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Post-transcriptional Regulation of the Pluripotent State

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Abstract

Pluripotency describes the developmental capacity to give rise to all cell types in the adult body. A comprehensive understanding of the molecular mechanisms that regulate pluripotency is important for both basic and translational research. While earlier studies mostly focused on signaling pathways, transcriptional regulation, and epigenetic modifications, recent investigations showed that RNA binding proteins, RNA processing machineries, and regulatory RNA molecules also play essential roles. Here, we provide a concise review on the latest findings and developments in post-transcriptional regulation of the pluripotent state.

Introduction

Pluripotency is defined as the developmental potential to give rise to all cell types formed by the three germ layers [1]. It is a unique property of the epiblast cells in blastocyst stage embryos, and it can also be captured in vitro in embryonic stem cells (ESCs), epiblast stem cells (EpiSCs), embryonic germ cells (EGCs), germline pluripotent stem cells (gPSCs), and induced pluripotent stem cells (iPSCs) [2–6]. Mouse ESCs cultured with MEK and GSK3 inhibitors (2i) show transcriptional and epigenetic similarity to the naïve pluripotent state in the pre-implantation epiblast [7], which can contribute to both blastocyst chimeras and the germline. In contrast, mouse EpiSCs derived from the post-implantation embryos represent the primed state. They are more primed for differentiation, and cannot integrate into the blastocyst or give rise to the germ cells The molecular mechanisms that regulate these pluripotent states have been extensively investigated, as they not only provide insights to early development but also facilitate the use of pluripotent stem cells in therapeutic applications.

From studies in mouse ESCs and other systems, it has been shown that the pluripotent state is controlled by a combination of signal-transduction pathways, transcription factors, epigenetic modifiers, RNA binding proteins (RBPs), RNA processing machineries, and regulatory RNA molecules [8–11]. However, most of the early research focused on the transcriptional and epigenetic regulation. In comparison, the role of post-transcriptional regulation on pluripotency has only begun to be revealed in more recent years. One study

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using genomic and proteomic-approaches suggested that post-transcriptional regulation may be responsible for a large proportion of protein level changes during ESC fate transition [12]. Indeed, with emerging technologies such as high-throughput sequencing, large-scale screening, and systematic identification of protein-RNA interactions or RBPs, more and more post-transcriptional mechanisms have been uncovered in the regulation of the pluripotent state. Here, we provide an overview of the latest findings and developments in the post-transcriptional regulation of pluripotency, and we propose that post-transcriptional regulation adds important layers of controls to fine-tune the gene expression program in pluripotent stem cells (Figure 1, Table 1).

Post-transcriptional regulation of pluripotency

Gene expression can be regulated at every stage during the making of the gene product. While transcriptional regulations often function as on-off switches, post-transcriptional mechanisms can act as rheostats to refine the output of gene expression. After transcription initiation, the primary transcripts undergo a series of steps including processing, export, modification, translation, and degradation to complete their life cycles. Almost all of these steps are subjected to regulation to influence the final production of the protein. In the following sub-sections, we review the intricate post-transcriptional regulations in pluripotent stem cells in a temporal order, based on the sequence of events that happen to an RNA molecule after its synthesis.

mRNA processing

The primary transcripts generated by the RNA polymerase must first be processed into mature mRNAs. RNA processing includes 5’-capping, splicing, and 3’-end processing. Both alternative splicing (AS) and alternative polyadenylation (APA, during 3’-end processing) can lead to the production of multiple mRNA variants from the same transcript, which in turn greatly increases the complexity of gene expression and facilitate cell type-specific gene regulation without editing the genome [13].

AS was found to play an important role in both the maintenance of the pluripotent state as well as the re-establishment of pluripotency during somatic reprogramming in mouse and human cells [14–16]. Many pluripotency factors, such as *Oct4*, *Nanog*, *Sall4*, *Tcf3*, *Foxp1*, *Mbd2*, and *Yy2* [16–22], have multiple isoforms that vary in expression, intracellular localization, stability, or function due to differences in their coding exons or untranslated regions from AS. Furthermore, AS regulators are differentially expressed in pluripotent stem cells and somatic cells [14]. They control the proper splicing of cell-state specific transcripts, and can rewire AS networks during cell fate transitions. Specifically, FOX2, SON, SFRS2, MYC, GCN5, ZCCHC24, and RBM47 facilitate pluripotency-specific AS of their target genes [14,21,23–25]. In contrast, MBNL1, MBNL2, RBM24, and SFRS11 promote differentiation-specific AS patterns for a large number of splicing events [16,26,27]. Together, these studies demonstrated an active role of AS in regulating both self-renewal and differentiation, and it will be important to further understand how specific AS signatures are established and maintained in different developmental states.

In addition to AS, the majority of mammalian genes also generate alternatively polyadenylated mRNAs. In most cases, APA leads to the production mRNAs with different 3'-untranslated regions, which can impact mRNA stability, translation, or intracellular localization [28,29]. Global profiling showed that widespread APA occurs during early mouse development, mouse ESC differentiation, and somatic cell reprogramming, suggesting that APA is tightly regulated during cell fate transitions [30]. Consistently, genes involved in 3'-end processing were implicated in mouse ESC maintenance in several genetic screens [31,32]. Furthermore, the FIP1 subunit in the cleavage and polyadenylation specificity factor (CPSF) complex was shown to promote mouse ESC self-renewal and somatic cell reprogramming [33]. It activates an ESC-specific APA pattern on a group of pluripotency-associated genes to enhance their expression. *Fip1* expression and the FIP1-dependent APA program change during ESC differentiation and are restored to an ESC-like state during somatic reprogramming. Thus, similar to AS, APA plays a significant role in regulating pluripotent stem cell fate specification. In this case, *Fip1* expression level is at least partly responsible for the pluripotency-specific APA patterns. However, there likely exist other factors that regulates APA in development and diseases.

mRNA modification

In addition to RNA processing, RNA can be chemically modified and RNA modifications serve as another layer of post-transcriptional control in gene expression [34,35]. There are more than 100 distinct RNA modifications, such as N^6-adenosine methylation (m^6A), N^1-methyladenosine (m^1A), 5-methylcytosine (m^5C), and pseudo-uridine (Ψ) [36]. Among them, m^6A is the most abundant internal modification in mRNAs and has been extensively investigated in recent years. m^6A is catalyzed by writers (METTL3, METTL14,), removed by erasers (ALKBH5 and FTO), and interpreted by readers (YTH domain family proteins, HNRNP proteins, and EIF3) [34,37]. In addition, m^6A level can also be regulated by miRNAs and transcription factors. m^6A is involved in multiple aspects of RNA metabolism, including degradation, splicing, transport, localization and translation, and its level is dynamically regulated in different cell types and during cell fate transitions.

It was initially shown that m^6A modification destabilizes developmental regulators in in mouse ESCs [38]. However, further studies showed that in both mouse and human ESCs, m^6A is enriched in pluripotency gene transcripts, and it promotes differentiation by facilitating pluripotency gene transcripts degradation [39,40]. In mouse EpiSCs, m^6A is also found in differentiation gene transcripts and promotes EpiSC maintenance by driving differentiation gene transcript removal [40]. Therefore, it appears that m^6A modification of mRNAs regulate pluripotent stem cell fate by acting on cell-type specific transcripts. It will be interesting to further dissect the underlying molecular mechanism to reconcile the results from different groups.

In mouse somatic cell reprogramming, the role of m^6A is also complicated. One study showed that elevated m^6A level resulted from human METTL3 overexpression enhances reprogramming efficiency of mouse embryonic fibroblasts [41]. However, a second study indicated that increased m^6A level accompanying *Zfp217* depletion impairs reprogramming [42]. This apparent discrepancy may be explained by how the m^6A level was manipulated

and which target mRNAs were involved. But these results suggested that m^6A deposition is exquisitely regulated by different factors, and the resulting phenotype is highly dependent on specific m^6A target mRNAs.

mRNA export

After processing, the mature mRNAs need to be exported from the nucleus to the cytoplasm for translation, and the mRNA export serves as the next step in post-transcriptional gene regulation. The main components of the mRNA export machinery have been well characterized, and a key player is the TRanscription and EXport (TREX) complex. Components of TREX are associated with other mRNA processing machineries, suggesting that mRNA export may act as an important interface in various processing steps to fine-tune gene expression [43]. The core of TREX is formed by the hexameric sub-complex THO. In mammals, THO is composed of THOC1, THOC2, THOC3, THOC5, THOC6, and THOC7, among which THOC2 functions as a scaffold for the complex and THOC5 acts as an adaptor for mRNA binding. THO plays a pivotal role in normal development and cellular differentiation, as its disruption leads to early embryonic lethality, as well as defects in hematopoietic progenitor survival, intestine stem cell homeostasis, and testis development [44,45].

THOC2 and THOC5 were identified as novel regulators of ESC maintenance in genetic screens [31]. It was further shown that THO preferentially interacts with pluripotency gene transcripts via THOC5, and regulates their export and expression [46]. During differentiation, THO loses its interaction with those transcripts due to reduced *Thoc5* expression, facilitating the down-regulation of pluripotency gene expression and the exit from the pluripotent state. Finally, THO is also important for the establishment of pluripotency, as its depletion inhibits somatic cell reprogramming and blastocyst development. Thus, by regulating the export and expression of pluripotency genes at the post-transcription level, THO provides an extra layer of fast and potentially non-committal control to fine-tune the balance between self-renewal and differentiation. Importantly, the specificity of THO toward pluripotency gene mRNAs can be partly explained by the expression level of the *Thoc5* subunit. This is reminiscent of the APA regulation, in which *Fip1* expression also contributes to substrate selectivity. As will be seen in more examples below, the expression and composition of the RNA processing machinery may play a critical role in carrying out

mRNA poly(A)-tail length

Once the mRNAs are exported into the cytoplasm, they are subjected to additional regulations, such as by the enzymes that control the poly(A)-tail length. Most mRNAs are polyadenylated by poly(A) polymerases, and the poly(A)-tail length can be modulated to influence mRNA stability or translation. Indeed, global measurement of poly(A)-tail length suggested that it is highly involved in development and cell fate transition [47,48]. Among the regulatory mechanisms controlling poly(A)-tail length, cytoplasmic deadenylation plays a pivotal role. The best characterized deadenylases so far include the CCR4-NOT complex, the poly(A)-specific ribonuclease (PARN), and the poly(A) nuclease (PAN), with CCR4-NOT being the predominant deadenylase in all eukaryote cells [49].

CCR4-NOT is a highly conserved multi-protein complex from yeast to human. It has been implicated in gene regulation at many steps throughout the lifetime of mRNAs. The CNOT1, CNOT2, and CNOT3 subunits of the CCR4-NOT complex were identified as regulators of the pluripotent state in mouse ESCs, and were shown to prevent differentiation into extraembryonic lineages [50]. Further, CNOT3 was found to be required for epiblast cell maintenance during embryonic development [51]. Mechanistically, *Cnot3* deletion results in increases in the poly(A)-tail lengths, half-lives, and steady-state levels of a subset of differentiation gene mRNAs. Consistently, the half-lives of these CNOT3 target mRNAs, but not those of housekeeping or pluripotency gene mRNAs, are shorter in ESCs and become longer during normal differentiation. Together, these results revealed that the CCR4-NOT complex maintains the pluripotent state by promoting differentiation gene mRNA deadenylation and degradation. More importantly, they strongly argued that poly(A) tail-length regulation is yet another critical post-transcriptional mechanism that controls pluripotency. In line with this notion, forced expression of CCR4-NOT components were shown to promote somatic cell reprogramming [52,53].

Intriguingly, other studies suggested that CCR4-NOT likely plays a much more complex role. First, the RNA binding protein PUM1 is required for the exit of the pluripotent state in ESCs [54]. It targets pluripotency gene mRNAs and accelerates their degradation at the onset of differentiation. As PUM1 is known to interact with CCR4-NOT, this study implied a potential involvement of CCR4-NOT in the regulation of pluripotency gene mRNAs. Second, in somatic cells the m^6A reader YTHDF2 directly interacts with CNOT1 and recruits CCR4–NOT to m^6A-containing mRNAs [55]. This recruitment is essential for the deadenylation and degradation of m^6A-containing mRNAs. As m^6A can mediate pluripotency gene mRNA degradation in ESCs, this study again suggested a possible connection between CCR4-NOT and pluripotency gene mRNAs. Integrating all the above findings, it is possible that different subunits in CCR4-NOT and/or different RBPs may facilitate the recognition of specific mRNA substrates by CCR4-NOT, allowing the complex to selectively target different functional groups of mRNAs for deadenylation.

mRNA translation

Finally, mRNA translation can profoundly regulate protein levels [56], and factors involved in translation control have been implicated in ESC biology. For example, the mechanistic target of rapamycin (mTOR) pathway is a master regulator of protein synthesis. It has been shown that protein synthesis and protein content significantly increases during mouse ESC differentiation. Such an increase can be partly attributed to the activation of the mTOR pathway and a hierarchy of translational regulators including 4EBP1, DAZL and GRSF1 [57]. Consistent with that, a recent study showed that mTOR inhibition can promote the maintenance of the pluripotent state both in vivo during embryonic diapause and in vitro in cultured ESCs [58]. Thus, mRNA translation clearly plays a critical role in pluripotent stem cells, and serves as a means for extracellular signals to influence cell fate. Intriguingly, mTOR inhibition was found to impair the long-term self-renewal of human ESCs [59], but in this case the function of mTOR was attributed to the transcriptional repression of developmental and growth-inhibitory genes.

Besides the mTOR pathway, additional translational regulators have also been implicated in ESC maintenance. The NAT1 protein binds to eukaryotic translation initiation factors and ribosomal proteins [60]. It promotes mouse ESC differentiation by enhancing the translation of genes involved in differentiation, mitochondrial oxidative respiration, and chromatin modification via a noncanonical, cap-independent mechanism [61]. The RNA-binding protein DAZL marks a sub-population of mouse ESCs [62]. It associates with *Tet1* mRNA and enhances its translation, promoting global cytosine hydroxymethylation. The DAZL-mediated translational control promotes the conversion between the naïve and primed pluripotent state. However, how NAT1 and DAZL act on selected target mRNAs to promote pluripotency remains to be elucidated.

Multi-functional RBPs

In addition to the above regulators that act at specific steps during the life cycle of an RNA, some RBPs have been shown to regulate gene expression at multiple levels in pluripotent stem cells. We discuss their functions separately below.

LIN28 is an RBP that can regulate both miRNA let-7 biogenesis and other mRNAs. It is highly expressed in mouse ESCs, further induced in EpiSCs, and down-regulated upon differentiation [63,64]. In human ESCs, LIN28 interacts with RNA helicase A and regulates *Oct4* mRNA translation to support the pluripotent state [65]. Consistently, it promotes the reprogramming of human somatic cells [64], and the conversion from the naïve to the primed pluripotent state in mouse cells [66,67]. On the molecular level, LIN 28 acts in both let-7-dependent and independent manner. In the let7-independent axis, it binds to metabolic gene mRNAs and represses their expression, conferring the metabolism characteristic of primed state pluripotency [67]. The let-7-independent function of LIN28 may be regulated via MAPK/ERK-mediated phosphorylation [68].

L1td1 (LINE-1 type Transposase Domain-containing 1) was originally identified as an ESC-associated transcript. It is highly expressed in the inner cell mass of mouse blastocysts, and is also rapidly activated during somatic cell reprogramming. While it is dispensable for mouse early development and iPSC derivation [69,70], it is required for human ESC self-renewal [71]. It is an RBP that interacts with LIN 28 via RNA, and may regulate pluripotency gene expression post-transcriptionally [71]. Further analysis of L1TD1 interactome revealed that it indeed binds to many RNA processing factors, adding additional support for its role in RNA regulation in pluripotent stem cells [72].

ESRP1 was initially shown to be a negative regulator of pluripotency. Its silencing inhibits mouse ESC differentiation, and it binds to Oct4 and Sox2 mRNA 5'-UTR, and prevents their efficient loading into the polysomes [73]. However, a later study found that ESRP 1 is differentially expressed during mouse somatic cell reprogramming [14]. Its overexpression promotes iPSC generation and facilitates the establishment of the pluripotent-specific AS of an epithelial specific transcription factor *Grhl1* transcript. These observations suggested that ESRP1 have multiple functions at different post-transcriptional steps, and it can influence pluripotent stem cell fate via different mechanisms.

Finally, two RNA interactome studies examined RBPs in mouse ESCs. One identified a list of novel RBPs that are selectively expressed in the pluripotent state [69], and the other provided a high-resolution mapping of RNA-binding regions of known and unknown RBPs [58]. Another study investigated the proteomic changes during somatic cell reprogramming, and uncovered many RNA processing factors that show stage-specific expression [74]. These systematic studies expanded the atlas of RBPs involved in pluripotency and provided a useful resource to study post-transcriptional gene regulation in pluripotent stem cells.

microRNAs

Beyond the protein factors, microRNAs (miRNAs) also play essential roles during post-transcriptional regulation in pluripotent cells. miRNAs are 20–21 base noncoding RNAs. They bind target mRNAs and regulate their stability or translation via the seed sequence in the 5’-region of the miRNAs. The involvement of miRNAs in ESCs maintenance and pluripotency was first revealed by the observations that the disruption of miRNA processing machineries led to impaired growth and differentiation [75,76]. In addition, the miRNA profile of pluripotent stem cells is well documented. In mouse ESCs, the polycistronic clusters miR-290–295 and miR-17–92b are dominantly expressed [77,78]. In mouse EpiSCs or human ESCs, the miR-302–367 cluster is highly expressed [79]. These pluripotency-specific miRNAs are activated by pluripotency transcription factors. Importantly, they all share the same seed sequence, and target cell cycle inhibitors p21, Lats2, and Rbl2 to maintain the distinct ESC cell cycle. In addition to these clusters of miRNAs, the let-7 family of miRNAs were shown to be important for ESC differentiation [80]. They target hundreds of pluripotency gene transcripts for degradation, and are required for the dismantlement of the pluripotent state. Consistent with results from ESCs, overexpression of many miRNAs, especially those pluripotency-specific ones, was shown to enhance somatic cell reprogramming in the presence of other reprogramming factors [81]. Furthermore, while still in debate, miRNA-only reprogramming has also been reported [82–84]. Finally, DDX5, a component of the Drosha miRNA processing complex, was recently reported to inhibit somatic cell reprogramming. DDX5 promotes miR-125b processing to repress the expression and function of the non-canonical polycomb complex 1 (PRC1) subunit RYBP, thereby impairing iPSC generation [85]. Together, these findings highlight the significance of miRNAs as a means for post-transcriptional regulation of pluripotency.

Concluding remarks and perspectives

Pluripotent cells have a sophisticated gene expression program that controls the delicate balance between self-renewal and differentiation. In addition to the transcriptional regulations, post-transcriptional mechanisms bring additional layers to fine-tune gene expression and cell fate. Significant advances have been made in understanding the fundamental roles of post-transcriptional regulation in governing the pluripotent state. However, many questions remain to be answered. How are specific mRNAs being recognized and targeted at each of the regulatory steps? How can the regulatory machineries regulate different groups of mRNAs in different or sometimes even the same cellular context? Can functionally related mRNAs be co-regulated? Is there any coordination across the different regulatory factors or processes? Is there any crosstalk between transcriptional

and post-transcriptional regulations? Along these lines, it has been proposed that functionally related mRNAs may be coordinately regulated as post-transcriptional RNA regulons by RBPs or RNA processing machineries [86]. Pluripotent stem cells appear to be an appealing system to further test this RNA regulon hypothesis. In addition to the conceptual challenges, technical improvements, such as those to identify protein-RNA interactions more convincingly, accurately measure poly(A)-tail length, and determine translation efficiency from limited materials, are also needed to move the research forward. Progress along these lines will provide a more comprehensive view of post-transcriptional regulation in the establishment, maintenance, and destabilization of the pluripotent state, as well as in other developmental and disease processes.

Finally, it may be interesting to look beyond the pluripotent stem cells to have a broader view on the post-transcriptional regulation of pluripotency. In particular, we propose that germ cells can provide a novel perspective. Germ cells harbor latent pluripotent potential, as they can re-acquire pluripotency via fertilization, teratocarcinogenesis, or spontaneous conversion during culture [87]. Furthermore, they express and require many of the same key post-transcriptional regulators, and may share similar regulatory mechanisms with pluripotent stem cells [47,48,51,54,62,78,88]. Therefore, a systematic investigation of post-transcriptional gene regulation in the pluripotency cycle between germ cells and pluripotent stem cells [87] will uncover new mechanistic and evolutionary insights to answer the conceptual questions listed above.

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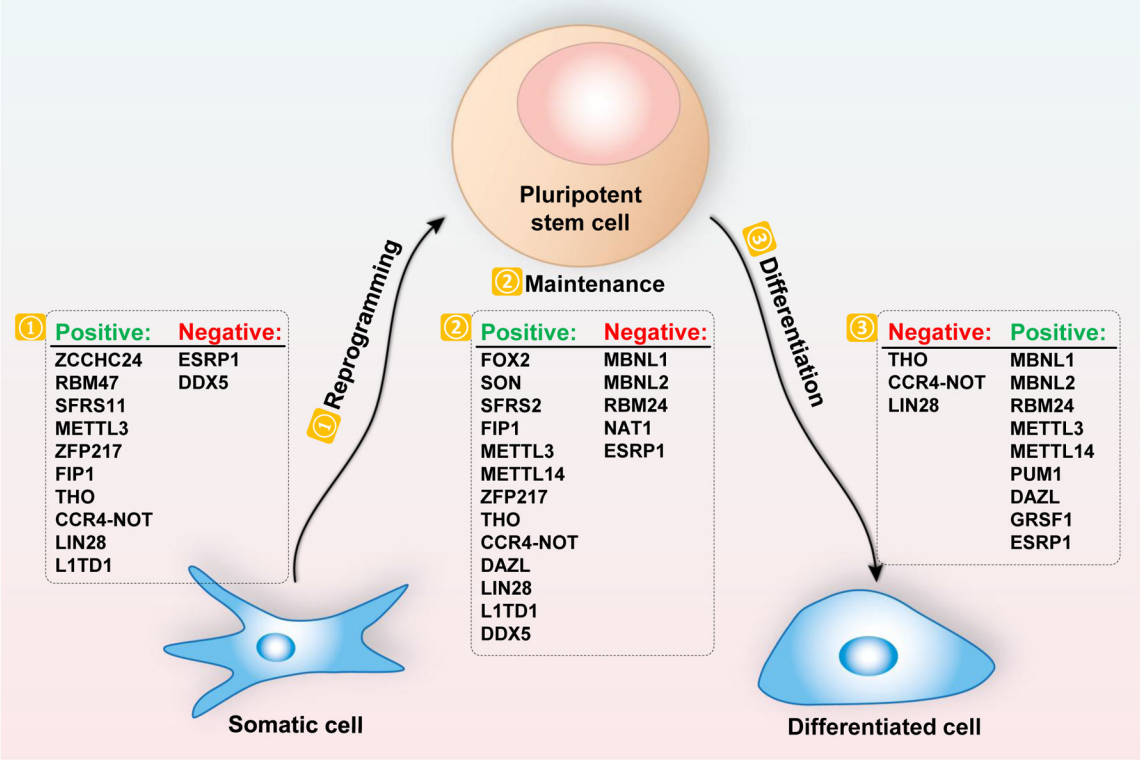

Figure 1.
Post-transcriptional Regulation of the Pluripotent State

Table 1.

Post-transcriptional Regulators of the Pluripotent State

Biological process	Regulators	Mechanisms	PMID
mRNA processing	FOX2	Facilitates pluripotency-specific AS in human ESC	19136955
	SON		24013217
	SFRS2	Regulates AS of *Mbd2* to support human ESC and iPSC self-renewal	24813856
	ZCCHC24, RBM47	Regulates phase-specific AS during reprogramming	27050523
	SFRS11	Regulates AS for genes that are critical for human somatic cell reprogramming	27292646
	MBNL1, MBNL2	Promotes differentiation-specific AS patterns in human ESC and reprogramming	23739326
	RBM24	Regulates AS events that favor cardiac specification during mouse ESC differentiation	26990106
	FIP1	Activates ESC-specific APAs for pluripotency-associated genes in mouse ESC and reprogramming	24596251
mRNA modification	METTL3, METTL14	Promotes degradation of target mRNAs via m6A in mouse ESC	25456834, 25569111, 24394384
	ZFP217	Promotes mouse somatic cell reprogramming via m6A deposition on target mRNAs	26526723
mRNA export	THOC2, THOC5	Facilitates pluripotency gene transcript nuclear export to support mouse ESC self-renewal and somatic cell reprogramming	24315442
poly(A) tail	CCR4-NOT	Promotes differentiation gene mRNA deadenylation and degradation to support mouse ESC self-renewal and epiblast maintenance; also supports somatic cell reprogramming and germ cell development	22367759, 27746116, 27037025, 28297718
		Promotes planarian stem cell differentiation via deadenylation and degradation of stem cell gene mRNAs	24367277
mRNA translation	NAT1	Promotes *Map3k3* and *Sos1* mRNA translation and mouse ESC differentiation	11032820, 28003464
	DAZL	Promotes *Tet1* mRNA translation and supports the naïve pluripotency state	26077710
		Suppresses *Oct4* mRNA translation in both human and mouse ESCs	27768780, 23298641
Multifunctional	LIN28	Promotes human somatic cell reprogramming and transition to the primed pluripotent state via *let-7*-dependent and independent pathways; couples MAPK/ERK signaling to post-transcriptional control	27320042, 27992407
		Inhibits *Hmga2* mRNA translation in mouse ESC	27920151
		Interacts with RHA to promote *Oct4* mRNA translation and human ESC self-renweal	19966271
	L1TD1	Interacts with post-transcriptional regulators and pluripotency factors in human ESC	25702638
		Interacts with LIN28 to regulate *Oct4* mRNA translation in human ESC	22162396
	ESRP1	Fine-tunes pluripotency gene mRNA translation in mouse ESC	24015231
		Promotes pluripotency-specific AS events during mouse iPSC generation	27050523
	DDX5	Represses the expression and function of RYBP via miR-125b during mouse somatic cell reprogramming	28111200