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Regulation of pluripotency and reprogramming by RNA binding proteins

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Abstract

Embryonic stem cells have the capacities of self-renewal and pluripotency. Pluripotency establishment (somatic cell reprogramming), maintenance, and execution (differentiation) require orchestrated regulatory mechanisms of a cell's molecular machinery, including signaling pathways, epigenetics, transcription, translation, and protein degradation. RNA binding proteins (RBPs) take part in every process of RNA regulation and recent studies began to address their important functions in the regulation of pluripotency and reprogramming. Here, we discuss the roles of RBPs in key regulatory steps in the control of pluripotency and reprogramming. Among RNA binding proteins are a group of RNA helicases that are responsible for RNA structure remodeling with important functional implications. We highlight the largest family of RNA helicases, DDX (DEAD-box) helicase family and our current understanding of their functions specifically in the regulation of pluripotency and reprogramming.

1. Pluripotency and reprogramming

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the blastocyst and they can be maintained indefinitely in culture. ESCs have two main characteristics: self-renewal, the ability of a cell to propagate indefinitely in the same state; and pluripotency, the potential of a single ESC to develop into any cell types of an embryo or an adult animal (Young, 2011).

During the mouse embryo development, at around embryonic day 3.5 (E3.5), the blastomeres compact into a blastocyst and the blastocyst has two different cell populations:

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the outer layer cells or the trophectoderm which will develop into the extra embryonic tissues; and the ICM which will develop into the primitive endoderm (hypoblast) and primitive ectoderm (epiblast). The primitive endoderm will give rise to the secondary extra embryonic tissues while the primitive ectoderm will produce the three germ layers of the embryo: ectoderm, mesoderm and endoderm (Morris et al., 2010).

At around mouse embryonic day E4.5, the blastocyst implants into the uterus to undergo the further development. ESCs are derived from the ICM of the pre-implantation blastocyst. It is also found that some of the post-implantation epiblast cells are capable of giving rise to all three embryonic germ layers, like ESCs. Based on the definition of pluripotency, these cells would be also considered pluripotent (Young, 2011). However, there are many differences between the cells derived from the ICM of the pre-implantation blastocyst and the cells from the post-implantation epiblast, such as the capacity to contribute to the chimeras and germ line transmission, the signaling to support cell's pluripotency. Besides, human ESCs, which are also derived from the ICM of human pre-implantation embryos (Thomson et al., 1998), display characteristics much closer to the mouse post-implantation epiblast stem cells (EpiSCs), than to the mouse ICM-derived mESCs. This observation suggests that hESCs correspond to a more differentiated developmental stage, or a primed pluripotency state (Brons et al., 2007; Tesar et al., 2007).

Research into molecules that control pluripotency has led to the landmark discovery in 2006 by Yamanaka's group who found a way to convert the mouse fibroblasts into a pluripotent ESC-like state through over expression of four transcription factors *Oct4*, *Sox2*, *Klf4*, and *c-Myc*. These reprogrammed cells are called induced pluripotent stem cells (iPSCs) and are highly similar to ESCs (Takahashi & Yamanaka, 2006). In 2007, Yamanaka's group also reported the reprogramming of human somatic cells to a pluripotent state with the same set of factors (Takahashi et al., 2007).

Human ESCs and iPSCs have tremendous therapeutic and regenerative potentials by providing a precious resource for drug testing, disease modeling, and cell replacement. A better understanding of the molecular regulatory mechanisms underlying pluripotency and reprogramming is a prerequisite for ESCs and iPSCs to be applied in disease therapeutics and regenerative medicine. An interplay of transcription factors, epigenetic factors, and signal transduction pathways are crucially important in the regulation of establishment, maintenance, and execution of pluripotency. While epigenetic and transcriptional regulation of pluripotency and reprogramming has been extensively studied and reviewed (reviewed by Chambers & Tomlinson, 2009; Gökbuget & Blelloch, 2019; Theunissen & Jaenisch, 2017; Yeo & Ng, 2013), post-transcriptional encompassing translational and posttranslational controls are relatively under-explored and are becoming the subjects of an ever increasing number of recent publications in the field of stem cell biology understanding pluripotency and reprogramming (Di Stefano et al., 2019; Freimer, Hu, & Blelloch, 2018; Li et al., 2017a; Yoffe et al., 2016; Zhang et al., 2020).

2. RNA binding proteins

RNA binding proteins (RBPs) are key factors in gene expression regulation by participating in every RNA-involved process, from transcription, RNA maturation, transport, stability, to translation and RNA degradation (reviewed by Guallar & Wang, 2014; Ye & Blelloch, 2014). RBPs are defined as proteins that contain one or multiple well-known RNA-binding domains (RBDs); or less commonly, proteins that reside within the ribonucleoproteins even if they don't directly interact with RNA (Gerstberger, Hafner, & Tuschl, 2014).

RBPs can be classified based on their target RNAs: mRNA-binding, tRNA-binding, pre-rRNA-binding, small nucleolar RNA (snoRNA)-binding, small nuclear RNA (snRNA)-binding, and other non-coding RNA (ncRNA)-binding. Notably, some RBPs can interact with different RNA types, such as the RNA exosome that regulates general RNA turnover. In these cases, researchers usually group the RBPs into their predominant target groups. Also, some RBPs with well-known RBDs are without available RNA target information (Gerstberger et al., 2014). Many published studies are focused on the mRNA-binding proteins (mRBPs), and relatively less is known about other RBPs subclasses such as the ncRNA-binding proteins. In the Online Mendelian Inheritance in Man (OMIM) database that links the known diseases to the relevant genes in the human genome, there are around 150 RBPs listed. In this list, only one-third of the RBPs are mRNA-binding, with the rest mostly targeting diverse ncRNAs (Hamosh, 2004), supporting the significance of studying the latter group.

Owing to the important roles that RBPs play in the gene expression regulation, it is not surprising that RBP families are well conserved across eukaryotes. Previous studies show that there are at least 200 distinct RBPs which are also present in the lowest common animal ancestor (Anantharaman, Koonin, & Aravind, 2002). Gerstberger et al. reported that in human, 50% of the RBP families are conserved in *S. cerevisiae* and even more are conserved in higher eukaryotes. The relative percentage of each RBP subclass based on its RNA targets is also maintained across phylogenies, 38% for mRBPs and 12% for tRNA-binding proteins (Gerstberger et al., 2014). Gerstberger et al. also showed that in human, 98% of paralogous RBP families are ubiquitously expressed across tissues while only 2% of paralogous families have tissue-specific expression patterns (Gerstberger et al., 2014). As evolutionary origin and tissue-specificity of gene expression often correlate with the protein function, highly evolutionary conservation and ubiquitous expression of RBPs support their critical roles in basic cellular functions (Freilich et al., 2005; Ramsköld, Wang, Burge, & Sandberg, 2009; Winter, Goodstadt, & Ponting, 2004).

RBD determines the specificity of binding of a certain RBP to its targets (MacKay, Font, & Segal, 2011). The following RBDs are some of the best characterized domains described in the literature:

> *RNA-Recognition Motif (RRM)*: 90–100 amino acids in length, present in up to six copies per protein, the most abundant and the most extensively studied RBD in higher vertebrates (Maris, Dominguez, & Allain, 2005). The RRM-RNA interaction is specific to single-stranded RNA, with low sequence-specificity. RRM has been shown to be also capable to interact with DNA and proteins.

K-Homology Domain (KH): around 70 amino acids in length. KH can recognize four single-stranded nucleotides with rather weak affinity, the stronger affinity or longer than four nucleotides target can be achieved by synergy in multiple copies (Beuth, Pennell, Arnvig, Martin, & Taylor, 2005).

Double-Stranded RNA-Binding Domain (dsRBD): around 70–75 amino acids, present in up to five copies per protein. The dsRBD recognizes double-stranded RNA in a sequence-independent way. The recognition covers 15 nucleotides with two minor grooves separated by a major groove. The additional functional domains modulate the binding specificity for various RNA shapes (Stefl, Skrisovska, & Allain, 2005).

DEAD-Box Domain: the name of DEAD-box is coming from their characteristic Asp-Glu-Ala-Asp (DEAD) motifs. DEAD-box proteins form the largest helicase family and they utilize ATP to bind or remodel RNA and ribonucleoproteins (Linder & Jankowsky, 2011). A major focus of this review (see more in Section 3).

PUF RNA-Binding Repeats: the PUF (formed by Pumilio and FBF) domain is around 36 amino acids, present six to eight tandem repeats per protein, packed in a curved structure, bind to single-stranded RNAs (Guallar & Wang, 2014).

PAZ Domain: the PAZ (Piwi/Argonaute/Zwille) domain is around 110 amino acids, recognizes the two-base 3′ overhang of dsRNA and ssRNA. The PAZ-domain RBPs function in the post-transcriptional gene silencing (Tian, Simanshu, Ma, & Patel, 2011).

Zinc-Finger Domains (ZnF): Znf domain is a classical DNA-binding domain, but it is also able to interact with RNA (Teplova & Patel, 2008). ZnFs present alone or in multiple copies per protein, they can also work in combination with other RBDs.

Even though above RBDs are well characterized in their association with RNA, many proteins have now been shown to interact with RNA in the absence of known RBDs. It is still necessary to determine whether those candidates directly interact with their RNA targets, and to characterize potential new RBDs. The high number of proteins that have been shown to bind RNA underscores the importance of both RBPs and RNA regulation in cellular function (Guallar & Wang, 2014).

Because RBPs are involved in every process of RNA regulation, from transcriptional to post-transcriptional as well as translational regulation, it is not surprising that they also play important roles in pluripotency and reprogramming (reviewed by Guallar & Wang, 2014; Ye & Blelloch, 2014). Therefore, categorization of RBPs in pluripotent stem cells provides an inroad to understanding their biology in pluripotency and reprogramming. Kwon et al. identified 555 proteins, including 283 novel RBP candidates, to constitute the mESC mRNA interactome. In this interactome, 68 proteins are preferentially expressed in ESCs by comparison to differentiated cells (Kwon et al., 2013). Bao et al. developed an approach to capture the newly transcribed RNA interactome using click chemistry (RICK) and applied it in mESCs. They identified 518 high-confidence proteins, 160 of which are overlapped with Kwon et al.'s interactome and the rest 358 are defined as RICK-exclusive mESC RBPs with RNA binding and polyA-RNA binding capacities. Among these 358 proteins, expression

levels of 95 proteins are higher in mESCs than in differentiated cells, suggesting their specific roles in ESC self-renewal and pluripotency (Bao et al., 2018). He et al. performed proteomic identification of RNA-binding regions in mESCs, and identified 803 nuclear RBPs, many of which are well-known transcriptional regulators and chromatin modifiers, such as NANOG and TET2 (He et al., 2016).

Mechanistically, RBPs participate in the regulation of pluripotency and reprogramming in many different regulatory layers (Fig. 1), which are discussed in detail below.

2.1 Epigenetic regulation

RBPs can also interact with ncRNAs to control chromatin activation or repression, the epigenetic control that serves as another important regulatory layer in the embryonic development. Examples of such RBPs are JARID2, an Xist-interacting RBP that promotes PRC2 recruitment for X chromosome inactivation in early female development and also during female ESC differentiation *in vitro* (da Rocha et al., 2014; Kaneko et al., 2014); and EZH2 and SUZ12, two catalytic subunits of PRC2 (polycomb repressive complex 2) that interact with lncRNAs (such as *HOTAIR* lncRNA (Brockdorff, 2013)) to function during embryonic development. *HOTAIR* lncRNA is also bound by an epigenetic modifier LSD1, a histone demethylase. LSD1 plays important roles in ESC differentiation through its H3 demethylase activity (Adamo et al., 2011; Whyte et al., 2012). Studies by Tsai et al. showed that *HOTAIR* promoted the bridging between PRC2 and LSD1 to facilitate their cooperation in regulating gene repression (Kaya & Higuchi, 2010). In addition, *HOTAIR* is induced during differentiation and its expression is also required in epithelial-to-mesenchymal transition (EMT) and metastasis in cancer cell lines (Gupta et al., 2010; Pádua Alves et al., 2013).

2.2 RNA modification

Posttranscriptional RNA modification provides a new layer of gene regulation at the RNA level. RNA modifications can be separated into two types: the addition of untemplated nucleotides and the chemical modification of the template nucleotides. One example for the former is the uridylation of *pre-let-7* miRNA: *let-7* is an important miRNA in facilitating ESC differentiation and repressing reprogramming of somatic cells (Melton, Judson, & Blelloch, 2010). Its formation can be regulated through uridylation in two opposite ways: LIN28A can direct 3′ terminal uridylyl transferases (TUTases) ZCCHC11 and ZCCHC6 to add a string of around 11 uridines to the *pre-let-7* miRNA (Hagan, Piskounova, & Gregory, 2009; Heo et al., 2008), then the oligouridylated *pre-let-7* would be targeted and degraded by the DIS3L2 exoribonuclease (Ustianenko et al., 2013); on the contrary, the addition of only one uridine to *pre-let-7* would facilitate the maturation of *let-7* (Heo et al., 2012).

The latter type of RNA modification can take several forms. One major class of RNA modifications is editing by deamination (Bass, 2002). Classical examples are Adenosine-to-inosine, A-to-I, catalyzed by the adenosine deaminase acting on RNA (ADAR) family (Eggington, Greene, & Bass, 2011), and cytidine-to-uridine, C-to-U, catalyzed by the AID-APOBEC enzyme (Powell et al., 1987). A-to-I RNA editing catalyzed by ADAR1 is

important in human embryogenesis and ADAR1 is required for hESC differentiation and neural induction (Chen et al., 2015; Shtrichman et al., 2012).

A second class of RNA modifications is methylation of adenosine to form *N*6-methyladenosine (m^6A), the most abundant modification of eukaryotic mRNA which is critical for pluripotency and reprogramming. METTL3, a m^6A methyltransferase, is required for m^6A in mRNAs of ESCs. While ESCs without *Mettl3* can preserve their naïve pluripotent identity, *Mettl3* knockout (KO) naïve ESCs cannot be transferred to primed state, and they lose differentiation competence, staying in a hyper-naïve pluripotency state. Such resistance to differentiation is because during the transition from naïve to primed pluripotent states, m^6A is required to timely destabilize the transcripts of pluripotency factors, which is necessary for proper lineage differentiation (Batista et al., 2014; Geula et al., 2015). In iPSC reprogramming and naïve ESCs, ZFP217 interacts with and sequesters METTL3, inhibiting m^6A deposition on the transcripts of the core stem cell network, such as *Nanog*, *Sox2*, and *c-Myc* (Aguilo et al., 2015). Apart from transcripts of these core pluripotency factors, a recent paper shows that in human pluripotent stem cells, m^6A is also important in the regulation of R-loops, the tripartite nucleic acid structures that are formed during transcription with an RNA:DNA hybrid and a non-hybridized single-stranded DNA. During cell cycles, m^6A-containing R-loops accumulate during G_2/M phases and are drastically depleted during G_0/G_1 phases. An m^6A reader, YTHDF2, interacts with RNA:DNA hybrids. The depletion of YTHDF2 or METTL3 leads to accumulation of RNA:DNA hybrids and increases γH2AX, a marker of DNA double-strand breaks, indicating genome instability (Abakir et al., 2019).

A third class of RNA modifications is oxidation of 5-methylcytidine (5mC) to 5-hydroxymethylcytidine (5hmC) on RNA that can be catalyzed by *Tet* enzymes (Delatte et al., 2016; Fu et al., 2014; Masiello & Biggiogera, 2017; Miao et al., 2016; Zhang, Xiong, Qi, Feng, & Yuan, 2016). In mESCs, TET2 can be recruited to actively transcribed *MERVL* RNAs through its physical association with another RBP PSPC1 and deposit 5hmC modification on *MERVL* RNAs, contributing to *MERVL* destabilization in mESCs (Guallar et al., 2018). Besides TET2, PSPC1 can also recruit HDAC1/2 (histone deacetylase complex) to silence *MERVL* transcriptionally (Guallar et al., 2018). Readers are encouraged to read this review (Frye & Blanco, 2016) to gain additional information on RNA modifications in development and stem cells.

2.3 Alternative splicing

In mouse and human, more than half of the genes can generate different transcripts through alternative splicing (Modrek, 2001). Many pluripotency-associated transcripts, including *OCT4* and *Nanog*, two of the most important pluripotency factors, have different isoforms generated through alternative splicing: in human, *OCT4A* is the key pluripotency transcription factor in ESCs while *OCT4B* expresses in nonpluripotent cells without known functions (Wang & Dai, 2010); in mouse, the three isoforms of *Nanog* contributes with various efficacies to maintaining ESC pluripotency (Atlasi, Mowla, Ziaee, Gokhale, & Andrews, 2008; Das, Jena, & Levasseur, 2011). Another example is *Sall4*, a transcription factor essential for pluripotency. It has two isoforms, *Sall4a* and *Sall4b*, which can form

either homodimers or a heterodimer with each other. The genomic binding loci of *Sall4a* and *Sall4b* are overlapped but not identical. *Sall4b* is relatively more important than *Sall4a* in the regulation of pluripotency as *Sall4b*, but not *Sall4a*, can partially rescue the loss-of-function phenotype of both isoforms (Rao et al., 2010). A fourth example is *FOXP1*. It has an ESC-specific isoform that promotes iPSC reprogramming and ESC maintenance by stimulating the expression of pluripotency factors (Gabut et al., 2011).

Apart from the RBPs involved in the core machinery of the spliceosome, specific RBPs are also needed to generate the ESC-specific splicing signature. For example, MBNL1 and MBNL2 are conserved negative regulators of cassette exon alternative splicing events that are differentially controlled among cell types. The alternative splicing event of aforementioned ESC-specific *FOXP1* isoform is inhibited by MBNL1/2 and consistent with such inhibitory control of ESC-specific *FOXP1* isoform, the depletion of *MBNL1*/*2* enhances iPSC reprogramming (Han et al., 2013). Another splicing regulator example is *FOX2*, which is critical for pluripotency in hESCs as its depletion drives hESCs into differentiation and death. The CLIP-seq (crosslinking immunoprecipitation with high-throughput sequencing) from Yeo et al. showed that FOX2 binding to one intron induced the inclusion of the upstream flanking exon and the exclusion of the downstream flanking exon. In addition, FOX2 also acts as an upstream splicing master regulator because it targets and regulates the alternative splicing of several other splicing regulators, such as *LIN28*, *FOX2* itself and serine/threonine kinases (Yeo et al., 2009). All these highlight the important functions of FOX2 in the splicing program to maintain the hESC pluripotency. For more examples, we direct readers to two related reviews of the subject (Chen, 2015; Cheong & Lufkin, 2011).

2.4 Alternative polyadenylation

Around 70% of the mammalian RNAs are subjected to alternative polyadenylation (APA), leading to different 3′UTR lengths of transcripts (Derti et al., 2012). The various 3′UTR lengthening can affect the stability, localization and translation of transcripts, leading to differential protein expression. Previous studies show that APA is closely related with cell states: somatic cell reprogramming is associated with 3′UTR shortening (Ji & Tian, 2009; Sandberg, Neilson, Sarma, Sharp, & Burge, 2008), whereas embryonic development and exit from pluripotency are accompanied by 3′UTR lengthening (Ji, Lee, Pan, Jiang, & Tian, 2009; Shepard et al., 2011). In transcript cleavage and polyadenylation, cleavage and polyadenylation specificity factor (CPSF) complex recognizes the polyadenylation signal flanking upstream of the cleavage site (Lackford et al., 2014). Lackford et al. demonstrated that *Fip1*, one subunit of CPSF, functioned as an mRNA 3′ processing factor in establishing ESC-specific APA profile. *Fip1* knockdown resulted in partial differentiation in mESCs and inhibited MEF reprogramming. Deep sequencing showed that *Fip1* depletion changed the APA profile of 374 genes with 3′UTR lengthening (Lackford et al., 2014). Further studies are needed to investigate how *Fip1* regulates the 3′UTR length in contributing to pluripotency. Another protein complex, known as cleavage factor Im (CFIm) complex, acts as an activator of transcript cleavage and polyadenylation. *Nudt21* (also called *Cpsf5*), a component of CFIm, regulates cell fates by manipulating alternative polyadenylation. *Nudt21* is a barrier to reprogramming as its depletion dramatically increases iPSC

reprogramming efficiency. Its depletion also enhances the transdifferentiation of MEFs to induced trophoblast stem cells but impairs ESC differentiation. Mechanistically, *Nudt21* knockdown facilitates alternative polyadenylation of chromatin regulators, such as *Rybp*, *Chd1*, and *Wdr5*, that play important roles in reprogramming (Ang et al., 2011; Brumbaugh et al., 2018; Gaspar-Maia et al., 2009; Li et al., 2017b). For additional information on APA in stem cell biology, readers are referred to this review (Mueller, Cheung, & Rando, 2013).

2.5 Nuclear retention and export of RNAs

Most RNAs need to be exported from nucleus to cytoplasm to function, so gene expression can also be regulated through controlling the access of RNA to the cytoplasmic machineries (*e.g.*, translation machinery). A study from Wang et al. showed this regulatory level played important roles in ESCs. The THO complex is a conserved complex regulating mRNA export from the nucleus to the cytoplasm. The depletion of two subunits of the THO complex, namely *Thoc2* or *Thoc5*, didn't change the overall transcripts level, however, resulted in the nuclear accumulation of a subset of pluripotency-related transcripts, including *Nanog*, *Esrrb*, *Klf4*, and *Sox2*. The interaction of THOC2 with these pluripotency-related mRNAs is THOC5-dependent. THOC5 is an adaptor protein, which is downregulated in normal development. The knockdown of *Thoc5* promotes ESC differentiation and inhibits somatic cell reprogramming, while overexpression of *Thoc5* delays the differentiation in ESCs (Wang et al., 2013). This example emphasizes the important role of RNA nuclear export control in pluripotency regulation (Saunders & Wang, 2014).

2.6 Translation

RBPs can also adjust the RNA/ribonucleoprotein structures to control the accessibility of the RNA to ribosomes or the movement of ribosomes along the mRNA to control protein synthesis. At this regulatory level, RBPs often bind to the 5′UTR of RNA and such 5′UTR-RBP interactions have been reported to regulate ESC proliferation and differentiation (Ye & Blelloch, 2014). For example, RBM35A was found to target the 5′UTR of *Sox2* and *Oct4* transcripts to prevent their loading into the polysomes, as demonstrated through RBM35A immunoprecipitation and polysome profiling. And *Rbm35a* depletion blocks ESC differentiation and facilitates somatic cell reprograming through promoting the expression of key pluripotency transcription factors, including *Oct4* and *Sox2* (Fagoonee et al., 2013). Another example is *NAT1* (also known as *EIF4G2*, *DAP5*, and *p97*), which is homologous to the C-terminal of eukaryotic translation initiation factor 4G (*EIF4G1*). In both mESCs and hESCs, its depletion results in the resistance to differentiation induction due partly to the translational block of NAT1-mediated translation of a specific subgroup of proteins that are critical for ESC differentiation (Sugiyama et al., 2017; Yoffe et al., 2016). Translational control in pluripotency and reprogramming is being increasingly recognized (Tahmasebi, Amiri, & Sonenberg, 2019), although much more work needs to be done to further unravel this important regulatory layer.

2.7 mRNA stability and degradation

During quality surveillance of RNA, RBPs can bind to aberrant RNAs and export them in the cytoplasm for degradation (Reed & Hurt, 2002), as well as modify RNA in nucleus for degradation (Houseley, LaCava, & Tollervey, 2006; LaCava et al., 2005). Some RBPs that

function in RNA quality control have been shown to be important for pluripotency and reprogramming. For example, TRIM71 can interact with miRNA-containing AGO2 and cooperate with ESC-specific *miR-290* and *miR-302* to target the 3′UTR of *Cdkn1a*, a repressor of the G_1-S transition, inhibiting its activity to promote the cell cycle process for optimal ESC self-renewal (Chang et al., 2012). Loedige et al. also demonstrated the binding of TRIM71 to the 3′UTRs of a subset of prodifferentiation genes, leading to the downregulation of mRNA levels in an AGO2-independent way (Loedige, Gaidatzis, Sack, Meister, & Filipowicz, 2013). In addition, Worringer et al. showed that overexpression of *TRIM71* promoted human somatic cell reprogramming, which was partly due to the post-transcriptional inhibition of the fibroblast-enriched *EGR1* transcripts to which TRIM71 binds and negatively regulates (Worringer et al., 2014).

In sum, RBPs can function in multiple regulatory layers to control pluripotency and reprogramming. Some RBPs can even work multi-functionally by controlling various molecular layers of RNA regulation. One example is coming from the study by Dardenne et al., demonstrating the multiple functions of two RNA helicases DDX5 and DDX17 in various regulatory layers controlling myogenesis and EMT (epithelial-to-mesenchymal transition) (Dardenne et al., 2014). We will further discuss this particular family of RBPs below.

3. RNA helicases and DEAD-box helicase family

RNA is one of the most important biological macromolecules that function in many biological processes. To be functional, RNA must fold into specific secondary or tertiary structures in three dimensions, and many proteins are involved in the RNA folding/remodeling to regulate the physical characteristics of RNA or form ribonucleoprotein complexes for further function (Jarmoskaite & Russell, 2014). For example, in the spliceosome assembly, Sub2 and Prp5, two DEAD-box helicases, are required to promote the rearrangements allowing the recognition base-pairing between the branchpoint and U2 snRNA (Ruby, Chang, & Abelson, 1993).

Helicases are the enzymes responsible for nucleic acids remodeling by using the energy from nucleoside triphosphate binding and hydrolysis (Hardwick & Luisi, 2013). Helicases function in almost every cellular process in which nucleic acids are involved. Until now, at least two mechanisms have been reported: canonical translocation-based duplex unwinding and duplex unwinding by local strand separation, which are employed by some viral RNA helicases and DEAD-box helicases, respectively (Jankowsky, 2011). Helicases are classified into six superfamilies (SFs) and all eukaryotic RNA helicases are found in six families belonging to SFs 1 and 2; the remaining families in eukaryotes are composed of DNA helicases. Some families consist of both RNA and DNA helicases and some helicases work on both DNA and RNA (Fairman-Williams, Guenther, & Jankowsky, 2010; Putnam & Jankowsky, 2013). The DEAD-box (DDX) is the largest family of RNA helicases, belonging to SF2 (Hardwick & Luisi, 2013). The name of DEAD-box reflects their characteristic Asp-Glu-Ala-Asp (DEAD) motifs. This family is present in all eukaryotes and also in many Archaea and bacteria. These highly conserved helicases are involved in virtually every RNA metabolism step, from ribosome biogenesis, to transcription, RNA maturation, microRNA

processing, translation, and RNA degradation. DDX proteins generally function as part of large multicomponent complex, like the spliceosome (Linder & Jankowsky, 2011). Because DDXs are widely involved in the RNA metabolism, it is not surprising that some DDX members also play important roles in pluripotency and reprogramming. To date, direct implication of DDX family members in stem cell pluripotency and somatic cell reprogramming came from the studies of following DDX factors.

3.1 DDX3

The expression level of DDX3 is highly enriched in human undifferentiated stem cells compared to differentiated cells. Inhibition of DDX3 reduces cellular proliferation in hESCs but doesn't decrease proliferation of human embryonic fibroblast cells. In hESCs, inhibition of DDX3 also downregulates critical pluripotency markers (OCT4, SOX2, and NANOG) and facilitates differentiation (Kerr, Bol, Vesuna, & Raman, 2019) (Fig. 2A). Interestingly, Cruciat et al. reported that DDX3 could bind and activate the casein kinase 1 isoform epsilon (CK1ε), which leads to the Wnt-dependent phosphorylation of Disheveled, enabling β-catenin's function in activating its target genes (Cruciat et al., 2013). As Wnt signaling plays important roles in both pluripotency and reprogramming, it remains to be determined whether DDX3's regulatory role as the subunit of CK1ε defined by Cruciat et al. is part of the mechanism.

3.2 DDX5/DDX17

A notable example for DDX's multi-functionality in the stem cell field comes from the study of DDX5 and DDX17 paralogs (Dardenne et al., 2014). Dardenne et al. show that DDX5 and DDX17 can cooperate with hnRNP (heterogeneous nuclear ribonucleoprotein) H/F splicing factors to express an epithelial- and myoblast-specific splicing subprogram. Also, DDX5 and DDX17 serve as transcriptional coregulators of key differentiation transcription factors to drive the transcription programs specific to the myogenesis and EMT (epithelial-to-mesenchymal transition), which in turn can produce differentiation-specific miRNAs resulting in the down-regulation of DDX5 and DDX17. Another example is the association of DDX3 and DDX5 with the Wnt/β-catenin signaling pathway (Cruciat et al., 2013; Yang, Lin, & Liu, 2006). This pathway is very important in the embryonic development and is directly linked to the pluripotency core transcription factors, playing essential roles in pluripotency and self-renewal regulation (Kim & Kimmel, 2006). In Yang et al. (2006) showed that the stimulated DDX5 could displace the inhibitor Axin from β-catenin, facilitating the transfer of β-catenin to the nucleus to activate the target gene expression instead of being phosphorylated and degraded.

DDX5 also inhibits iPSC reprogramming. The depletion of *Ddx5* results in the dysregulation of dozens of miRNAs, including downregulation of *microRNA-125b*, which inhibits the expression of non-canonical polycomb complex 1 (PRC1) subunit *Rybp*. RYBP upregulation upon *Ddx5* depletion not only facilitates the deposition of inhibitory H2AK119ub1 at lineage-specific genes through PRC1 but also activates the OCT4-KDM2B network to enhance pluripotency-associated gene expression independently of PRC1 (Fig. 2B) (Li et al., 2017b).

3.3 DDX6

DDX6 has been shown to be necessary for the maintenance of adult progenitor cell functions (Wang, Arribas-Layton, Chen, Lykke-Andersen, & Sen, 2015). On one hand, to maintain self-renewal, DDX6 facilitates the translation of proliferation and self-renewal transcripts by recruiting them to translation initiation factor EIF4E; on the other hand, to prevent differentiation of progenitor cells, through association with mRNA degradation proteins, DDX6 targets and destabilizes the mRNA of *KLF4*, a differentiation-inducing transcription factor that is required for the activation of the epidermal differentiation and the conversion of fibroblasts to keratinocyte-like cells (Fig. 2C) (Chen, Mistry, & Sen, 2014; Mistry, Chen, Wang, Zhang, & Sen, 2014; Segre, Bauer, & Fuchs, 1999; Wang et al., 2015). In mESCs, *Ddx6* is required to maintain normal mESC cell morphology and proliferation. The loss of *Ddx6* produces a similar downstream consequence as the depletion of *Dgcr8*, which is essential for miRNA biogenesis. Instead of miRNA-induced RNA degradation, *Ddx6* is important in miRNA-induced translational repression in mESCs (Freimer et al., 2018). Recently, Di Stefano et al. showed that DDX6 is an important regulator of pluripotency in both human and mouse ESCs as its depletion leads ESCs to a differentiation-resistant state. Suppression of *DDX6* also promotes the reprogramming of primed hESCs to a naïve state. DDX6 was also found to regulate the differentiation potential of adult somatic progenitors in a context-dependent manner. Mechanistically, DDX6 is associated with critical P-body proteins and mediates the translational suppression of the target transcripts in P-bodies. DDX6 loss results in dissolvement of P-bodies, which releases mRNAs encoding key cell fate transcription regulators and chromatin factors to the translational machinery in promoting pluripotency and reprogramming (Fig. 2D) (Di Stefano et al., 2019).

3.4 DDX18

Zuo et al. (2009) constructed a protein interaction network encompassing hESC-enriched proteins in hESCs and found that DDX18 is among the top 5% highly connected nodes, suggesting that DDX18 may have important functions in hESCs. Very recently, *Ddx18* was reported to be required for mESC maintenance and embryonic development. DDX18 directly interacts with PRC2 and modulates the formation of PRC2 complex. Such interaction prevents PRC2 from accessing and marking ribosomal DNA (rDNA) with repressive H3K27me3. rRNA (ribosomal RNA, the product of rDNA transcription) is highly expressed in ESCs and becomes downregulated upon differentiation. *Ddx18* depletion increases PRC2 occupancy at rDNA loci to inhibit rDNA transcription, leading to reduced ribosomal protein level and global translation level (Fig. 2E) (Zhang et al., 2020). Owing to the alternative pluripotent states between mouse and human ESCs, it remains to be addressed whether the human ortholog DDX18 may play a similar or distinct role in regulating human pluripotency and reprogramming.

3.5 DDX21

RNA helicase DDX21 functions in multiple steps of ribosome biogenesis by coordinating transcription and rRNA processing. DDX21 was found associated with actively transcribed ribosomal genes as well as rRNAs and snoRNAs, facilitating rRNA modification (Calo et al., 2015). The multifaceted function of DDX21 in ribosome biogenesis suggests its

potential role in ESCs as both ribosomal genes and rRNAs are highly expressed in ESCs and properly downregulated/repressed during early differentiation (Ingolia, Lareau, & Weissman, 2011; Savić et al., 2014; Woolnough, Atwood, Liu, Zhao, & Giles, 2016), although a definite functional contribution to pluripotency and reprogramming has yet to be tested.

3.6 DDX47 and DDX52

DDX47 and DDX52 are subunits of small subunit processome (SSUP), which mediates 18S rRNA biogenesis (Phipps, Charette, & Baserga, 2011; Tafforeau et al., 2013). The components of SSUP are highly expressed in stem cells and important to maintain the protein levels of pluripotency factors. As SSUP subunits, both DDX47 and DDX52 are validated to be necessary for ESC maintenance and efficient iPSC reprogramming: (1) depletion of either of them in mESCs induced differentiation; (2) they help to sustain the protein levels of labile pluripotency factors NANOG and OCT4 in mESCs; and (3) Both are required for efficient reprogramming of iPSCs (Fig. 2F) (You, Park, & Kim, 2015).

4. Conclusions

Understanding molecular mechanisms underlying pluripotency and reprograming is highly significant both scientifically and clinically. The posttranscriptional regulation by RBPs constitutes an important regulatory layer for controlling pluripotency and reprogramming. Although RBPs have been studied widely because of their involvement in a broad range of cellular processes, their regulatory functions in stem cell field are only just beginning to be appreciated. As post-transcriptional regulation enables cells to quickly respond by adjusting protein abundance, future studies are warranted to dissect mechanistic actions of RBPs during cell fate transitions and further our understanding of their roles in pluripotency and reprogramming. The potential multifaceted functions of RBPs on both RNA and DNA targets at transcriptome/epitranscriptome and genome/epigenome levels should be more carefully examined in light of their dual DNA/RNA binding capacities. Finally, as many RBPs contain intrinsically disordered regions, the roles of RBPs in the regulation of phase separations and gene expression, which are only recently recognized (A & Weber, 2019; Shorter, 2019; Xiao et al., 2019; Youn et al., 2019), await more future investigations at both physiological and pathological conditions.

Together, RBP studies would provide a platform and new framework for better understanding of molecular mechanisms underlying pluripotency and reprogramming, which would bring us closer to the practical applications of pluripotent ESCs/iPSCs for regenerative medicine, tissue engineering, and disease therapeutics.

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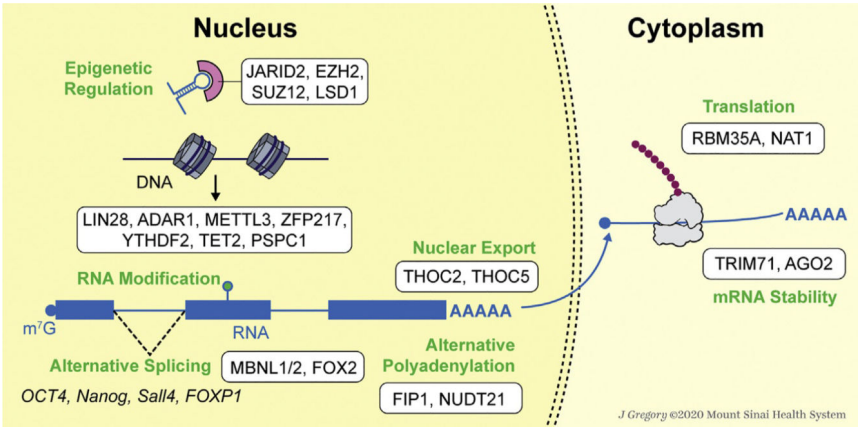

Fig. 1.

RBPs participate in multiple regulatory layers to control pluripotency and reprogramming. See Sections 2.1–2.7 for details.

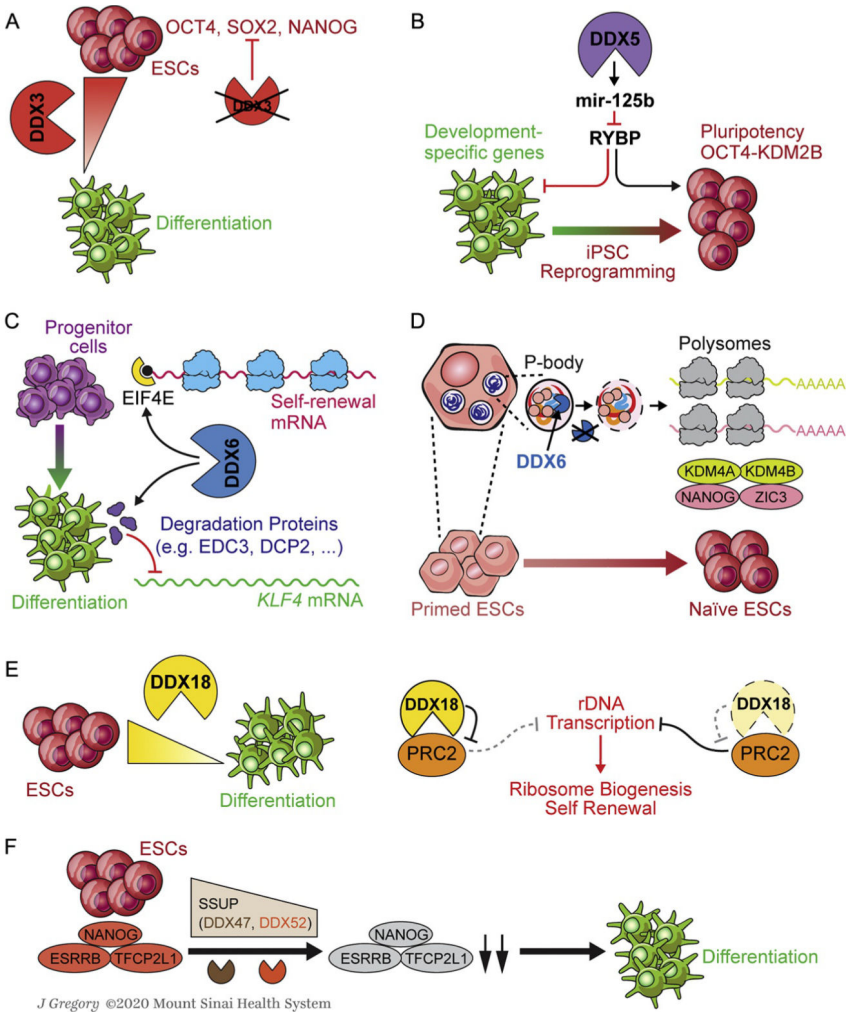

Fig. 2.

DDX proteins that are reported to be important in pluripotency and reprogramming. (A) DDX3 expression is highly enriched in human undifferentiated stem cells and decreases during differentiation. The inhibition of DDX3 in hESCs downregulates core pluripotency factors. (B) DDX5 inhibits iPSC reprogramming. Depletion of *Ddx5* downregulates miRNA-125b, leading to the increase of RYBP. RYBP upregulation inhibits development-specific gene expression, and facilitates pluripotency through the activation of OCT4-KDM2B network. (C) DDX6 is necessary for the maintenance of adult progenitor cell functions, through facilitating the translation of proliferation and self-renewal transcripts, and the degradation of differentiation-inducing *KLF4* mRNA. (D) Depletion of *DDX6* promotes the reprogramming of primed hESCs to a naïve state. DDX6 interacts with P-body proteins and suppresses the target transcripts in P-bodies. Depletion of *DDX6* leads to dissolvement of P-bodies, releasing target mRNAs that encode key cell fate transcription regulators and chromatin factors. The released mRNAs are translated to promote pluripotency and reprogramming. (E) *Ddx18* expression is highly enriched in mESCs and decreases along differentiation. DDX18 interacts with PRC2, preventing it from accessing and marking rDNA with H3K27me3. DDX18 downregulation leads to inhibition of rDNA

transcription and reduced ribosomal protein level as well as global translation level. (F) DDX47 and DDX52, as the components of SSUP, are highly expressed in ESCs and important for maintaining the protein levels of pluripotency factors. Downregulation of DDX47 or DDX52 leads degradation of pluripotency factors and consequent differentiation of mESCs. The references for each group are listed in the main text.