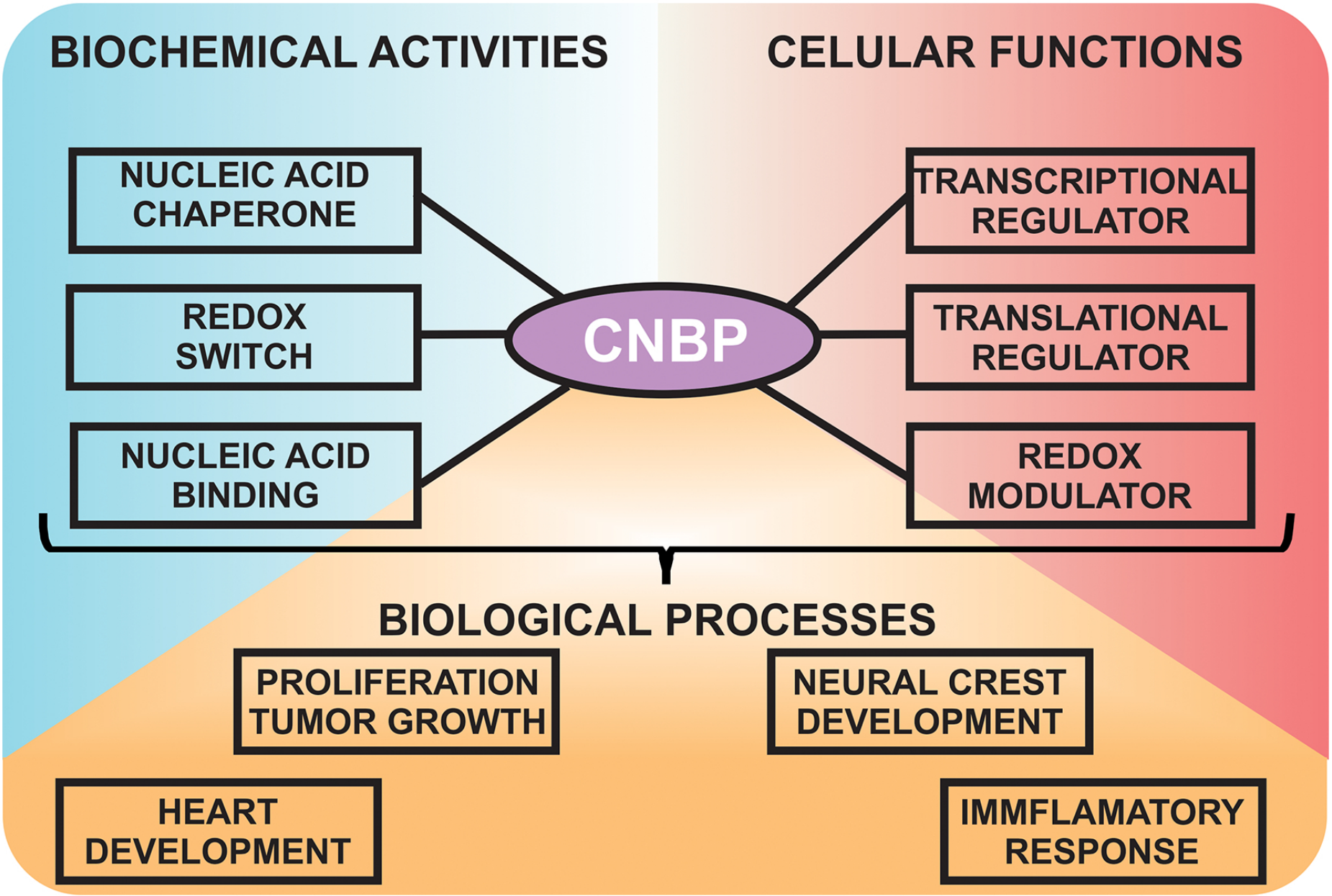
BIOCHEMICAL ACTIVITIES
CELULAR FUNCTIONS
NUCLEIC ACID CHAPERONE
REDOX SWITCH
NUCLEIC ACID BINDING
CNBP
TRANSCRIPTIONAL REGULATOR
TRANSLATIONAL REGULATOR
REDOX MODULATOR
BIOLOGICAL PROCESSES
PROLIFERATION TUMOR GROWTH
NEURAL CREST DEVELOPMENT
HEART DEVELOPMENT
IMMFLAMATORY RESPONSE

Figure 5