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REVIEW

Regulatory roles of vertebrate Nocturnin: insights and remaining mysteries

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ABSTRACT

Post-transcriptional control of messenger RNA (mRNA) is an important layer of gene regulation that modulates mRNA decay, translation, and localization. Eukaryotic mRNA decay begins with the catalytic removal of the 3′ poly-adenosine tail by deadenylase enzymes. Multiple deadenylases have been identified in vertebrates and are known to have distinct biological roles; among these proteins is Nocturnin, which has been linked to circadian biology, adipogenesis, osteogenesis, and obesity. Multiple studies have investigated Nocturnin's involvement in these processes; however, a full understanding of its molecular function remains elusive. Recent studies have provided new insights by identifying putative Nocturnin-regulated mRNAs in mice and by determining the structure and regulatory activities of human Nocturnin. This review seeks to integrate these new discoveries into our understanding of Nocturnin's regulatory functions and highlight the important remaining unanswered questions surrounding its regulation, biochemical activities, protein partners, and target mRNAs.

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Introduction

Cells must sense and respond appropriately to a variety of external cues including hormones, nutrients, stress, and circadian inputs. These cues may trigger broad changes in the gene expression program to promote survival, such as those seen during nutrient starvation; alternatively, these changes may be targeted to specific pathways, e.g., the inflammatory response following injury or infection. Changes in gene expression may be exerted at multiple levels including messenger RNA (mRNA) synthesis, processing, and translation. Although regulation of mRNA transcription has received significant attention in the past, the importance of post-transcriptional control over gene expression has become increasingly apparent in recent years [1–4]. A multitude of cis- and trans-acting factors exert influence at this level, including those that modulate RNA stability and translation by shortening mRNA poly-adenosine (poly(A)) tails [5,6].

In vertebrates, Nocturnin (NOCT; also known as Noct, Noc, CCR4L, CCRN4L, and Ccr4c) was originally identified in *Xenopus* retina as a circadian-expressed RNA with homology to the yeast deadenylase CCR4 [7,8]. NOCT orthologs have been identified in invertebrates and vertebrates including *Drosophila*, fish, amphibians, and mammals, suggesting NOCT has an evolutionarily conserved function in metazoans [8–17]; however, it is absent from other organisms such as *C. elegans* and yeast. NOCT has been most extensively studied in mice, where loss of NOCT confers resistance to high-fat diet-induced obesity [9]. In addition to regulation of body mass, NOCT affects the regulation of intestinal lipid trafficking, and the differentiation of bone marrow mesenchymal stem cells into either adipocytes or osteoblasts (Fig. 1) [18–21]. As a putative RNA decay factor, NOCT has been proposed to contribute to these processes by destabilizing mRNAs encoding proteins with important metabolic and developmental functions. Several studies sought to understand NOCT function by characterizing its biochemical activity and identifying the mRNAs whose misregulation may explain *NOCT* knockout mouse phenotypes. Thus far these studies have had limited success and our understanding of NOCT function has remained unclear; however, recent reports provide new insights into NOCT biology, structure, and function. Consequently, it is now necessary to integrate these new results with previous data and to clearly define the remaining unanswered questions surrounding NOCT biology.

Post-transcriptional control of mRNA abundance and stability by specialized deadenylases

The terminal 5′ 7-methylguanosine cap and 3′ poly(A) tail modifications found on mature mRNAs serve to both promote transcript association with the translation machinery and protect transcripts from exonuclease-mediated decay [22], and their enzymatic removal inhibits translation and destabilizes mRNAs. As the first and rate limiting step in mRNA decay, deadenylation is therefore an important regulatory nexus. Poly(A) tail removal is catalyzed by members of the deadenylase class of enzymes, twelve of which have been identified in mammals through sequence homology to either the yeast POP2 or CCR4 deadenylases. Six deadenylases are related to POP2 and share a DEDD-type catalytic domain (Pfam PF04857), while the remaining deadenylases including NOCT share homology with CCR4, a member of the exonuclease-endonuclease-phosphatase (EEP) superfamily (Pfam PF03372) [13].

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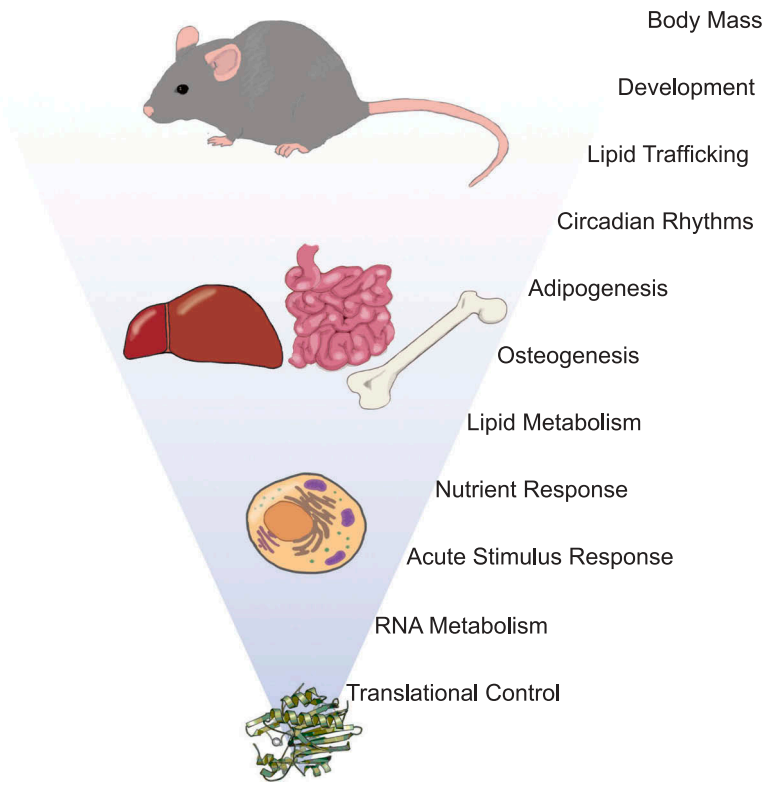

Figure 1. Molecular through physiological roles of NOCT. NOCT-mediated regulation of mRNA translation and decay at the molecular level affects multiple biological processes and modulates tissue development and function, circadian processes, and ultimately regulation of body mass.

Since all of the deadenylases could theoretically catalyze the same 3′-5′–directed removal of adenosines from an mRNA substrate, it is unsurprising that functional redundancy has been observed [13,23]. However, the collection of deadenylases is larger than expected if all deadenylases were fully interchangeable. Although a comprehensive review of the specific functions attributed to each deadenylase is beyond the scope of this article (readers are instead referred elsewhere [13,21,24–26]), several lines of evidence support functional specialization amongst these proteins, even among the most closely-related of these enzymes. The strongest support for this idea comes from analyses of vertebrate NOCT, CNOT7, and CAF1z loss-of-function models that exhibit distinct phenotypes, indicating unique functions for these enzymes [9,27–30].

Functional specificity among deadenylases could arise in part through tissue-, cell type- and developmental stage-specific expression as has been observed for the CCR4-NOT (CNOT) complex nuclease subunits CNOT6 and CNOT6L, which form mutually exclusive heterodimer complexes with CNOT7 and CNOT8, and have distinct roles even when co-expressed [23,26,31–34]. Additional functional specialization among deadenylases is likely mediated by divergent amino acid motifs within each protein, even among those sharing catalytic domain structure. Alignment of the amino acid sequences of human CCR4-type deadenylases reveals that although the EEP motif defining this class of enzymes is shared, the CNOT6/CNOT6L and ANGEL1/ANGEL2 paralog pairs exhibit the most extensive conservation while others members are more divergent (Fig. 2). The unique domains of each protein likely mediate subcellular localization, substrate, and regulatory partner specificity, as has been observed for CAF1z [30,35,36], PDE12 [37–39], PARN [36,40–42], and PNLDC1 [43–46]. NOCT biological function is likely further defined by its expression pattern and subcellular localization.

NOCT expression and biological functions

As in *Xenopus*, circadian fluctuations in mRNA abundance have been observed for *NOCT* orthologs in sea sponge [15], zebrafish [17], mice [10–12,47,48], and humans [49]. NOCT expression has been best characterized in mice, where *NOCT* mRNA abundance varies by tissue, and by time of day in several tissues [12,19,47]. Circadian variations in *NOCT* mRNA abundance are most dramatic in liver, followed by smaller fluctuations in heart and kidney; in each case, peak expression occurs at or soon after light offset [12,47,48]. These daily changes in transcript abundance suggested that NOCT may have functions relating to the circadian clock (Fig. 1). Consistent with this idea, the *Drosophila* NOCT ortholog *curled* has been implicated in the circadian light response [50]; however, *NOCT* knockout mice exhibit normal circadian behaviors, indicating that NOCT may function as a downstream effector of circadian signals in vertebrates [9].

The circadian clock may regulate *NOCT* transcription; however, the mechanism and consequences of this regulation are unclear. The core transcriptional regulators CLOCK and BMAL1 are recruited to the *NOCT* promoter in humans and mice through the action of a conserved E-box transcriptional element. Evidence from human cells suggests that CLOCK and BMAL1 associate with the *NOCT* promoter regardless of circadian time [51]; while BMAL1 ChIP-seq analysis in mice revealed time-dependent BMAL1 association with the

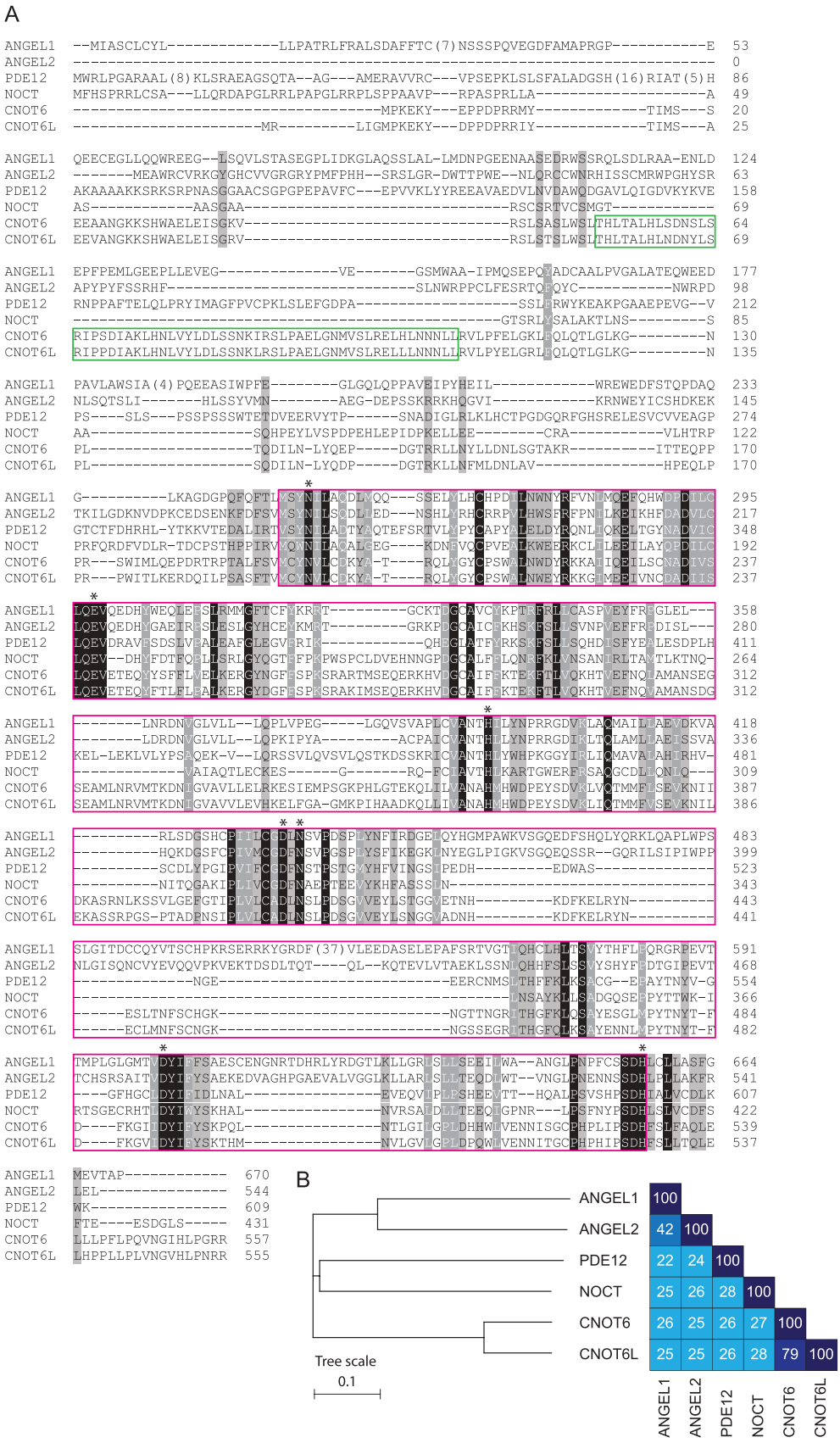

Figure 2. NOCT is related to CCR4-type deadenylases and contains a conserved EEP catalytic domain. A) Multiple sequence alignment of human CCR4-type deadenylases. The shared characteristic EEP domain is boxed in pink; leucine-rich repeats found in CNOT6 and CNOT6L (boxed in green) are absent from NOCT. Putative NOCT catalytic residues (*) are conserved among EEP family members. Amino acid sequences were aligned using Clustal Omega and colored according to extent of chemical property similarity (charge, hydrophobicity, etc.) using Chroma [107,108]. Darker background shading indicates greater similarity, with strictly conserved positions indicated by black background and white text. Uniprot accession numbers: ANGEL1 Q9UNK9, ANGEL2 Q5VTE6, PDE12 Q6L8Q7, NOCT Q9UK39, CNOT6 Q9ULM6, CNOT6L Q965LI5. B) Phylogenetic tree demonstrating the relationships between human CCR4-type deadenylases and pair-wise amino acid identities. Phylogenetic relationships were determined using Clustal Omega and visualized using Interactive Tree of Life [107,109].

NOCT promoter [52]. Interestingly, *NOCT* mRNA abundance in liver remains rhythmic in *clock*-deficient mice that lack a global circadian clock, as well as in the absence of a local circadian clock, suggesting that *NOCT* transcription is regulated by both local and global cues [53,54]. Furthermore, *NOCT* mRNA levels do not appear to be rhythmic in white adipose tissue from *ad lib* fed mice, suggesting that other cues may override clock function in certain contexts [55]. Consistent with this, there is evidence that mammalian *NOCT* transcription can be controlled by other transcription factors. Sequence analysis indicates the presence of binding sites for CRE [55], RevERBα[51], NFκB [10,56], and PPARγ [57]; and functional data support regulation by FoxO [58], PPARγ [57,59], STAT3 [60] and Nanog [60–62]. Furthermore, CLOCK-independent control of *NOCT* transcription is known in *Xenopus* photoreceptor cells and is mediated in part by CREB through a cis-regulatory element termed the Nocturnin Element [63,64]; however, this activity has not been extensively characterized.

The temporal alignment of peak liver *NOCT* mRNA abundance and mouse feeding behavior, and the increased NOCT protein and mRNA abundance in cultured cells following serum shock led to the hypothesis that NOCT may regulate gene expression in response to nutrient availability (Fig. 1) [65]. Surprisingly, the temporal profile of liver *NOCT* mRNA expression is not affected by fasting, nor is it altered in response to fasting followed by daytime refeeding when *NOCT* mRNA levels are low [55]. These data suggest that *NOCT* mRNA expression in liver is typically independent of nutrient availability; however, the timing of peak *NOCT* mRNA abundance in liver does shift when food availability is limited to daytime, suggesting *NOCT* mRNA levels can be regulated by nutrient availability under some circumstances [55]. Consistent with this idea, changes in *NOCT* mRNA abundance in response to feeding may be tissue specific. *NOCT* mRNA levels are constant in white adipose tissue from *ad lib* fed mice but become rhythmic in response to daytime feeding, and dietary fat induces *NOCT* mRNA expression in the proximal small intestine [19,55]. The connection between feeding and *NOCT* mRNA expression in certain contexts appears to be evolutionarily conserved, as several tissues in the goldfish *Carassius auratus* exhibit increased *noc-a* mRNA expression following a meal [16].

In some contexts, *NOCT* mRNA expression can also be induced under certain starvation conditions: in mice subject to daytime feeding, *NOCT* mRNA levels increase in white adipose tissue – but not liver – upon missing a feeding [55]. Starvation has also been connected with increased *curled* mRNA abundance in *Drosophila* [14], as well as time- and tissue-dependent induction of *noc-b* and repression of *noc-a* mRNA expression in *C. auratus* [16]. The links between *NOCT* expression and nutrient availability suggest a role for NOCT in modulating metabolic activity, perhaps regulating the expression of genes involved in energy uptake, storage, and utilization [9,15,16,19,55]. Whether nutrient availability and feeding regulate NOCT expression in humans remains to be explored.

NOCT has also been implicated in dietary lipid absorption and trafficking in the small intestine of mice (Fig. 1) [19]. In addition to induction in response to dietary fat, *NOCT* mRNA exhibits circadian fluctuations in abundance in the proximal portion of the small intestine where dietary lipids are absorbed, and nighttime NOCT protein abundance is highest in this region. Consistent with a role for NOCT in mediating lipid absorption and trafficking, intestines from *NOCT*-deficient mice accumulate excess lipids, and isolated enterocytes exhibit defects in lipid absorption and trafficking [19]. Together, these data indicate that NOCT activity may promote dietary lipid absorption and secretion, potentially forming the basis of *NOCT* knockout mouse resistance to high-fat diet-induced obesity and liver steatosis [9]; however, the mechanistic details of NOCT involvement in these processes is unclear. Moreover, the fate of unabsorbed dietary lipids is unclear as fecal lipid contents were reportedly unchanged between wild-type and *NOCT* knockout mice [19]. This finding remains paradoxical in light of the decreased intestinal lipid absorption and secretion [19] and reduced body temperature of *NOCT* knockout mice [9], as well as the absence of differences between *NOCT* knockout and wild-type mouse metabolic activity [9].

NOCT also functions during embryo development and later during mesenchymal stem cell differentiation (Fig. 1). Although NOCT is dispensable for mouse embryo viability, depletion of *NOCT* mRNA temporarily slows early development while overexpression leads to developmental arrest [66]. Later in development, STAT3 and Nanog-mediated *NOCT* transcription may promote stem cell pluripotency by repressing differentiation along mesoderm and endoderm lineages [60]. Developmental requirements for NOCT function have also been observed in *Xenopus* embryos, where manipulation of *NOCT* mRNA expression affects somite size, number, and organization [67,68]. Currently, NOCT's function during *Xenopus* somitogenesis is unclear, as is whether NOCT is required for embryo viability. These data suggest that NOCT modulates the stability of developmentally important mRNAs in both mouse and *Xenopus* embryos; consequently, identification of NOCT target transcripts in embryos would provide significant insight into its function during early development.

NOCT also functions during adipose and bone tissue differentiation (Fig. 1). Adipocytes and osteoblasts are derived from a common mesenchymal stem cell precursor [69], and NOCT has been suggested to influence the balance of these cell types through its pro-adipogenic and anti-osteogenic activities. Mice lacking NOCT have reduced fat pad mass [9], and work in cultured cells demonstrated that NOCT expression promotes adipocyte differentiation from pre-adipocyte precursors and bone marrow mesenchymal stem cells [18,70]. Consistent with these roles, NOCT mRNA and protein expression increase during early and late adipogenesis in 3T3-L1 preadipocytes [18,70]. The mechanism by which NOCT promotes adipogenesis is currently unclear, but has been suggested to occur through increased nuclear entry of peroxisome proliferator-activated receptor gamma (PPARγ), the key transcriptional regulator of fat metabolism and adipogenesis, via an association between NOCT and PPARγ at the nuclear periphery [18]. Mutation of a conserved Mg^{2+} coordinating residue, E193, did not affect this interaction, leading the authors to conclude that this role is independent

of NOCT catalytic activity [18]; however, mutation of the corresponding residue in human NOCT does not abrogate mRNA decay activity in cultured cells, indicating retention of mRNA regulatory activity by this mutant [71]. Another hypothesis for NOCT's involvement in adipogenesis is that NOCT facilitates mitotic clonal expansion (MCE) of pre-adipocytes during adipocyte differentiation. In cultured mouse cells, NOCT depletion is associated with reduced MCE and Cyclin D1 abundance [70]; however, how NOCT depletion leads to these reductions is unclear. In addition to reduced adiposity, mice lacking *NOCT* exhibited increased bone mass, suggesting that NOCT negatively regulates osteogenesis [18]. Consistent with an anti-osteogenic role, *NOCT* mRNA and protein expression is downregulated during osteogenic differentiation, and studies in bone marrow mesenchymal stem cells demonstrated an inverse relationship between NOCT expression and osteogenic differentiation [18]. NOCT-mediated repression of osteogenesis is thought to occur in part through downregulation of osteogenic transcripts [18,72,73]. Moreover, *NOCT* knockout mice are protected against reductions in bone density induced by rosiglitazone (a PPARγ agonist), although it is not clear if this results from the lost induction of pro-adipogenic genes and/or lost repression of pro-osteogenic genes [73].

NOCT may also function during cellular responses to acute stimuli. Following stimulation of mouse cells with serum [65], mitogens [65], or lipopolysaccharide [56], *NOCT* mRNA abundance rapidly increases and displays expression dynamics similar to those observed for primary response genes [65,74,75]. Mitogen-inducible *NOCT* mRNA and protein expression may be unusual among deadenylases; however, only four other deadenylases have been tested for changes in mRNA abundance in response to serum stimulation, and protein levels have only been measured for one additional deadenylase [65]. The functional consequences of increased NOCT expression in these cases are almost entirely untested; however, these observations suggest that induction of NOCT expression by acute stimuli would result in degradation of cohort of mRNAs, thereby remodeling the transcriptome. Thus far, NOCT protein expression has only been linked with increased stability of *inducible nitric-oxide synthase* (*iNOS*) mRNA, and knockout mice injected with LPS exhibit increased survival compared to wild-type mice [56]; at present, the mechanisms underlying these outcomes are unstudied.

Limited data is currently available regarding NOCT function and regulation in humans. Rhythmic changes in *NOCT* mRNA expression are observed in synchronized human liver cells [51]; however, only two reports have suggested rhythmic expression of *NOCT* mRNA in human oral mucosa [76] and skeletal muscle [49]. Currently, insight into potential biological roles for human NOCT is limited to findings from genome-wide association studies (GWAS) and mRNA expression studies. One GWAS in Chinese men observed a correlation between the intronic tag SNP rs9684900 and body mass index [77]; however, it is not clear whether this SNP affects NOCT function, or whether this SNP or a closely-linked locus is the source of the association. *NOCT* mRNA abundance has been correlated with increased body mass index, suggesting a possible but unverified role in adipogenesis similar to that observed in mice [77]. Another GWAS study suggested a link between the intronic *NOCT* SNP rs3805213 and non-small cell lung cancer [78], while yet another study observed a correlation between *NOCT* expression and increased survival among small cell lung cancer patients [79]. These associations are interesting, but whether or not NOCT activity directly causes these phenotypes remains untested.

In contrast with transcriptional regulation, post-transcriptional control of NOCT expression has received little attention. This is surprising because circadian and non-circadian inputs result in substantial *NOCT* mRNA induction exceeding the subsequent increases observed for the protein [56,65], suggesting that *NOCT* mRNA may be subject to post-transcriptional regulation or posttranslational control of protein stability. Further, the observed oscillations in NOCT mRNA level necessitate control of mRNA decay in addition to the aforementioned transcriptional control. Indeed, a recent study indicated that *NOCT* mRNA levels may be regulated by both rhythmic transcription and degradation in mouse liver [80]. Thus far only the liver-specific microRNA miR-122 has been shown to fine-tune the *NOCT* mRNA abundance profile in mouse liver [81].

Nocturnin has unique structure and regulatory activity

Sequence alignment of CCR4 orthologs reveals that they share substantial amino acid sequence identity and similarity within the EEP catalytic domain; in contrast, the N-terminus is less conserved and is unique to NOCT orthologs (Fig. 2 and 3). At present the function of the N-terminus is unclear; however, it may be important for NOCT-specific functions including protein-protein interaction domains, and/or contain sites for posttranslational modifications that may modulate catalytic activity. Recently reported NOCT crystal structures (6BT1 and 6BT2; Fig. 4) [71] and an independently determined structure (6DIP) [82] revealed that the catalytic domain structure closely resembles the CNOT6L and PDE12 catalytic domains, which all possess the characteristic hydrolase fold found in EEP family members and are active in vitro [39,83,84]. Conserved residues that are predicted to have catalytic functions cluster in the active site (Fig. 4B), and their arrangement is nearly identical to that of corresponding residues in CNOT6L and PDE12 [71]. Together, these data suggest that NOCT possesses exoribonuclease – and potentially deadenylase – activity. Beyond the active site, the structures of CNOT6L, PDE12, and NOCT are more divergent. One striking feature is that NOCT possesses a basic patch near the active site that has been suggested to function in substrate binding (Fig. 4C, D) [71,82]; however, this hypothesis has not yet been experimentally tested.

Despite the high degree of active site conservation among CNOT6L, PDE12 and NOCT (Fig. 2), new data indicate that highly purified, recombinant human and mouse NOCT are inactive against poly(A) RNA substrates in biochemical assays, whereas CNOT6L purified under the same conditions exhibited robust exonuclease activity [71]. These new observations contradict earlier studies performed using partially

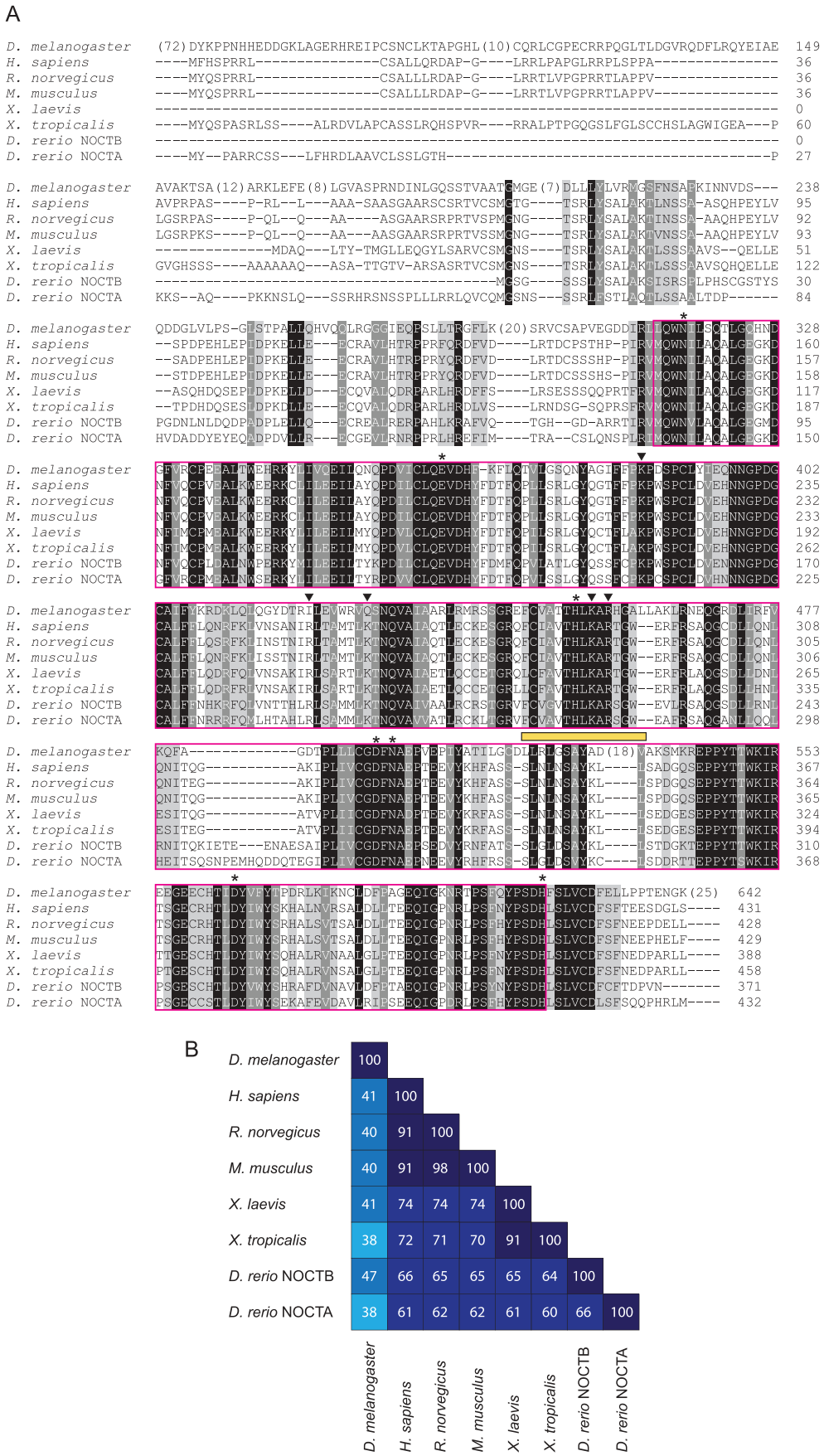

Figure 3. NOCT orthologs share a conserved EEP domain but have divergent N-termini. A) Amino acid sequence alignment of NOCT orthologs from various model organisms. Sequences were aligned and colored as in Fig. 2; darker shading indicates greater functional group similarity (charge, hydrophobicity, etc.). Strictly conserved positions are in white text with black background. Putative hNOCT catalytic (*) and basic patch residues (inverted triangles) are indicated. The putative PPARγ-interacting peptide is highlighted by a yellow bar. Uniprot accession numbers: *D. melanogaster* A8JQX3, *H. sapiens* Q9UK39, *R. norvegicus* Q9ET55, *M. musculus* O35710, *X. laevis* P79942, *X. tropicalis* Q28CV0, *D. rerio* NOCTB A0A0G2KRI0, *D. rerio* NOCTA E7F177. B) Pair-wise amino acid identities among NOCT orthologs.

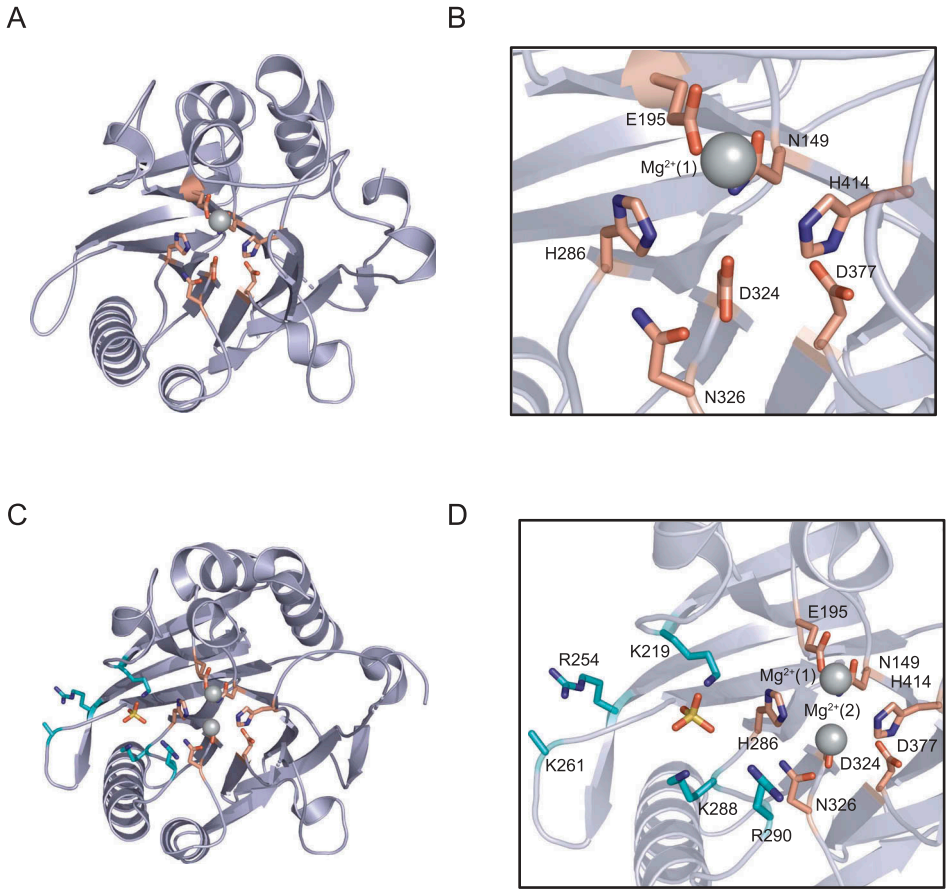

Figure 4. The structure of the hNOCT EEP catalytic domain (aa 120–431) reveals a conserved hydrolase fold. A) Overview of the 1.48 Å resolution structure (6BT1) [71]. Active site residues are shown; one Mg^{2+} ion (gray) is coordinated to E195 and ordered water molecules (not pictured). B) Magnified view of active site from (A). C) Overview of 2.48 Å resolution structure (6BT2) [71] with active site (salmon) and basic patch (cyan) residues shown. Two Mg^{2+} ions (gray), coordinated to E195 and D324, and one SO_4^{2-} ion (yellow) bound to lysine side chains adjacent to the active site are shown. D) Magnified view of active site and adjacent basic patch shown in (C).

purified recombinant mouse and *Xenopus* NOCT that suggested that these enzymes catalyze degradation of poly(A) RNAs [56,65,85]. This discrepancy may be explained by differences in the purification strategies used to obtain the recombinant proteins, as RNA degradation assays are highly susceptible to RNase contamination [71]. Indeed, recent efforts to characterize mouse and human NOCT exonuclease activity under published reaction conditions were unsuccessful in detecting activity [71]. Moreover, NOCT substrate specificity remains unknown, and recombinant NOCT is not active against a range of phosphorylated substrates including nucleic acids, phospholipids, or phosphosugars [71]. Therefore, the enzymatic activity of pure recombinant NOCT in biochemical assays remains unknown.

While NOCT is not active in biochemical assays, the ability of NOCT to regulate mRNAs is supported by in vivo experiments. When experimentally directed to a target reporter mRNA in a tethered function assay in HEK293 cells, human NOCT reduced reporter protein and mRNA expression [71]. Repression of reporter protein expression is diminished by mutations in the conserved residues N149, D377, and H414, which are located in the NOCT active site (Fig. 4A, B), while mRNA degrading activity is diminished by mutation of H414 [71]. Together these data indicate that NOCT catalytic function contributes to mRNA translational repression and degradation in cells. Surprisingly, mutation of a conserved glutamate in the human NOCT active site (E195A) does not relieve reporter repression [71]. This result is unexpected because this residue is involved in Mg^{2+} coordination (Fig. 4) [18,65,71,85], and the corresponding residue in CNOT6L (E240) is required for catalytic activity [71,83]. The basis for this unexpected result is presently unclear, but may indicate that other residues can provide compensatory Mg^{2+} coordination. The observation that the NOCT E195 mutant retains RNA decay activity also is significant because mutation of the analogous residue in mouse NOCT was previously used as a tool for analyzing exoribonuclease-independent activity [18]. In light of human NOCT E195A activity, it is uncertain whether the mouse E193A mutation can accurately discriminate exoribonuclease-dependent and -independent activities, confounding the interpretation of data suggesting that NOCT performs a deadenylase-independent function [57].

NOCT activity in vivo against substrate mRNAs requires the presence of an accessible 3′ end, as NOCT does not repress expression of a reporter mRNA terminating in a highly-structured MALAT1 triple helix [71]. Lack of repression in this context, as well as the absence of RNA decay intermediates, is consistent with exoribonuclease activity. At this time it is unclear whether NOCT activity requires that the

accessible 3′ end be composed of poly(A), or whether NOCT can act on any accessible 3′ sequence. In summary, cell-based analyses indicate that NOCT association with an mRNA can cause translational repression and mRNA decay, and that the active site residues contribute to these activities. Important insights will be gained by further exploring NOCT activity in vivo through the identification of its natural substrate RNAs and any protein partners that may be necessary for NOCT regulatory activity.

Do NOCT protein partners control its function?

Many RNA-degrading enzymes function in multisubunit complexes, and their enzymatic activity and substrate specificity can be dramatically altered by protein partners [13,86,87]. The predicted involvement of partner molecules – particularly RNA-binding proteins (RBPs) – in controlling NOCT activity is intriguing because such a partner could modulate the specificity and affinity of NOCT for target mRNAs as has been observed for other deadenylases (Fig. 5) [13,88]. Moreover, the discrepancy between NOCT in vivo and in vitro activity suggests that one or more protein partners is required for NOCT function; such a requirement has been observed previously for the PAN2 deadenylase [89,90]. Thus far, NOCT-interacting partners have not been comprehensively characterized, although one report using co-immunoprecipitation experiments in mammalian cells overexpressing tagged proteins demonstrated a physical interaction between mouse NOCT and PPARγ1 and PPARγ2 [18]. Subsequent analysis indicated that NOCT and PPARγ2 coexpression enhances PPARγ2 nuclear entry, and a ten amino acid region of NOCT (aa341–351) was proposed to mediate this interaction. Introduction of this peptide into cultured cells disrupts this interaction [18]; however, the NOCT-PPARγ interaction has not been directly tested via mutational analysis, nor is it clear whether this is a direct protein-protein interaction. Mapping this peptide onto the human NOCT catalytic domain structure reveals that few amino acid side chains in this peptide are surface-exposed and accessible for protein-protein interactions (Fig. 6), although structural rearrangements could theoretically increase their accessibility. Moreover, the functional effects of NOCT expression on PPARγ-regulated genes are also unclear. Interestingly, a genetic interaction was recently reported between *Drosophila curled* and the nuclear receptor *HR4* [91], suggesting that NOCT orthologs may have a conserved role in modulating nuclear receptor-mediated gene expression; however, it is not known if this interaction occurs at the protein level.

NOCT may also interact with the multimeric CNOT complex. Biochemical evidence from *Drosophila* and mammalian cell culture experiments using overexpressed, tagged proteins suggests that components of the CNOT deadenylase complex associate with NOCT [31,35,92]. Like *NOCT* knockout mice, *CNOT3*$^{+/-}$ and *CNOT7*$^{-/-}$ mice resist high-fat diet-induced obesity [33,93]; however, it is difficult to assess the extent of phenotypic similarity among these mice as they have not been directly compared under common conditions. The structural basis for any potential interaction between NOCT and members of the CNOT complex is also unclear, because NOCT lacks the leucine-rich repeats that mediate interaction of other CCR4 orthologs with the CNOT complex [11,94] (Fig. 2). Consequently, any interaction between NOCT and the CNOT complex would likely involve a novel mode of interaction and potentially represent a new subclass of CNOT complexes. An understanding of the full repertoire of NOCT-interacting proteins will be essential to our understanding of NOCT function, as such partners may control target mRNA selection and regulatory activities, as well as protein localization and stability. In the future, characterization of these interactions should include analysis of the effects of depleting these partners on NOCT-mediated regulation and whether the interaction is direct or indirect (Fig. 5).

Is NOCT activity spatially restricted?

Several deadenylases exhibit specific subcellular distribution [35,36–38,40–43], raising the question of whether NOCT activity may be spatially restricted. Studies of overexpressed tagged constructs found *Xenopus* NOCT to be cytoplasmic in photoreceptor cells [95], while mouse NOCT is perinuclear in human HEK293 cells [57] and is excluded from stress granules in mouse NIH3T3 cells [56]. Importantly, overexpression and the inclusion of tags could potentially lead to aberrant localization; consequently, it is important to also examine

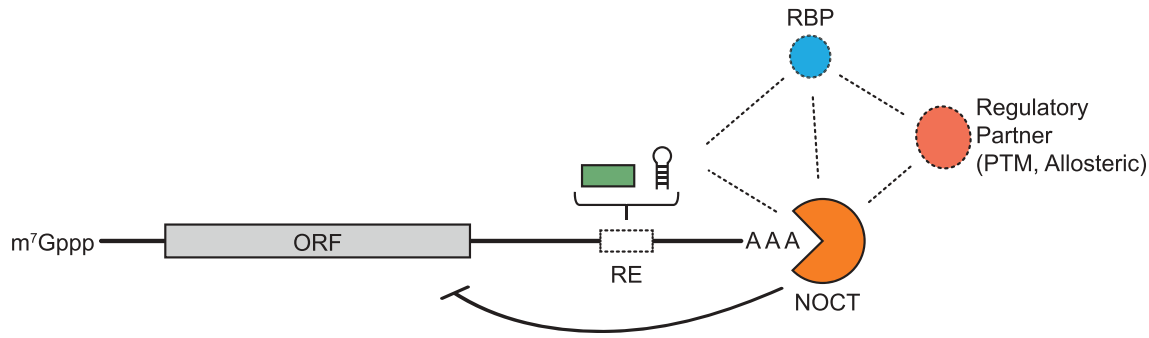

Figure 5. Hypothetical model for NOCT-mediated regulation of target mRNA transcripts. NOCT (orange) association with target transcripts may be direct or indirect. RNA-binding proteins (blue) and/or additional trans-acting factors (red) or post-transcriptional modifications (PTM) may be required for NOCT-mediated repression of targets. Cis-acting sequences regulatory element (RE) and/or structural elements may recruit NOCT or RNA-binding proteins (RBPs) to target transcripts. Association of NOCT complexes with substrate mRNAs represses expression of the encoded gene via RNA decay and possibly translational repression.

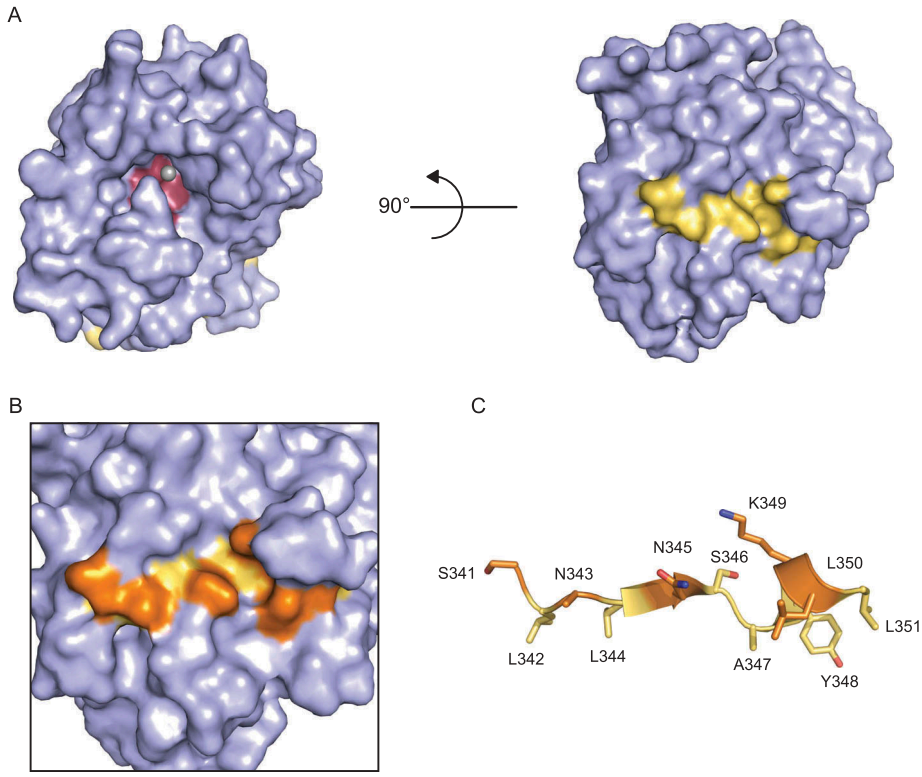

Figure 6. Amino acid side chains in the proposed PPARγ-interacting peptide are largely inaccessible. A) Surface rendering of NOCT 120–431 (6BT1; 1.48 Å resolution) [71]. The PPARγ-interacting peptide is highlighted in yellow and the active site is colored in red. One Mg^{2+} ion is coordinated in the active site. B) Magnified view of putative PPARγ-interacting peptide with solvent-accessible amino acid side chains highlighted in orange. C) Cartoon diagram view of the PPARγ-interacting peptide with solvent-exposed amino acid side chains colored in orange.

endogenous protein localization. Analysis of endogenous NOCT localization indicated both nuclear and cytoplasmic distribution in mouse embryos [66], and cytoplasmic localization in *Xenopus* retina [85]. Importantly, none of these studies were performed with sufficient resolution to confidently assign NOCT to a particular subcellular compartment, and its localization has not been confirmed by subcellular fractionation.

We investigated the potential for NOCT localization using sequence analysis tools to identify potential localization signals in NOCT. Analysis of the NOCT orthologs in Fig. 3 using NucPred [96], cNLS Mapper [97–99], and SeqNLS [100] tools did not predict nuclear localization, whereas PSORTII [101,102] predicted nuclear localization for *Drosophila, X. laevis*, and *D. rerio* NOCTB orthologs. Mitochondrial localization is predicted for several NOCT orthologs. PSORTII [101,102], TargetP [103], and MitoProt [104] all predict mitochondrial localization of *X. tropicalis, R. norvegicus, H. sapiens, M. musculus*, and *D. rerio* NOCTA. TargetP and MitoProt predictions indicate that *D. rerio* NOCTB is likely to be mitochondrial; however, this conflicts with the nuclear localization predicted by PSORTII. Neither TargetP nor MitoProt identify *D. melanogaster* or *X. laevis* NOCT as mitochondrial [103,104]. Although useful in predicting possible NOCT functions, these programs do not account for shuttling among subcellular compartments. Furthermore, it will be necessary to experimentally evaluate these predictions using multiple methods including high-resolution microscopy and cellular fractionation to examine endogenous NOCT distribution, as well as the identification and mutagenesis of localization signals.

What are the direct targets of NOCT?

A major remaining hurdle to understanding NOCT function is the identification of its target RNAs. As a putative deadenylase, NOCT is predicted to associate with target transcripts and catalyze removal of the poly(A) tail, triggering transcript decay. If NOCT functioned as a general deadenylase, NOCT depletion would be expected to result in widespread poly(A) tail length changes; however, no such effects have been observed [105], suggesting that NOCT activity is directed towards specific transcripts. Selection of target mRNAs may occur through direct target recognition by NOCT, or through an interaction with an RBP (Fig. 5). Regardless of the mechanism of target recognition, it is reasonable to expect that bona fide NOCT exoribonuclease targets should meet two general criteria regarding their abundance and association with NOCT. First, trends in target transcript abundance and/or poly(A) tail length should be inversely correlated with NOCT protein expression. Second, NOCT should associate with the target transcript in vivo either directly or indirectly via interaction with an RBP or other partner.

Initial attempts to identify NOCT targets utilized candidate-based approaches that were informed by the lean phenotype of *NOCT* knockout mice, and examined the

rhythmicity of *PPARγ* and *sterol regulatory element binding transcription factor 1c* (*srebp-1c*) transcripts and the overall abundance of *stearoyl-Coenzyme A desaturase 1* (*scd1), sterol regulatory element binding transcription factor 1a* (*srebp-1a*), and *liver specific fatty acid binding protein 1* (*l-fabp*) in livers from wild-type and knockout mice fed standard and high-fat diets. Unexpectedly, none of these transcripts exhibited changes consistent with direct NOCT targeting; instead, *PPARγ* expression remained constant in *NOCT* knockout mice fed a high-fat diet, whereas *scd1* and *l-fabp* levels were either unchanged or reduced in *NOCT* knockout mice fed either diet [9]. Similarly, analysis of candidate transcripts involved in regulating small intestine lipid dynamics revealed only transcripts that were either unaffected or decreased in abundance in *NOCT* knockout mice [19]. At present, physical associations between NOCT and these transcripts have not been tested. As the effects of NOCT-depletion on these transcripts are inconsistent with those expected of direct targets, it is likely that these transcripts are indirectly regulated by NOCT.

Other candidate-based approaches have sought to clarify NOCT pro-adipogenic and anti-osteogenic activities by identifying relevant target mRNAs. One study in mice examined *Igf1* mRNA, an important factor in regulating bone density, and found that *Igf1* may be regulated by NOCT in tissues outside the liver [72]. NOCT protein expression is inversely correlated with *Igf1* mRNA abundance in cultured cells and in vivo, reporter constructs bearing *Igf1* 3′ untranslated region fragments were responsive to NOCT levels, and *Igf1* mRNA physically associated with FLAG-tagged NOCT. Interestingly, NOCT differentially regulates *Igf1* reporter constructs bearing long and short *Igf1* 3′ UTRs, indicating mRNA isoform-specific targeting. Although these data support classification of *Igf1* as a NOCT target transcript, reporters bearing analogous *Igf1* 3′ UTR fragments from a different mouse strain are not regulated by NOCT, suggesting that *Igf1* is not universally regulated by NOCT. Other studies of NOCT anti-osteogenic activity observed an inverse correlation between NOCT expression and abundance of *alkaline phosphatase* [18], *osteocalcin* [18,73], *runt-related transcription factor 2* (*Runx2*) [18,73], *activating transcription factor 4* (*atf4*) [73]; and *osterix* [73]; however, physical associations between NOCT and these transcripts have not been tested.

Several transcriptome-wide studies in mice have sought to identify NOCT target RNAs on the basis of differential mRNA abundance, poly(A) tail length, and circadian expression following NOCT depletion. Microarray analysis of differentiated mouse 3T3-L1 adipocytes with and without *NOCT*-directed shRNA identified 273 mRNAs with more than two-fold change in abundance in response to NOCT depletion; however, only 89 were upregulated in the absence of NOCT [70]. At this time, there have been no further studies validating these 89 transcripts as potential NOCT targets. Another study compared transcript abundance among mRNAs with long and short poly(A) tails in wild-type and *NOCT* knockout mice at times of high and low NOCT protein abundance [105]. This analysis identified 319 mRNAs with altered poly (A) tail length in *NOCT* knockout livers; among these transcripts, 213 had longer poly(A) tails in *NOCT* knockout livers at either timepoint but only ten transcripts had longer poly(A) tails coincident with peak NOCT abundance [105]. Moreover, most transcripts identified in this analysis did not exhibit circadian changes in abundance or polyadenylation in wild-type mice as might be expected of a transcript subject to circadian deadenylation and/or decay [105].

The most recent study investigating NOCT targets used RNA-seq to compare the change in abundance between minimum and maximum circadian expression in wild-type and *NOCT* knockout mice [106]. A group of mRNAs encoding proteins involved in cholesterol and lipid biosynthesis exhibited a greater increase in abundance in *NOCT* knockout livers compared with wild-type, and had a peak abundance coincident with peak NOCT protein levels. Close analysis of these transcripts revealed changes in abundance, modest changes to poly(A) tail length distribution, and one transcript with significantly altered median poly(A) tail length. Consistent with the involvement of these target genes in lipid biosynthesis, *NOCT* knockout mice have increased plasma triglyceride concentrations at the time of peak NOCT protein expression; in contrast, plasma cholesterol is not significantly affected. Interestingly, although expression of these mRNAs coincided with *NOCT* mRNA expression in response to fast and refeeding, they were not significantly more abundant in refed *NOCT* knockout mice as would be expected for NOCT targets. Even so, the increased abundance of these mRNAs in the absence of NOCT coincident with peak NOCT protein expression in wild-type mice establishes these transcripts as the best candidate targets to date. Future studies aimed at validating these transcripts as direct NOCT target mRNAs should include analysis of NOCT interaction with the mRNAs and measurement of NOCT mediated repression of the encoded proteins. Moreover, the cis-acting sequences and/or secondary structures within these transcripts that may serve as NOCT specificity determinants should be explored.

Despite multiple attempts to identify NOCT targets using candidate and genome-wide approaches, at this time only *Igf1* meets both requirements for identification as a direct NOCT target; however, the strain-dependent nature of targeting indicates that it is not a universal target. One explanation for the apparent difficulty in identifying NOCT targets may the underlying assumption that because *NOCT* mRNA levels vary over the course of a day, NOCT protein levels and activity must also do so. Circadian changes in *NOCT* mRNA abundance have been well-documented for several species; however, NOCT protein levels are rarely examined and are less substantial than changes in mRNA abundance [56,72,81]. Additionally, whether NOCT catalytic activity is constitutive or dynamically regulated remains unknown. Alternatively, perhaps NOCT acts to degrade other RNA species. Recent reports have shown that certain deadenylases – although capable of deadenylating mRNAs – actually target noncoding RNAs [36,39]. If NOCT orthologs do indeed target mRNAs, it is likely that differential mRNA isoform and NOCT partner protein expression will intersect to produce tissue- and cell type-specific NOCT targeting; consequently, de novo identification of NOCT targets in relevant contexts will be necessary.

Concluding remarks

Over the last two decades, our understanding of NOCT function has expanded dramatically from its identification as a circadian-expressed transcript to its implication in metabolic regulation, development, and differentiation. While substantial progress has been made, many questions about NOCT's in vivo roles remain, and human NOCT remains largely unstudied. Based on the phenotype of *NOCT* knockout mice, it is probable that NOCT regulates a subset of metabolically and developmentally important transcripts in relevant tissues including the digestive tract, liver, and adipose tissues. Specificity in NOCT targeting may be achieved either through direct or indirect recognition of cis-acting sequences and/or structures in target mRNAs (Fig. 5). Upon association with these targets, NOCT may inhibit mRNA translation and promote transcript decay, most likely in collaboration with additional partner proteins. At present, the identities of such specificity determinants and protein partners are unknown, and are likely to be unique to specific tissues and conditions. Furthermore, the contributions of NOCT catalytic site residues to target regulation remain unclear and will require testing within the context of endogenous NOCT repression complexes with natural substrate RNAs. Detailed analysis of factors controlling *NOCT* ortholog transcription, stability, and translation will also provide substantial insight into NOCT function and may aid in the design of experiments aimed at identifying NOCT targets and interacting partners. Similarly, it will be important to determine whether NOCT is subject to post-translational control, as such modifications could modulate NOCT activity and stability in response to various stimuli. Insights into these aspects of NOCT biology will not only deepen our understanding of deadenylase functions in gene regulation, but also the molecular mechanisms underlying diet-induced obesity, potentially revealing therapeutic targets for preventing and/or reducing obesity.

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