CNOT3-Dependent mRNA Deadenylation Safeguards the Pluripotent State

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http://dx.doi.org/10.1016/j.stemcr.2016.09.007

SUMMARY

Poly(A) tail length and mRNA deadenylation play important roles in gene regulation. However, how they regulate embryonic development and pluripotent cell fate is not fully understood. Here we present evidence that CNOT3-dependent mRNA deadenylation governs the pluripotent state. We show that CNOT3, a component of the Ccr4-Not deadenylase complex, is required for mouse epiblast maintenance. It is highly expressed in blastocysts and its deletion leads to peri-implantation lethality. The epiblast cells in Cnot3 deletion embryos are quickly lost during diapause and fail to outgrow in culture. Mechanistically, CNOT3 C terminus is required for its interaction with the complex and its function in embryonic stem cells (ESCs). Furthermore, Cnot3 deletion results in increases in the poly(A) tail lengths, half-lives, and steady-state levels of differentiation gene mRNAs. The half-lives of CNOT3 target mRNAs are shorter in ESCs and become longer during normal differentiation. Together, we propose that CNOT3 maintains the pluripotent state by promoting differentiation gene mRNA deadenylation and degradation, and we identify poly(A) tail-length regulation as a post-transcriptional mechanism that controls pluripotency.

INTRODUCTION

Pluripotency is defined as the ability of a single cell to give rise to all the cell types formed by the three germ layers (Posfai et al., 2014). It is a unique property of the epiblast cells in early embryos, and can also be captured in embryonic stem cells (ESCs) in culture (Boroviak and Nichols, 2014; Martello and Smith, 2014). ESCs provide an invaluable platform from which to investigate the molecular mechanisms that regulate pluripotency. It has been shown that the pluripotent state in ESCs is controlled by a combination of signal-transduction pathways, transcription factors, epigenetic modifiers, RNA binding proteins, and regulatory RNA molecules (Dejosez and Zwaka, 2012; Hackett and Surani, 2014; Martello and Smith, 2014). Although extensive research has focused on the signaling, transcriptional, and epigenetic regulation in ESCs (Ng and Surani, 2011; Wang et al., 2014; Young, 2011), how post-transcriptional mechanisms can influence the ESC gene expression program and the pluripotent state has only begun to be revealed. Indeed, recent studies showed that post-transcriptional regulation, such as alternative splicing (Gabut et al., 2011), alternative polyadenylation (Lackford et al., 2014), RNA export (Wang et al., 2013), and RNA modification (Geula et al., 2015), plays critical roles in ESC maintenance and pluripotency (Wright and Ciosk, 2013; Ye and Belloch, 2014).

Most eukaryotic mRNAs are polyadenylated, and poly(A) tail lengths are important for post-transcriptional gene regulation (Eckmann et al., 2011; Norbury, 2013). It has been shown that mRNA deadenylation is a main determinant of mRNA poly(A) tail length, and can influence mRNA half-life and/or translation efficiency in different cellular contexts (Eckmann et al., 2011; Norbury, 2013). A recent study showed that the poly(A) tail length appears to have a more profound impact on mRNA stability than mRNA translation in cells with active transcription (Subtelny et al., 2014).

To investigate whether and how mRNA poly(A) tail length and deadenylation regulates embryonic development and pluripotent cell fate, we focused on the Ccr4-Not complex. Ccr4-Not is the main mRNA deadenylase complex in eukaryotic cells that shortens mRNA poly(A) tails (Collart and Panzenko, 2012; Shirai et al., 2014; Xu et al., 2014). It has been implicated in various physiological and developmental processes, such as spermatogenesis (Berthet et al., 2004), heart development (Neely et al., 2010), energy metabolism (Morita et al., 2011), B cell differentiation (Inoue et al., 2015), osteoporosis (Watanabe et al., 2014), reprogramming (Kamon et al., 2014), and ESC self-renewal (Hu et al., 2009; Zheng et al., 2012). However, its role in early development and the mechanism by which it regulates pluripotency remain to be fully elucidated. In this study, we focus on the CNOT3 subunit in the complex and present evidence that the poly(A) tail-length regulation by CNOT3 serves as a post-transcriptional regulatory mechanism governing early development and ESC maintenance.
RESULTS

\textbf{Cnot3 Expression Is Upregulated in the Blastocysts}

The Ccr4-Not complex is the main deadenylase complex in eukaryotic cells and regulates mRNA poly(A) tail length. To test the roles of Ccr4-Not and mRNA poly(A) tail length in mouse embryonic development, we focused on the \textit{Cnot3} subunit because its silencing resulted in prominent phenotypic and gene expression changes in ESCs (Zheng et al., 2012). We first examined Cnot3 expression during pre-implantation development. By qRT-PCR, we found that \textit{Cnot3} mRNA level is high in one-cell embryos, presumably from maternal expression, and is elevated again in blastocysts during pre-implantation development (Figure 1A). Immunofluorescence staining showed that Cnot3 protein expression is in agreement with the above pattern (Figure 1B). Furthermore, Cnot3 is enriched in the inner cell mass at the blastocyst stage. It predominantly localizes in the cytoplasm (Figure 1B), consistent with the notion that it is a part of the Ccr4-Not complex that regulates mRNAs.


**Cnot3 Is Required for Epiblast Maintenance**

To test its role in embryonic development, we generated a Cnot3 conditional deletion mouse model by conventional gene targeting (Figures S1A–S1D). We confirmed the successful depletion of the Cnot3 protein in the null embryos by immunofluorescence staining (Figure 1C). Because Cnot3 is required for ESC maintenance, we hypothesized that it may play important roles in the specification or maintenance of the epiblast. Consistent with the hypothesis, we found that Cnot3 deletion resulted in early embryonic lethality, as we were not able to recover any viable null pups or embryos with normal morphology at embryonic day 6.5 (E6.5) to E7.5 (Figures 1D–1F, S1F, and S2A). At E3.5 and E4.5, Cnot3 deletion embryos appear normal and were recovered at a Mendelian ratio (Figure 1F).

Furthermore, the expression pattern of the epiblast (Nanog), trophectoderm (Cdxi2), and primitive endoderm markers (Gata4, Gata6, Pdgfra) was comparable between the null and wild-type (WT) embryos (Figures 2A and S2B). Thus, Cnot3 may not be required for the formation of the epiblast.
Figure 3. CNOT3 C-Terminal Domain Is Required for ESC Maintenance

(A) Domain structure of mouse CNOT3.

(B and C) Induction of Cnot3 deletion in Cnot3 cKO ESCs. Cells were treated with or without 4-OHT, and Cnot3 expression was determined by qRT-PCR (B) and western blot (C) at the indicated time points. Values were plotted as mean ± SEM from three independent experiments.

(D) Interaction between CNOT3 fragments and CNOT1 or CNOT2. HA-tagged CNOT3 fragments were expressed in Cnot3 cKO ESCs and affinity purified by HA beads. Co-purified endogenous CNOT1 and CNOT2 were detected by western blot. Whole images of the same blots are shown in Figure S4B.

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of the blastocysts and the specification of the epiblast lineage, although we cannot rule out the possible contribution from maternal expression (Figures 1A and 1B).

To further test the role of Cnot3 in the maintenance of the epiblast, we used the embryonic diapause model. During diapause, the embryos are arrested in utero at the late blastocyst stage and the pluripotent state is maintained in the epiblast cells for an extended period of time (Fenelon et al., 2014). We found that Cnot3 was clearly required for the maintenance of the blastocysts during diapause, as the deletion embryos show significant compromise in morphology and reduction in size (Figures 1F and 2A; Movie S1). Quantitatively, Cnot3 deletion led to a decrease in the total cell number in the embryos. More importantly, it led to a reduction in the percentage of cells expressing epiblast markers Oct4 and Nanog, but not those expressing the trophectoderm marker Cad2 (Figures 2A and 2B), suggesting that epiblast cells were lost in the null embryos. To further support these findings, we carried out epiblast outgrowth studies. As expected, epiblast cells from Cnot3 null blastocysts failed to expand and grow into colonies, while trophectoderm cells continued to survive (Figure 2C). Together, our data support the notion that Cnot3 is required for the maintenance of the pluripotent epiblast cells in vivo.

CNOT3 C-Terminal Domain Is Required for ESC Maintenance
To understand how CNOT3 regulates the pluripotent state, we carried out structure-function analysis to determine the functional domain(s) in Cnot3. Based on sequence and structural information, the Cnot3 protein can be divided into the N-terminal, middle (NM), and C-terminal domains (Figure 3A) (Boland et al., 2013). We generated Cnot3 conditional knockout (cKO) ESCs in which Cnot3 mRNA and protein can be quickly depleted upon tamoxifen treatment (Figures S3A, 3B, and 3C). Cnot3 deletion led to ESC differentiation as indicated by obvious changes in cellular morphology and lineage marker expression (Figures S3B and S3C). Importantly, its deletion induced upregulation of differentiation gene markers even in the 2i + LIF medium that supports the ground state (Figure S3D). However, it did not induce obvious changes in caspase-3 cleavage (Figures S3E and S3F), suggesting that CNOT3 does not directly regulate apoptosis. In the Cnot3 cKO ESCs, we overexpressed CNOT3 full-length (FL), N-terminal and NM, and C-terminal domains (Figure S4A). Consistent with previous results from other cell types (Boland et al., 2013), we found that the C-terminal domain in CNOT3 is responsible for its interaction with CNOT1 and CNOT2 in the Ccr4-Not complex in ESCs (Figure 3D, and whole blot images in S4B). More importantly, the overexpression of either the CNOT3 full-length or C-terminal domain led to normal morphology and marker expression after the depletion of the endogenous CNOT3 by tamoxifen treatment (Figures 3E and 3F). Furthermore, the overexpression sustained the formation of normal ESC colonies at clonal density (Figures 3G and S4C). In contrast, overexpression of the CNOT3 N-terminal and NM domains (Figure S4A) had no effect (Figures 3E–3G), even though this fragment retained the interaction with Cnot1 (Figure 3D). Therefore, our results showed that the CNOT3 C-terminal domain is required for the maintenance of the pluripotent state, possibly via the interaction with CNOT1, CNOT2, and other unknown factors.

CNOT3 Negatively Regulates Differentiation Gene Expression
Because the Ccr4-Not complex regulates mRNA deadenylation, we hypothesize that CNOT3 may regulate the gene expression program in pluripotent cells post-transcriptionally. Indeed, immunofluorescence staining showed that CNOT3 localizes predominantly in the cytosol in both epiblast cells in vivo and ESCs in vitro (Figures 1B and S5A). Moreover, CNOT3 remained in the cytosol in ESCs even after nuclear export was blocked by leptomycin B treatment. These results are consistent with the idea that CNOT3 likely acts on mRNAs in the cytosol.

Gene expression analysis showed that Cnot3 deletion led to the upregulation of 840 genes and downregulation of 436 genes at the mRNA level (Figure 4A and Table S1; fold change >2, false discovery rate [FDR] <0.05). Gene ontology (GO) analysis showed that genes upregulated after Cnot3 deletion are highly enriched for those that are involved in normal differentiation and development (Figure 4B). Together, these results support the notion that CNOT3 inhibits differentiation and development of gene expression.

CNOT3 Promotes mRNA Degradation
To determine its role in the gene expression program in pluripotent cells, we first tested whether Cnot3 deletion has an impact on mRNA translation. We carried out polysome fractionation (Sampath et al., 2011) in both control and tamoxifen-treated Cnot3 cKO ESCs, and isolated...
polysome-associated RNAs. By RNA sequencing (RNA-seq), we found that Cnot3 deletion resulted in very similar changes in total RNAs versus polysome-associated RNAs (Figure S5B), suggesting that Cnot3 deletion did not have a significant impact on mRNA translation.

Next, we tested whether Cnot3 deletion affects mRNA stability. We inhibited transcription in the control and tamoxifen-treated Cnot3 cKO ESCs with actinomycin D, and determined the decay rate of all transcripts by carrying out RNA-seq at defined time points. We found that Cnot3

Figure 4. Cnot3 Deletion Leads to Increases in mRNA Half-Life and Steady-State Level
(A) Gene expression changes after Cnot3 deletion in ESCs. Cnot3 cKO ESCs were treated with (KO) or without 4-OHT (WT). Cells were collected 72 hr after treatment and total RNAs were prepared for RNA-seq.
(B) GO analysis of upregulated genes after Cnot3 deletion. Only selected GO categories were plotted. For complete list of enriched GO categories, see Table S2.
(C and D) Increase in mRNA half-life in a subset of genes after Cnot3 deletion. Cnot3 cKO ESCs were treated with (KO) or without 4-OHT (WT). Actinomycin D was added 48 hr after treatment, and cells were collected at 0, 4, and 8 hr after actinomycin D addition for RNA-seq.
(C) Frequency distribution and box plot for mRNA half-life in WT or KO samples. (D) Changes in mRNA half-life for genes down- or up-regulated after Cnot3 deletion.
(E) Venn diagrams showing the overlaps between genes with extended mRNA half-lives and those that are down- or upregulated after Cnot3 deletion.
deletion led to an increase in mRNA half-life (Figure 4C). Importantly, while the increase in half-life (fold change >2, FDR <0.05; see Experimental Procedures for details) was detected for many mRNAs that were upregulated after Cnot3 deletion, there was little change in half-life for those mRNAs that were downregulated (Figure 4D). Indeed, 194 mRNAs show increases in both stability and expression (Figure 4E, p < 3.9 × 10^{-12}) after Cnot3 deletion, which are likely direct targets of CNOT3. In contrast, there is no significant overlap between mRNAs with extended half-life and those with reduced expression. Notably, CNOT3 was recently suggested to regulate cell death-inducing genes in mouse embryonic fibroblasts (MEFs) (Suzuki et al., 2015). However, CNOT3 does not appear to regulate such cell death-inducing genes in ESCs, and there is no significant overlap between the 194 CNOT3 target genes in ESCs and those determined by similar methods in MEFs (Figure S5C). Together, these results suggest that CNOT3 specifically promotes the degradation of a subset of mRNAs in ESCs.

**Cnot3-Dependent Differentiation Gene mRNA Degradation Plays an Important Role in the Maintenance of the Pluripotent State**

Gene set enrichment analysis (GSEA) showed that the 194 CNOT3 target genes are highly enriched for those that are upregulated during normal ESC differentiation (Figure 5A), suggesting that CNOT3 promotes differentiation gene mRNA degradation. To further test this idea, we compared the gene expression changes caused by Cnot3 deletion with those caused by the depletion of pluripotency genes and overexpression of differentiation genes in ESCs. Unsupervised hierarchical clustering analysis revealed that the pluripotency and differentiation genes clustered separately and formed two distinct modules. Consistent with our hypothesis, Cnot3 deletion clustered with the overexpression of differentiation genes (Figure S5B), including those that are likely its direct targets such as Foxa1 and Sox9 (see below).

Intriguingly, the CNOT3 target genes showed shorter half-life compared with pluripotency genes in ESCs (Figure S5C), and their half-lives were extended during normal differentiation (Figure 5D). Indeed, the half-lives of differentiation gene mRNAs in general are shorter in ESCs and become longer during differentiation, while pluripotency gene mRNAs do not show the same trend. These results suggest that CNOT3-dependent regulation of differentiation gene mRNA stability may play an important role in the maintenance of the pluripotent state. Finally, we found that the CNOT3 target genes tend to be bivalently modified by H3K4me3 and H3K27me3 near their transcription start sites (Figure 5E), suggesting that CNOT3 further dampens the expression of poised developmental genes in pluripotent cells at the post-transcriptional level.

**Cnot3 Promotes Differentiation Gene mRNA Deadenylation**

To test whether CNOT3 may regulate the poly(A) tail length of its target mRNAs, we first examined global polyadenylation in WT and KO ESCs. We found that Cnot3 deletion led to a subtle increase in global polyadenylation (Figure S5D), suggesting that only a subset of mRNAs were affected. To further test the impact of Cnot3 deletion on specific mRNAs, we fractionated total RNAs from control or tamoxifen-treated Cnot3 cKO ESCs based on poly(A) tail length using oligo(dT) beads (Meijer and de Moor, 2011) (Figure 5A). To assess the fractionation efficiency, we added in vitro synthesized mRNA standards with defined poly(A) tail lengths (Figure S5E) to both WT and KO total RNAs before the fractionation. By qRT-PCR, we found that the added poly(A) tail standards were recovered from the different fractions with expected efficiency, confirming the validity of the procedure (Figure 5B). Using this method, we tested the impact of Cnot3 deletion on poly(A) tails of several genes, including the housekeeping gene Actin, the pluripotency genes Sox2, Oct4, Nanog, Esrb, Rex1, and the differentiation genes Cited1, Foxa1, Hand1, and Sox9. We found that Cnot3 deletion led to an increase in mRNA poly(A) tail length in differentiation genes Cited1, Foxa1, Hand1, and Sox9, without affecting the poly(A) tail length of housekeeping or pluripotency genes (Figures 5C and S5F). Interestingly, poly(A) tails appear to be longer in pluripotency than differentiation gene mRNAs (Figures 5C and S5F), consistent with our finding that pluripotency gene mRNAs tend to have longer half-lives (Figure 5C).

Consistent with the above data, qRT-PCR showed that while Cnot3 deletion led to increases in the stability of Hand1, Foxa1, Sox9, and Cited1 mRNAs, it had no impact on the stability of Actin or Sox2 mRNAs (Figure 5D). Furthermore, Cnot3 deletion led to an upregulation of the steady-state level of the differentiation gene mRNAs, but not that of Actin or Sox2 (Figure S3C). As we have previously shown that Cnot1, Cnot2, and Cnot3 from the Ccr4-Not complex are all required for ESC maintenance (Zheng et al., 2012), we also tested the effect of Cnot1 and Cnot2 silencing on the half-life of the differentiation gene transcripts. Unlike Cnot3 deletion, Cnot1 and Cnot2 silencing by small interfering RNAs showed very little impact (Figure S5G), suggesting that Cnot3 may play a unique role in post-transcriptional gene regulation in ESCs. This is consistent with our previous finding that Cnot3 silencing resulted in the most prominent phenotypic and gene expression changes in ESCs (Zheng et al., 2012), and is also consistent with the notion that individual subunits in the Ccr4-Not complex may differentially regulate its function (Azzouz et al., 2009). Finally, overexpression of Hand1, Foxa1, Sox9, and Cited1 induced ESC differentiation based on changes in cellular morphology (Figure S5H). Taken
Figure 5. Cnot3 Deletion Impairs Differentiation Gene Degradation

(A) GSEA showing the enrichment for differentiation genes in the 194 CNOT3 target genes from Figure 2E.
(B) Hierarchical clustering analysis showing the similarity in gene expression profiles after depletion of pluripotency genes and overexpression of differentiation genes (see Table S3 for gene expression datasets used in this plot).
(C) Box plot for the half-lives of CNOT3 target genes, pluripotency genes, differentiation genes, and all genes in ESCs.
(D) Box plot showing the changes in half-lives for CNOT3 target genes, pluripotency genes, differentiation genes, and all genes in ESCs during differentiation.
(E) Metagene analysis and box plots (based on yellow highlighted regions in the metagene analysis) for H3K4me3 and H3K27me3 occupancy at the transcription start sites (TSS) of the CNOT3 target genes and all genes.
together, we propose that CNOT3 promotes the deadenylation and degradation of the differentiation gene mRNAs to maintain the gene expression program in pluripotent cells (Figure 6E).

DISCUSSION

In pluripotent cells, the delicate balance between self-renewal and differentiation is tightly governed by a complex gene expression program. While transcription may function as an on-off switch to activate pluripotency genes and repress differentiation genes, post-transcriptional regulations can provide extra layers of control to refine the transcriptional output. In this study, we show that CNOT3, possibly via the Ccr4-Not complex, maintains pluripotency by promoting the deadenylation and degradation of differentiation gene mRNAs. Our finding reveals a previously uncharacterized mechanism in the maintenance of pluripotency, and provides additional evidence that post-transcriptional regulation is an integral part of the pluripotency regulatory network.

The length of poly(A) tails plays an important role in controlling mRNA degradation and translational silencing, and is therefore dynamically regulated during development and disease (Eckmann et al., 2011; Norbury, 2013). A recent study showed that poly(A) tail length predominantly correlated with mRNA stability in transcriptionally active cells. Furthermore, it showed that, during embryonic development, poly(A) tail length initially regulated translational efficiency and gradually shifted to regulate mRNA stability as zygotic transcription started (Subtelny et al., 2014). Our results are consistent with the above observations, and suggest that poly(A) tail-length regulation by Ccr4-Not may start to affect mRNA stability as early as at the blastocyst stage in mammals.

The Ccr4-Not complex is one of the main deadenylasers in mammalian cells that remove poly(A) tails. It can catalyze deadenylation in both gene- and context-specific manners to allow control of poly(A) tail lengths (Collart and Panasenko, 2012; Shirai et al., 2014; Xu et al., 2014). It has been proposed that different RNA binding proteins and/or different subunits in the Ccr4-Not complex may mediate the recruitment and regulation of different target mRNAs. For example, in germ cell development, NANOS2 and NANOS3 can interact with different Ccr4-Not subunits to regulate different mRNA targets (Suzuki et al., 2014). In ESCs, PUM1 facilitates the exit from the pluripotent state by binding to pluripotency gene mRNAs and promoting their degradation (Leeb et al., 2014), presumably via the interaction with CNOT7/8 in the Ccr4-Not complex (Van Etten et al., 2012). Thus, we propose that there may exist specific RNA binding proteins that facilitate the regulation of differentiation gene mRNAs by CNOT3 and Ccr4-Not in pluripotent cells. This is consistent with the “RNA Regulon” hypothesis, which suggests that mRNAs encoded by functionally related genes may be coordinately regulated by specific RNA processing machineries (Keene, 2007). Along similar lines, it was recently reported that m6A mRNA methylation reduces pluripotency gene mRNA stability to facilitate the exit from the pluripotent state in ESCs and that m6A depletion led to post-implantation lethality (Geula et al., 2015). Although it is not clear whether Ccr4-Not is involved in the m6A-mediated mRNA regulation, these and our results highlight the critical role of post-transcriptional regulation in the maintenance of the pluripotent state both in vitro and in vivo.

Finally, we found that CNOT3 target gene mRNAs have shorter half-lives in ESCs and acquire longer half-lives during differentiation. This result suggested that mRNA stability may indeed be a critical regulatory step, and that CNOT3, as well as Ccr4-Not, plays an important role in such a regulation to control pluripotent cell-fate specification. In addition, we found that CNOT3 target genes possess both active and repressive histone markers at their promoters. Promoter regions with bivalent histone modifications were initially discovered in ESCs at developmental genes, and were later shown to be present in epiblast cells during mouse development (Voigt et al., 2013). These bivalent genes are transcriptionally poised and can be activated upon suitable developmental cues to facilitate the exit from pluripotency. We propose that in addition to the transcriptional repression, differentiation genes may be further silenced by the Ccr4-Not complex at the mRNA level. Regulation of mRNA poly(A) tail length and stability may provide a quick and potentially reversible means to influence differentiation gene expression. Thus, by controlling the timely expression of differentiation genes, CNOT3 plays an essential role in maintaining the responsiveness of pluripotent cells to developmental signals.

EXPERIMENTAL PROCEDURES

Antibodies

Mouse anti-CNOT3 (H00004849-M01, Abnova), goat anti-OCT3/4 (SC-8628, Santa Cruz Biotechnology), mouse anti-OCT3/4 (SC-5279, Santa Cruz), rabbit anti-Cleaved Caspase-3 (ASP175, Cell Signaling Technology), goat anti-GATA6 (AF1700, R&D Systems), rat anti-PDGFRα (14-1401, eBioscience), goat anti-GATA4 (SC-1237, Santa Cruz), rabbit anti-NANOG (RCA0052-P, Cosmo Bio), mouse anti-CDX2 (CDX-88, Biogenex) and mouse anti-Cyclin B1 (AS4135, Cell Signaling).

Donkey anti-goat-493 (N0003, R&D), donkey anti-mouse-594 (A21203, Life Technologies), donkey anti-rabbit-647 (A31573, Life Technologies), goat anti-mouse-594 (A11005, Life Technologies), goat anti-mouse-488 (A11001, Life Technologies), goat
et al., 2010).

Whole embryos (Strumpf et al., 2005). Image data were acquired using standard protocols for fluorescence staining was carried out using standard methods for WT ESCs were treated with 10 nM leptomycin B for 4 hr. For measurement of mRNA stability, WT ESCs were treated with 10 µg/mL actinomycin D (Sigma, A-9415), and cells were harvested at 0, 4, and 8 hr after the treatment. The Cnot3 flox/+ mice were backcrossed into the 129 strain background and bred with the UBC-Cre/ERT2 transgenic mice. Individual blastocyst embryo was plated, and the resulting ESC lines were genotyped by PCR and Southern blot. Multiple Cnot3 flox/flox, UBC-Cre/ERT2 clones were established, and clone 8-4 was used for all the experiments. To induce Cnot3 deletion, we added 4-hydroxy-tamoxifen (4-OHT, Sigma) to the medium at 0.1 µM.

Figure 6. Cnot3 Domain Cloning and Expression

Mouse CNOT3 fragments corresponding to amino acids 1–751 (full length), 1–636 (NM), and 605–751 (C) were cloned from Mouse CNOT3 fragments corresponding to amino acids 1–751 (full length), 1–636 (NM), and 605–751 (C) were cloned from striatal embryonic day 18 (E18) embryonic day 18 (E18) regions. For rescue of the Cnot3 cKO ESCs to create stable expression clones, expression of the exogenous CNOT3 fragments was verified by qRT-PCR and western blot. Two to three clones expressing the same fragment were tested and behaved similarly in the rescue of the Cnot3 deletion phenotype, and the results from one clone were included in the figures.

Immunoprecipitation

Cnot3 cKO lines stably expressing hemagglutinin (HA)-tagged CNOT3 domain fragments were lysed in 1% NP-40 buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1% NP-40) with EDTA-free protease inhibitors (Roche). Lysates were sonicated, centrifuged, and pre-cleared with protein-A Sepharose beads. HA-tagged fragments were immunoprecipitated with HA-affinity matrix (Roche), and protein was eluted with 2× lithium dodecyl sulfate buffer with 2-mercaptoethanol and boiling. Proteins were resolved on 4%–12% Bis-Tris gels (Life Technologies) and transferred to nitrocellulose membranes. Western blots were conducted using an HA antibody from Cell Signaling and Cnot1, Cnot2 antibodies from Proteintech.
**Colony Formation Assay**

Cnot3 cKO ESCs stably expressing Cnot3 domain fragments were treated with 4-OHT at 0.1 μM for 48 hr. Cells were replated at 1,000 cells/cm² in 6-cm plates and used for growth to allow 7 days. The colonies were stained for alkaline phosphatase activity (AP Staining II kit, Stemgent). One hundred colonies were counted and scored as being normal, partially differentiated, or fully differentiated based on intensity of alkaline phosphatase staining and cellular morphology.

**qRT-PCR and RNA-Seq**

Total RNAs were extracted using the GeneJET RNA Purification Kit (Thermo), and reverse transcribed using the iScript (Bio-Rad, for qRT-PCRs) or the SuperScript II kit (Life Technologies, for poly(A) tail-length determination). qPCRs were performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on CFX 384 (Bio-Rad).

For RNA-seq, sequencing libraries were prepared using the TruSeq RNA sample preparation kit v2 using the low sample protocol (Illumina). Sequencing was performed on the MiSeq or NextSeq sequencer (Illumina).

**Polysome-Associated RNA Purification**

Polysome-associated RNAs were purified as previously described (Sampath et al., 2011). In brief, Cnot3 cKO ESCs were treated with or without 4-OHT at 0.1 μM for 72 hr. Cytosolic proteins were extracted and loaded onto linear sucrose gradients of 15%–45% (w/w) and centrifuged for 2.5 hr at 30,000 rpm in an SW-41 rotor (Beckman). The gradients were fractionated into 0.4-ml fractions on a Gradient Master (BioComp) and polysome fractions were pooled. RNAs were extracted from both pooled polysome fractions and total cell lysis and used for RNA-seq.

**Poly(A) Tail-Length Analysis**

DNA templates used for poly(A) tail-length standards were cloned by PCR into the PCR-blunt II vector. Poly(A) tail-length standards were generated by first synthesizing mRNAs from the DNA templates by in vitro transcription (RiboMax, Promega) and then adding poly(A) tails using the poly(A) polymerase tailing kit (Epicenter) with different reaction times. Products from the poly(A) tailing reactions were purified by denaturing PAGE and analyzed on Agilent’s Bioanalyzer to determine the poly(A) tail lengths.

Separation of mRNAs based on poly(A) tail length was performed based on a published protocol (Meijer and de Moor, 2011) with modifications. In brief, 1 μg of total RNA was first mixed with nuclease-free water and then incubated with Oligo-dT Dynabeads (Life Technologies) at room temperature for 10 min. The supernatant (the A0 fraction) was kept, and the elution solution was added to the beads. The elution was carried out at 25°C for the A1 fraction, and then again at 75°C for the A2 fraction. RNAs in each fraction were ethanol precipitated, and reverse transcribed with the SuperScript II kit using random primers (Life Technologies). The percentage of individual transcript in each fraction relative to that in 1 μg of total RNA was determined by qRT-PCR.

**Bioinformatics Analysis**

Sequenced reads were aligned to the mouse genome (mm9) using the latest version of STAR (Dobin et al., 2013) by allowing a maximum of three mismatches and retaining only unique alignments. Gene expression raw counts were quantified by counting aligned reads using the htseq-count program (Anders et al., 2015) with Ensembl gene annotations. Raw gene counts were subsequently normalized using the DESeq2 R package (Love et al., 2014) for each replicate of the WT and Cnot3 KO comparison experiment; and for each time point (0 hr, 4 hr, and 8 hr) and each replicate of the WT and Cnot3 KO half-life experiment. For the WT and Cnot3 KO comparison experiment, normalized gene counts were used to identify differentially expressed genes using DESeq2. Genes with an FDR smaller than 0.05 and a fold change greater than 2 were deemed as differentially expressed.

For RNA half-life estimation, genes that have fewer than six normalized read counts at all three time points (0, 4, and 8 hr) in either WT or Cnot3 KO were deemed as lowly expressed and were removed. For genes that passed the filtering, their half-lives were estimated as described in Sharova et al. (2009). First, since the same amount of each sample was sequenced for each time point, gene expression at 4 hr and 8 hr was corrected by timing a scaling factor to account for relative increase of stable mRNA due to the degradation of unstable species after block of transcription. Then a linear regression of the form \( y = a - bt \) was performed on each gene through the three time points, where \( y \) is the log-transformed (base 10) read count, \( t \) is the time, \( b \) is the slope, \( a \) is intercept, and \( d = b \times \ln(10) \) is the instantaneous decay rate. Half-life of each gene was estimated and projected as \( H = \min(24, \ln(2)/d) \) for positive \( d \) and \( H = 24 \) hr for negative \( d \). Genes that displayed significant difference of half-life in WT and Cnot3 KO were determined by requiring a fold change greater than 2 and an FDR smaller than 0.05, calculated as \( z = (d_1 - d_2)/\sqrt{s_1^2 + s_2^2} \), where \( d_1 \) and \( d_2 \) are decay rates estimated from regression for WT and Cnot3 KO, respectively, and \( s_1 \) and \( s_2 \) are standard errors of the estimated decay rates.

Fisher’s exact test was performed on overlap of gene sets that were significant in the half-life experiment and in the differential expression experiment, and on overlap of gene sets that are significant in WT versus Cnot3 KO in ESCs and MEFs (Suzuki et al., 2015). GSEA was performed to test for enrichment of gene set against EB differentiation data at day 9 (Hailesellasse Sene et al., 2007). Hierarchical clustering was performed to generate the heatmap profile and clustering global expression change after knockout, knockdown, or overexpression of each given gene (Correa-Cerro et al., 2011; Zheng et al., 2012). ESC-associated genes and differentiation-associated genes were defined by taking the top 5% and the bottom 5% of the genes ranked in Cinghu et al. (2014). Tag densities of H3K4me3 and H3K27me3 were generated by aligning H3K4me3 (Agarwal and Jothi, 2012) and H3K27me3 (Ho et al., 2011) sequencing data to mm9 genome using Bowtie (Langmead et al., 2009) allowing two mismatches and retaining only unique alignment.

**ACCESSION NUMBERS**

Sequencing data have been uploaded to the GEO database with the accession number GEO: GSE84953.
SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, four tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.09.007.

AUTHOR CONTRIBUTIONS

G.H. and X.Z. conceived the study. X.Z., B.L., L.W., H.L., L.M., Y.W., Y.M., J.E.F., and G.H. carried out the experiments. P.Y. and B.D.B. analyzed the data. G.H., R.J., C.J.W., D.C.F., and Y.J. interpreted the data. G.H. and X.Z. wrote the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Anna-Katerina Hadjantonakis for helpful discussions. We thank the NIEHS Animal, Epigenomics, Bioinformatics, Protein Expression, Imaging, and Histology core facility for assistance with various techniques and experiments. This study was supported in part by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences Z01ES102745 (to G.H.), Z01ES102985 (to C.J.W.), and Z01ES102625 (to R.J.).

Received: April 12, 2016
Revised: September 14, 2016
Accepted: September 15, 2016
Published: October 13, 2016

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Zheng_Figure S1

(A) Diagram of the genomic structure of the Cnot3 locus in different alleles:
- **Wild type**: KpnI sites at 17.0 kb and 4.1 kb.
- **Targeting vector**: Frt-SA-IRES-LacZ-Puro-Frt-LoxP and LoxP-Frt3-Neo-Frt3.
- **LacZ allele**: KpnI at 10.5 kb.
- **Conditional KO allele**: Primer1 and primer4 sites.
- **KO allele**: KpnI at 13 kb.

(B) Gel analysis of different genotypes:
- +/+ and LacZ+.

(C) Genotyping by PCR:
- Cnot3\textsuperscript{flf}, Cre-ER.
  - +/+ and +/F.
  - -/-.

(D) Southern blot analysis:
- 650bp, 300bp, and 100bp bands.
  - KO and WT.
(A) E7.5

(B) GATA6/NANOG/Dapi

(C) PDGFA/NANOG/Dapi

(D) GATA4/NANOG/Dapi
Zheng_Figure S3

(A) 24 hr 48 hr

|        | Cnot3\(^{3\text{ff}}\), Cre-ER | Cnot3\(^{3\text{ff}}\), Cre-ER | Cnot3\(^{3\text{ff}}\) |
|--------|---------------------------------|---------------------------------|---------------------|
| Cre    | ![Image](image1)                 | ![Image](image2)                 | ![Image](image3)    |
| LoxP1  | ![Image](image4)                 | ![Image](image5)                 | ![Image](image6)    |
| LoxP2  | ![Image](image7)                 | ![Image](image8)                 | ![Image](image9)    |
| Cnot3 KO | ![Image](image10)          | ![Image](image11)                | ![Image](image12)   |
| B2M    | ![Image](image13)                 | ![Image](image14)                 | ![Image](image15)    |
| 4OHT   | ![Image](image16)                         | ![Image](image17)                           | ![Image](image18) |

(B) -4OHT +4OHT

72 hours

(C) Serum + LIF

(D) 2i +LIF

(E) -4OHT +4OHT

(F) 72 hours

C-CASP3
CNOT3
RAN
(A) Cnot3 Relative mRNA Expression

-4OHT +4OHT

C3 Fragment Overexpression

C3 cKO cKO+FL cKO+N cKO+C

(B) Lysate IP Lysate IP Lysate IP Lysate IP

C3 cKO FL NM C

IP:HA

MW (kD)

Un-differentiated Partially differentiated Fully differentiated

*non-specific bands

(C) Un-differentiated Partially differentiated Fully differentiated
**Zheng_Figure S5**

**A**
Control vs Leptomycin B

**B**

![Polysome (WT vs KO)](image)

Mass Total RNA (µg)

Total log2 FC (WT vs KO)

**C**

![Graph showing ESC and MEF cells](image)

**D**

![Relative Oligo-dT Hybridization Signal](image)

**E**

![Graph showing Mass Total RNA (µg)](image)

**F**

![Relative abundance](image)

**G**

![Relative abundance over time](image)

**H**

![Images of GFP, Hand1, Foxa1, Sox9, and Cited1](image)
Supplemental Figure Legends

Figure S1. Cnot3 gene deletion strategy. (A) Gene targeting strategy for Cnot3. (B-D) Genotyping of Cnot3 cKO mice (B) and ESCs (C) by Southern blotting, and Cnot3 deletion embryos by genomic PCR (D).

Figure S2. Cnot3 deletion does not impact trophectoderm and primitive endoderm formation. (A) Hematoxylin and eosin stain of E7.5 WT and Cnot3 KO embryo sagittal sections (white bar = 200 µm). Genotypes of the embryos were determined by laser capture microdissection. (B-C) Immunofluorescence staining of primitive endoderm markers GATA6, PDGFRα, GATA4 and epiblast marker NANOG in E4.5 WT and Cnot3 deletion embryos (white bar = 20 µm).

Figure S3. Cnot3 is required for ESC maintenance. (A) Genotyping by genomic PCR in Cnot3 cKO ESCs treated with (KO) or without (WT) 0.1 µM 4-OHT for 24 and 48 hours. B2M primers amplify a genomic regions present in both WT and KO cells and serves as a control for PCR. (B) Morphological changes of Cnot3 cKO ESCs 72 hours after 4-OHT treatment (white bar = 200 µm). (C-D) Changes in marker gene expression 72 hrs after 4-OHT treatment in the serum+LIF medium (C) or the 2i+LIF medium (D), as determined by RT-qPCR. Expression was normalized by Actin, and values were plotted as Mean ± SEM from 3 independent experiments. (E) Immunofluorescence staining (white bar = 10 µm) and western blot (F) of cleaved-Caspase3 in WT and KO ESCs.
**Figure S4. C-terminal domain in CNOT3 is required for ESC maintenance.** (A) Overexpression of CNOT3 fragments in Cnot3 cKO ESCs. Cells were treated with or without 4-OHT for 48 hrs, and the expression of total Cnot3 (endogenous Cnot3 and exogenous Cnot3 fragment) was determined by RT-qPCRs. Expression was normalized by Actin, and values were plotted as Mean ± SEM from 3 independent experiments. (B) Interaction between CNOT3 fragments and CNOT1 or CNOT2. HA-tagged CNOT3 fragments were expressed in Cnot3 cKO ESCs and affinity purified by HA-beads. Co-purified endogenous CNOT1 and CNOT2 were detected by western blot. Cropped images of the same blots were shown in Figure 3D. (C) Representative images of alkaline phosphatase stained colonies from Figure 3G (white bar = 100 µm).

**Figure S5. CNOT3 regulates differentiation gene mRNA expression and deadenylation.** (A) CNOT3 localization in ESCs. ESCs were treated with or without leptomycin B, and protein localization was determined by immunofluorescence staining. Localization of Cyclin B1 served as a positive control (white bar = 20 µm). (B) Comparison of changes in polysome-associated vs. total RNAs between WT and Cnot3 KO ESCs. Cnot3 cKO ESCs were treated with (KO) or without 4-OHT (WT) for 72 hrs, and both total and polysome-associated RNAs were extracted and sequenced. Log2 fold-changes were calculated for all detected total or polysome-associated RNAs in WT and KO cells and plotted. (C) Venn diagram comparing CNOT3 target genes in ESCs (194 genes) and MEFs (486 genes). p-value represents the test for depletion in overlapping genes, and was calculated by the Fisher Exact test. (D) Quantitation of global polyadenylation in WT and Cnot3 KO ESCs. Cnot3 cKO ESCs were treated with (KO) or without 4-OHT (WT) for 48 hrs, and global polyadenylation in total RNAs were determined by biotinylated Oligo-dT
hybridization. Relative hybridization signal intensity values were plotted as Mean ± SEM from 3 independent experiments. (E) Standards used for poly(A)-tail length determination. Each poly(A) standard was synthesized by in vitro transcription followed by poly(A) tailing, and the length of the poly(A) tail was estimated by the size difference between the in vitro transcribed and the polyadenylated standard using bioanalyzer. The estimated poly(A)-tail length for the standards are: p(A)-SD1 = 0 A; p(A)-SD2 = 30 A; p(A)-SD3 = 100 A. (F) Measurements of poly(A)-tail length of pluripotency gene mRNAs based on the Oligo-dT fractionation method. Values were plotted as Mean ± SEM from 3 independent experiments. (G) Examination of mRNA stability for the indicated genes. E14 ESCs were transfected with Cnot1 or Cnot2 siRNAs. Actinomycin-D was added to the cells 48 hrs after transfection, and mRNA level was measured by RT-qPCR at the indicated time points after Actinomycin-D treatment. Relative expression values were plotted as Mean ± SEM from 3 independent experiments. (H) Overexpression of CNOT3 target genes in ESCs. E14 cells were transfected with plasmids expressing the indicated genes using Lipofectamine 2000. Cell morphology was imaged 72 hrs after transfection (white bar = 200 µm).
Supplemental Tables

Table S1: Changes in mRNA half-life and steady-state level after Cnot3 deletion (excel attached)
Table S2: Gene ontology analysis for up-regulated genes in *Cnot3* KO ESCs

| GO term                                                   | p-value     | -LOG(p-value)          |
|-----------------------------------------------------------|-------------|------------------------|
| developmental process                                     | 8.58E-12    | 11.06662882            |
| multicellular organismal development                      | 2.07E-11    | 10.68417222            |
| system development                                        | 2.71E-10    | 9.566792523            |
| organ development                                         | 4.15E-10    | 9.381632278            |
| anatomical structure development                          | 1.04E-09    | 8.981101734            |
| cell differentiation                                      | 7.84E-09    | 8.105426255            |
| cellular developmental process                            | 2.06E-08    | 7.685927377            |
| Wnt receptor signaling pathway, calcium modulating pathway| 2.80E-08    | 7.553361502            |
| placenta development                                      | 8.41E-06    | 5.075135325            |
| anatomical structure morphogenesis                        | 9.44E-06    | 5.024928977            |
| embryonic placenta development                            | 2.59E-05    | 4.586413727            |
| heart development                                         | 3.12E-05    | 4.505344342            |
| embryonic organ development                               | 1.04E-04    | 3.981376088            |
| tissue development                                        | 1.55E-04    | 3.809826638            |
| anatomical structure formation involved in morphogenesis  | 1.56E-04    | 3.805510393            |
| vasculature development                                   | 1.81E-04    | 3.743337562            |
| angiogenesis                                              | 2.79E-04    | 3.55503242             |
| cell adhesion                                             | 2.93E-04    | 3.533834593            |
| biological adhesion                                       | 3.00E-04    | 3.522609248            |
| blood vessel development                                  | 3.31E-04    | 3.479932407            |
| cell development                                          | 4.46E-04    | 3.351019422            |
| organ morphogenesis                                       | 4.77E-04    | 3.321027603            |
| cellular amino acid derivative biosynthetic process       | 5.74E-04    | 3.241170939            |
| cellular process                                          | 6.69E-04    | 3.17483647             |
| negative regulation of cellular process                   | 7.12E-04    | 3.147793718            |
Table S3: Gene expression datasets used for data analysis

| Citation            | PMID    | Accession | Gene(s)                        |
|---------------------|---------|-----------|--------------------------------|
| Yamaji et al. (2013)| 23333148| GSE42580  | Prdm14 KO                      |
| Oldfield et al. (2014)| 25132174| GSE56840  | NF-YA,B,C triple KD            |
| Cinghu et al. (2014)| 24711389| GSE47872  | Ncl KD                         |
| Loh et al. (2006)   | 16518401| GSE4189   | Oct4 KD                        |
| Leeb et al. (2010)  | 20123906| GSE19076  | Eed, Ring1B KO                 |
| Shen et al. (2008)  | 19026780| GSE12982  | Ezh2 KO                        |
| Jiang et al. (2008) | 18264089| GSE9775   | Klf2,4,5 triple KD             |
| Ho et al. (2009)    | 19279218| GSE14344  | Brg KO                         |
| Ho et al. (2011)    | 21785422| GSE27708  | LIF withdrawal (Stat3)         |
| Freudenberg et al. (2012)| 22210859| GSE34887  | Tet1 KD                        |
| Ivanova et al. (2006)| 16767105| GSE4679   | Esrrb, Nanog, Sox2, Tbx3, Tcl1 KD |
| Merrill et al. (2011)| 21685894| GSE27455  | Tcf3 KO                        |
| Ding et al. (2009)  | 19345177| GSE12078  | Paf1 KD                        |
| Lim et al. (2008)   | 18804426| GSE12482  | Sall4 KD                       |
| Yamamizu et al. (2011)| 25371362| GSE31381  | Gata2, Gata3, Dtx3, Eomes, T, Ascl2, Cdx2, Sox9, Ascl1, Rhox6, Foxa1, Sox7, Tbx5 OE |
### Table S4: Primers used in this study

| qPCR primers  | Primer Name      | Forward Sequence                  | Reverse Sequence                   |
|---------------|------------------|-----------------------------------|------------------------------------|
| mCnot3        | Cnot3-F          | AGAGGCCGATCTACAGATAGTGA           |                                    |
|               | Cnot3-R          | GACAGGCTTGGAGCCAATTT              |                                    |
| mHand1        | Hand1-F          | TCTGGCTCGCTCTCTCGTCC              |                                    |
|               | Hand1-R          | CTGGAGAAGCATCAGGGTA               |                                    |
| mCited1a      | Cited1a-F        | TCGAGGCTGCACTTGATGTCGAAG          |                                    |
|               | Cited1a-R        | ATCCCTCACTCCAAAGTTGGAGTAG         |                                    |
| mSox9         | Sox9-F           | ATCTGCAACACCGGAGCTCA              |                                    |
|               | Sox9-R           | CTCTTCTCGCTCTGCAGCA              |                                    |
| mFoa1         | Foa1-F           | GAACCTCATCCGCCACTCAGTG           |                                    |
|               | Foa1-R           | GCGAAGTAGCACAGCCGTTCTCG           |                                    |
| mOct4         | Oct4-F           | CCTCTCTGAGCCCTGTGC               |                                    |
|               | Oct4-R           | CTCTTCTCGAGGGCTTTCAT             |                                    |
| mSox2         | Sox2-F           | TCCTGCTCCTTTAAGACTAGG            |                                    |
|               | Sox2-R           | TGCTGGCTCCTTCTCTCAG              |                                    |
| mActin        | Actin-F          | AAGGCCAACCCTGAAAGAT              |                                    |
|               | Actin-R          | GAGGTAGCAAGAGACGATCAGCTA         |                                    |
| mCdax2        | Cdax2-F          | CTTGGCAGTGGGAGTCGGGAAG           |                                    |
|               | Cdax2-R          | CTCCAGCTCAGGCGCTGA               |                                    |
| mActin2       | Actin2-F         | AGTACTCTGTCTGGAGTGGGCTC          |                                    |
|               | Actin2-R         | TCCTGCTGATTCTCTGGGTGACT         |                                    |
| mCnot3-N-term | Cnot3-N-term-F   | GAGGGCTGACCTAAAGGAAGAGA          |                                    |
|               | Cnot3-N-term-R   | TCATTGTAGCTCAGCATTCTGA          |                                    |
| mCnot3-C-term | Cnot3-C-term-F   | GAGTTCTACACCGGCGCTGTCA          |                                    |
|               | Cnot3-C-term-R   | AATCGGCAAGGACTGCTTCT            |                                    |

**Poly(A) standards** (*Primers for cloning poly(A) standards also work as qPCR primers*)

| Poly(A) standards | Primer Name      | Forward Sequence                  | Reverse Sequence                   |
|-------------------|------------------|-----------------------------------|------------------------------------|
| Hygro*            | P(A)-SD1-F       | GTATTTGACTGGAGCGAGCGAG            |                                    |
|                   | P(A)-SD1-R       | CTGGCTGCTCATAACAGCCAACCA         |                                    |
| GFP*              | P(A)-SD2-F       | GAACCGGCATCAAAGTAGAAGAAG          |                                    |
|                   | P(A)-SD2-R       | GTGTCTCGCTGATGTGGTCCGGGA         |                                    |
| Luciferase*       | P(A)-SD3-F       | GTGCCAGAGTCCTCGTACAGGGAAC         |                                    |
|                   | P(A)-SD3-R       | CGACACCTTTAGGCAGACAGTACGA         |                                    |

**Cloning primers for Cnot3**

| Cnot3 aa249      | pDNR5            | GGGGACAAGTTTTGTACAAAAAACAGCAGCTCCGCCACCATGGAGGACGAGATCTTCAACCAAG |                                    |
| Cnot3 aa605      | pDNR5            | GGGGACAAGTTTTGTACAAAAAACAGCAGCTCCGCCACCATGGAGGACGAGATCTTCAACCAAG |                                    |
| Cnot3 aa636      | pDNR3            | GGGGACCACCTTTGTACAAGAAAGACTGTCGGGAGGTACTGCGCAGATCAGGCG |                                    |
| Cnot3 pDNR5      | pDNR3            | GGGGACAAGTTTTGTACAAAAAACAGCAGCTCCGCCACCATGGCGCAGAAAGCAAGCAGCTC |                                    |
| Cnot3 pDNR3      | pDNR3            | GGGGACAAGTTTTGTACAAAAAACAGCAGCTCCGCCACCATGGCGCAGAAAGCAAGCAGCTC |                                    |