SURVEY AND SUMMARY

Nonsense-mediated mRNA decay: a ‘nonsense’ pathway makes sense in stem cell biology

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Abstract

Nonsense-mediated mRNA decay (NMD) is a highly conserved post-transcriptional regulatory mechanism of gene expression in eukaryotes. Originally, NMD was identified as an RNA surveillance machinery in degrading ‘aberrant’ mRNA species with premature termination codons. Recent studies indicate that NMD regulates the stability of natural gene transcripts that play significant roles in cell functions. Although components and action modes of the NMD machinery in degrading its RNA targets have been extensively studied with biochemical and structural approaches, the biological roles of NMD remain to be defined. Stem cells are rare cell populations, which play essential roles in tissue homeostasis and hold great promises in regenerative medicine. Stem cells self-renew to maintain the cellular identity and differentiate into somatic lineages with specialized functions to sustain tissue integrity. Transcriptional regulations and epigenetic modulations have been extensively implicated in stem cell biology. However, post-transcriptional regulatory mechanisms, such as NMD, in stem cell regulation are largely unknown. In this paper, we summarize the recent findings on biological roles of NMD factors in embryonic and tissue-specific stem cells. Furthermore, we discuss the possible mechanisms of NMD in regulating stem cell fates.
malian embryonic and tissue-specific stem cells and biological roles of NMD in mammals.

Diversity of NMD targets in a cell transcriptome

mRNAs with PTCs are classical targets of the NMD machinery. Recent findings suggest that transcripts of genes with physiological significances in cell functions are regulated by the NMD machinery (5). Since NMD affects the mRNA half-life, inhibition of NMD results in high levels of NMD target gene transcripts in a cell. In this regard, identification of highly represented DEGs (Differential Expression Genes) in NMD deficiency conditions is one of the major strategies in defining NMD targets (12,19–21).

Recently, transcriptome-based strategies, such as microarray and RNA-Seq, have enabled the identification of new groups of NMD targets with features of upstream open reading frame (uORF), long 3′ UTR, introns in 3′ UTR, etc. (2,5). Combing gene knockdown and microarray/RNA-Seq, Mendell et al. (21) and others found that NMD targets are enriched in mRNAs with features of PTC, 5′ uORFs, long 3′ UTRs and introns in 3′ UTRs (12,22). An integrated bioinformatic analysis on the RNA-Seq data generated from human cells with NMD factors UPF1, SMG6 or SMG7 gene knockdowns and rescue experiments further showed that SMG6 mediated endonucleolytic decay route and SMG5/7 mediated exonucleolytic decay route are largely redundant in degrading mRNAs with the feature of introns in 3′ UTR regions (21). This study further proposed that long non-coding RNAs, and transcripts from miRNA and snoRNA host genes could be regulated by the NMD machinery (21,23).

Although studies with DEG based bioinformatic analysis from microarray and RNA-Seq data have identified a series of NMD targets, it is difficult to distinguish whether an upregulated gene transcript upon NMD deficiency is a direct NMD target. To overcome this obstacle, Tani et al. developed BRIC-Seq (5′-bromo-uridine immunoprecipitation chase—deep sequencing analysis), a transcriptome wide approach to directly determine NMD targets with prolonged RNA half-lives in NMD factor depleted cells (24). By conducting BRIC-Seq in UPF1 knockdown human cells, new UPF1 targets with increased half-lives have been identified and experimentally validated (24). In recent years, PAR-CLIP (photoactivatable ribonucleotide enhanced crosslinking and immunoprecipitation) has been shown to be advantageous in the large scale and unbiased identification of RNA targets of RNA binding proteins (RBPs) (25). PAR-CLIP allows for a direct capture of RNA species bound to an RBP. With this method, serial studies have identified UPF1 specific RNA targets in mouse ESCs (25), HEK293 cells (26) and HeLa cells (27). Of note, the efficiency of PAR-CLIP relies largely on the specificity of antibody against RBPs. Thus, abundances of RNA targets as revealed by PAR-CLIP could be underestimated. Taken together, studies with these new techniques further confirm that NMD targets are beyond PTC containing mRNAs.

NMD is not a linear pathway, but rather with branches mediated by different NMD factors (5,8). For example, NMD branches mediated by SMG6 and SMG5/7 have preferences in their RNA targets (28), indicating NMD factors may selectively bind and degrade RNA targets through recognizing unique sequence motifs on RNAs. XRN1 degrades RNAs with its 5′-3′ exoribonuclease activity (29). Knockdown of XRN1 in mammalian cells leaves intact 3′ fragments of decay intermediates, which allows for the direct capture of sequence features of NMD targets. Ottens et al. employed a 3′ fragment capture and degradome sequencing technique on human cell samples with XRN1 single and XRN1/SMG6 double knockdown and found that the SMG6-mediated endonucleolytic route degrades mRNAs with features of PTC, 5′ uORFs and long 3′ UTRs, while the SMG5/7-mediated exonucleolytic route mainly targets mRNAs with features of 5′ uORFs and long 3′ UTRs (30). Schmidt et al. used a similar method, namely ‘Parallel Analysis of RNA Ends’ MaxSeq (PARE MaxSeq), and identified the 5′ termini sequence composition of SMG6 mediated decay intermediates (31). More than 60% of SMG6 targets harbor a degenerate pentameric sequence motif [(U/A)-(G/A)/(A/C)-N-(C/U)] at 5′ termini. Recently, by datamining on published datasets, including the RNA footprinting profile of phosphorylated UPF1, two sets of UPF1 PAR-CLIP data and the UPF1 target dataset generated with BIRC-Seq (24,32–34), Imamauchi et al. found that UPF1 preferably binds RNAs with high GC-rich motifs embedded in 3′ UTRs (35).

The identifications of NMD targets from different cell lines extend our understandings on how NMD shapes the transcriptome. These studies with different sequencing and analyzing strategies show that NMD functions beyond an RNA surveillance machinery in degrading PTC containing mRNAs, but plays essential roles in post-transcriptional gene expression regulation in degrading mRNAs implicated in various cellular functions. Of note, NMD targeted mRNAs with 5′ uORFs and long 3′ UTRs are enriched in the categories of oncogenes, transcriptional factors and stress response genes (5,36). Thus, NMD could coordinate the activation or repression of different signaling pathways and function in every level of cell dynamics (37).

Functional complexity of NMD factors: hints from cellular studies

In line with the diversity of NMD targets in cells, RNAi-based knockdowns on NMD factors in human cells cause severe defects in cell viability (5,38,39). These studies reveal that NMD functions in cell cycle regulation (40,41), cell viability (42,43) and amino acid starvation response (44), through modulating RNA stability. Furthermore, NMD factors possess NMD-independent roles (Table 1 and Figure 1), which include DNA damage response (41,45,46), telomere integrity maintenance (38,47), intracellular calcium dynamics (11,48), virus infection and replication (49–52) and tumorigenesis (Figure 1 and Table 1). SMG1, UPF1, UPF2, SMG5, SMG6 and SMG7 are involved in DNA damage response and telomere integrity maintenance (20,38,40,41,45,46,53–55), both of which are essential for cell viability. SMG1, together with ATM and ATR, belongs to the family of PI3Ks (56). Upon DNA damage induction, SMG1 phosphorylates p53 and induces p53-dependent cell cycle arrest (41,45,55). Accordingly, knockdown of SMG1 renders human cells sensitive to ionizing radiation (55).
SMG5, SMG6 and SMG7 are mammalian homologs of yeast telomere length regulator Est1 (Ever shorter telomere 1) (57). The yeast Est1 mutant shows a progressive loss of telomere length and the premature ageing phenotype (57). In human immortal cells, knockdown or overexpression of SMG5 and SMG6 compromises telomere integrity and cell viability (38,58,59). UPF1, with our current understanding, is an NMD factor with the most distinct functions reported. UPF1, as a core NMD factor, is involved in the degradation of PTC containing mRNAs and RNA species with other NMD features (12). UPF1 also participates in a variety of NMD independent processes, such as: (i) UPF1 is a downstream factor of activated PI3K signaling and could be phosphorylated by SMG1, ATM and ATR, indicating that UPF1 may directly take part in DNA damage response signaling (38,60); (ii) UPF1 is loaded onto chromatin in cell cycle S-phase and regulates S-phase progression (40,46); (iii) ATR-UPF1 axis is responsible for the degradation of replication-dependent histone mRNAs (61,62), indicating a role of UPF1 in balancing the DNA synthesis and histone mRNA translation during S-phase progression; (iv) UPF1 is associated with telomeres and maintains telomere integrity (54,63); (v) UPF1 is a component of ubiquitin system and has E3 ubiquitin ligase activity (64); (vi) UPF1 sits at the intercross of Staufen1-mediated mRNA decay (SMD) and NMD through a competitive binding by Staufen 1 or UPF2 (65). Therefore, UPF1 may selectively participate in SMD or NMD, depending on cellular states (66). Taken together, NMD factors function beyond NMD machinery. Thus, it is not clear whether the severe cellular phenotypes as shown with RNAi-based strategies are mediated by NMD.

Due to the functional complexity of NMD factors (Table 1), defining the biological roles of NMD per se with cell and animal models could be challenging (67,68). For example, DNA damage response, one of the NMD factors’ common functions, has been proposed in regulating stem cell dynamics, i.e. self-renewal and differentiation (56,69,70). Genomic instability imposed by defective DNA damage repair machinery occasionally results in early embryonic lethality of mammals (71). Thus, using an NMD factor with minimum NMD-independent functions could be optimal for elucidating roles of NMD. Among these NMD factors that have been reported with established mouse models, SMG6, UPF2 and UPF3A could be ideal for dissecting in vivo roles of NMD per se since these NMD factors possess minimum NMD-independent functions (Table 1). For example, SMG6 has been implicated in the NMD and telomere maintenance through stimulating telomerase activity to elongate telomeres (38). Since mouse chromosomes have longer telomere reservoirs as compared with humans, telomere dysfunctions do not generate any developmental

Table 1. Functions of NMD factors

| NMD factors | NMD functions | NMD independent functions |
|-------------|---------------|---------------------------|
| SMG1        | • Phosphorylate UPF1 | • Member of the PI3K family |
|             | • Promote the binding of SMG5/7 and SMG6 to UPF1 | • Telomere integrity maintenance |
|             | • Participate in DNA damage response by phosphorylating p53 and activating p53 dependent cell cycle arrest |
| SMG5        | • Form complex with SMG7 and recruit deadenylase complex | • Telomere integrity maintenance |
|             | • Interact with PP2A and play a role in UPF1 dephosphorylation | • Participate in DNA damage response in human immortal cells (but not in murine cells) |
| SMG6        | • Possess the endoribonuclease activity and cleave NMD targets near PTCs | • Telomere integrity maintenance |
|             | • Interact with PP2A and play a role in UPF1 dephosphorylation | |
| SMG7        | • Form complex with SMG5 and recruit deadenylase complex to NMD targets | • Telomere integrity maintenance |
|             | • Interact with PP2A and play a role in UPF1 dephosphorylation | • Participate in DNA damage response by regulating p53 protein stability |
| UPF1        | • Recognize the stalled ribosomes on PTC containing mRNAs | • DNA damage response |
|             | • Interact with UPF2/UPF3B to initiate the assembly of decay complex | • Bind to chromatin and regulate cell cycle S phase progression |
|             | • Bind to mRNAs with long 3’ UTRs and initiate mRNA decay | • Histone mRNA decay |
|             | • DNA damage response | • Telomere integrity maintenance |
|             | • UPF1 is an E3 ligase of the ubiquitin system | • UPF1 is an E3 ligase of the ubiquitin system |
|             | • Staufen1-mediated mRNA decay | |
| UPF2        | • Bind to UPF1 and UPF3B | • Telomere integrity maintenance |
|             | • Facilitate the UPF1 phosphorylation by SMG1 | • DNA damage response |
| UPF3B       | • Minimum NMD activity | • N.D. |
|             | • Interact with UPF2 and stimulate the ATPase and helicase activity of UPF1 | |
| UPF3A       | • Compete with UPF3B in binding to UPF2 | • N.D. |

Note: N.D., not defined.
The essential role of NMD in animal development

The development of an animal starts from a fertilized totipotent egg. In mouse, a fertilized egg undergoes at least four rounds of symmetric cell divisions to form a blastocyst at embryonic stage 3.5 (E3.5) (73), which contains an outer layer, the trophectoderm and an inner cell mass (ICM) with around 32 pluripotent cells. The pluripotent cells in the ICM divide quickly to reach around 100 cells, namely epiblast, at E4.5. During this period, the multipotent primitive endoderm cells are formed to cover the blastocoelic surface of the epiblast. The epiblast cells still maintain pluripotency, and could give rise to embryo proper by differentiating into three germ layers, i.e. ectoderm, endoderm and mesoderm (74). After the implantation of mouse embryos in the uterus finishing around E6.5, the gastrulation starts (73). During gastrulation (E6.5–E7.5), precursors of different germ layers are formed from the epiblast, and experience complicated patterning steps in the embryo to form a framework for further mouse organogenesis (75). From E8.0, mouse organogenesis starts at different ontological sites of the embryo and finally forms different tissues with specific functions (76). The progression of developmental stages is strictly controlled by temporal and spatial regulations in gene expression, abnormalities of which could generate developmental defects and embryo lethality.

The activity of NMD is a dynamic process during animal development (77–79). Accordingly, in zebrafish embryos, knockdown of any of the NMD factors, such as Upf1, Upf2, Smg1, Smg5 and Smg6, results in massive cell death in embryonic tissues and animal lethality (80). In mice, genetic ablations of NMD factors, such as Smg1 (19), Smg6 (20), Upf1 (81), Upf2 (82) and Upf3a (83), as well as EJC component Rbm8a (84) and Magoh (85), cause early embryonic lethality (Figure 2). The Upf3b knockout mouse generated with gene trap strategy is a viable NMD-deficient mouse model, possibly due to the fact that Upf3b deletion does not completely abolish the NMD activity (86,87). Of note, although NMD factors have different roles as mentioned above (Table 1), NMD factor knockout mice die around a similar developmental stage (Figure 2). Smg1 gene-trapped mouse embryos have normal Mendelian ratios before E10.5, but all die before E12.5 (19); Upf1 knockout mouse embryos are viable at E3.5, but die before E7.5 (81); The relative frequency of Upf2 knockout mouse embryos shows a great reduction at E3.5, and no Upf2 knockout embryos could be detected at E9.5 (82). Furthermore, Smg6 knockout embryos are viable at E3.5, but no viable mutant embryos could be isolated around E12.5. Most of Smg6 knockout embryos die around E7.5–E9.5 (20). Recently, Shum et al. found that Upf3a (an NMD repressor) knockout mice die between E4.5 and E8.5 (83). Taken together, these studies reveal that embryos of NMD factor mutant mice could successfully develop to E4.5 before the start of the primitive streak, and die between E5.5 and E9.5, during which gastrulation and early stages of organogenesis occur. These data strongly suggest that NMD per se is not essential for the maintenance or proliferation of murine pluripotent stem cells (PSCs) from the ICM and epiblast. The fact that all NMD factor mutant embryos die during gastrulation or early stages of organogenesis indicates that NMD is essential for the early development of mice, when the pluripotent cells start to specify into three different germ layers. In the future, in-depth analysis by dissecting NMD mutant mice embryos at different developmental stages and immunocytochemistry investigations with lineage-specific markers will resolve the biological roles of NMD in early mammalian development.

NMD in embryonic stem cell self-renewal and differentiation

The embryonic stem cell (ESC) is an important model to study the fundamental mechanisms of developmental biology (88,89). The establishment of ESC lines from ICMs of blastocysts greatly facilitates our investigation on the mechanisms of ESC identity maintenance during self-renewal...
and cell fate commitment to ectoderm, endoderm and mesoderm (89). The successful reprogramming of human patient derived somatic cells to PSCs enlightens the promise for applications of ESCs and PSCs in regenerative medicine (16,90). Transcriptional regulation and epigenetic modulation mechanisms are implicated in ESC self-renewal and differentiation (91–95). Recently, NMD has emerged as an important regulatory mechanism in ESC biology.

Although mouse embryos with NMD factor deficiency are viable at E3.5 (96), none succeeds in establishing a stable NMD deficient mouse ESC (mESC) line by outgrowing ICMS from blastocysts. Gene knockdown approaches with siRNAs or shRNAs are widely used to dissect NMD roles in mESCs (78,86). By analyzing the function of Upf1 in a mouse embryonic carcinoma cell line (P19), Lou et al. found that overexpression of Upf1 promotes self-renewal of P19 cells (78) (Table 2 and Figure 3A, upper panel). Knockdown of Upf1 reduces the expression of stemness markers (Nanog and Oct4), and simultaneously enhances the expression of neuronal lineage markers (Sox2 and Nestin), indicating that Upf1 knockdown accelerates P19 embryonic carcinoma cell differentiation toward ectoderm (78). Mechanistically, Lou et al. showed that gene transcripts of TGF-β signaling pathway members, such as Smad6 and Smad7, are targeted by Upf1-NMD (78). Intriguingly, using the E14.1 mESC line, which is widely used in gene targeting studies (97), Li et al. found that NMD safeguards the proper differentiation of mESCs (20). Stable knockdowns of NMD factors, such as Smg1, Smg5, Smg6, Upf1, and Upf2, in E14.1 mESCs generate variable mESC clones with different extents of NMD defects, indicating that NMD is dispensable for the cellular viability of mESCs (20) (Table 2). Furthermore, by transient expression of Cre recombinase in mESCs with two conditional alleles of Smg6 gene (Smg6F/− mESCs), Li et al. generated several lines of Smg6 knockout mESCs. Smg6 knockout mESCs behave indistinguishably from wild-type mESCs in cellular proliferation, cell cycle distribution and cell death index (20). The major defect of Smg6 knockout mESCs and Smg1, Smg5, Upf1 and Upf2 stable knockdown mESCs is the differentiation failure. Mechanistically, Smg6-NMD, by targeting 3′ UTR of c-Myc mRNA, destabilizes pluripotent gene c-Myc mRNAs and thus promotes mESCs differentiation (Figure 3A, upper panel). Furthermore, ectopic expressions of NMD proficient Smg6 truncated proteins in Smg6 knockout mESCs could rescue differentiation defects. These findings strongly suggest that: (i) Smg6-NMD activity is dispensable for the cell viability of mESCs, but essential for the pluripotency exit; (ii) The embryonic lethality of NMD factor knockout mice is most likely due to an essential role of NMD in mESC differentiation.

Compared to mESCs, NMD plays distinct roles in human ESCs (20,78,98). Lou et al. employed a human ESC line (H9) and found that NMD factors are highly expressed in human PSCs, indicating that NMD promotes pluripotency in human ESCs (98). Knockdown of UPF1 drives H9 cells differentiation toward endoderm lineage by promoting the TGF-β signaling, and suppresses mesoderm lineage through inhibiting the BMP/WNT signaling (Table 2 and Figure 3A, lower panel). Thus, UPF1 mediated NMD may be functionally involved in human ESC fate commitment toward different germ layers (98). Taken together, current studies in human and mouse ESCs reveal a dynamic requirement of NMD during ESC differentiation, but NMD functions in stem cell self-renewal and differentiation remain controversial, which could be attributed to the following facts. First, human and mouse ESCs in different culture condition may require distinct maintenance and differentiation programs, which could generate distinct responses and biological outputs upon NMD deficiencies (98–100). Second, different gene manipulation strategies are used in these studies. Li et al. used the complete gene knockout strategy in mESCs; Lou et al. applied an RNAi knockdown strategy, which may not completely deplete NMD factors in ESCs. Thirdly, different NMD factors are investigated in these studies and the NMD-independent functions of these NMD factors could be partially responsible for these discrepancies (20,78,98). Since gene editing tools, such as TALEN and CRISPR-Cas9, are routinely used to generate gene knockout human and mouse ESCs.
Figure 3. NMD function in ESCs (A) and NSCs (B). (A) Essential roles of NMD factors in the self-renewal and differentiation of mouse (upper panel) and human (lower panel) ESCs. Upper panel: NMD factor Upf1 promotes the self-renewal of mouse ESCs; During differentiation, Smg6 mediated NMD destabilizes mRNA transcripts of pluripotency gene c-Myc, and thus safeguards mouse ESC differentiation into three germ layers. Lower panel: by destabilizing mRNA transcripts of key components in BMP/WNT and TGF-β pathways, UPF1 regulates human ESC fate commitment between mesoderm and endoderm. (B) NMD function in mouse NSC self-renewal and differentiation. RNA transcripts and protein levels of NMD factors (Upf1 and Upf3a) decrease during NSC differentiation and neuronal maturation. NMD factor Upf1 promotes NSC self-renewal and is repressed by miR128 during NSC differentiation; However, Upf3a-NMD is required for NSC differentiation and neuronal maturation, such as dendritic spine outgrowth.

(101–104), direct comparisons on different NMD factor deficient ESCs within same species could efficiently elucidate roles of NMD in ESC self-renewal and differentiation.

NMD in neural stem cell self-renewal and differentiation

Neural stem cells (NSCs) are originated from mammalian neuroepithelium (105). During the early development of mammalian brain, NSCs proliferate at the ventricular zone of the neocortex and differentiate into different types of functional neurons to build up the mammalian brain and establish proper brain functions (105,106). The mammalian neurogenesis relies on transcriptional regulation, epigenetic modulation, genomic stability maintenance and tight cell cycle control of NSCs (107–111). Abnormalities in neurogenesis may generate neurological diseases in humans. UPF3B is the first NMD factor found to be associated with human neurodevelopmental disorders (112). Patients with UPF3B mutations manifest neurological symptoms, such as intellectual disability, schizophrenia and autism (112,113). Recent studies by genomic sequencing in human patients with neurodevelopmental disorders identify mutations in UPF2, UPF3A, SMG6, SMG9 and EJC component RBM8A, indicating that NMD is essential for mammalian brain development (115,114,115). Furthermore, since NMD participates in synapse architecture maintenance and synaptic vesicle recycling and is thus required for functions of mature neurons, the neurological symptoms in patients with NMD factor mutations could be due to synergistic effects of prenatal brain development abnormalities and postnatal neuronal dysfunctions (87,116,117).

How does NMD safeguard the neurogenesis? Recent studies from mammalian NSCs reveal some mechanistic clues. mRNA transcripts of Upf1, Upf2, Smg1 and Smg6 were reduced during the differentiation process of mouse NSCs and human neural progenitors (78) (Figure 3B). Furthermore, protein levels of NMD factors, such as Upf1 and Upf3b, decrease during differentiation of cultured mouse and rat NSCs (78,118,119), indicating NMD may promote NSC self-renewal (Figure 3B). In accordance with this hypothesis, using a siRNA approach, Lou et al. found that Upf1 knockdown or Upf3b deficiency in NSCs could stimulate mouse NSC differentiation with upregulation of neuronal markers (78). Of note, in Lou et al.’s study, Upf1 knockdown enhances the expression of NSC markers Sox2 and Nestin as well as the mature neuron marker DCX (78). These data are paradoxical since mammalian neurogenesis is accompanied with downregulation of NSC markers and upregulation of neuronal markers. Since Sox2 and Nestin have been widely used as stem cell markers for NSCs as shown by many groups (107,120,121), the co-upregulations of NSC markers (Sox2 and Nestin) and neuronal maker (DCX) in NSCs with Upf1 knockdown may generate a transcriptome chaotic state in NSCs and consequently compromise NSCs fate toward mature neurons. In line with this hypothesis, Alrahbeni et al. found that NMD inhibition by Amlexanox or overexpression of UPF3B missense mutation compromises the differentiation of NSC toward mature neuron (118). With mouse NSCs, Jolly et al. and Huang et al. further showed that Upf3b deficient NSCs are reluctant to differentiation (87,119). Upf3b deficient NSCs are hyper-proliferative and show higher self-renewal capacity in differentiation culture conditions (87,119). These data indicate that NMD promotes NSC differentiation in vitro and in vivo. Taken together, these data suggest that: (i) abundances of mRNAs and protein expression levels of NMD factors in cells may not reflect the magnitude of NMD activity; (ii) functions of NMD in NSC self-renewal and differentiation are under debate. Thus, future studies on NSCs with deficiencies in other NMD factors, such as Upf1, Smg5 and Smg6 will solve the discrepancies of current knowledge on NMD functions in NSCs. Since NSC differentiation is a...
Table 2. Biological roles of NMD factors in embryonic and tissue specific stem cells

| Cell type               | Targeted gene | Stem cell and mouse phenotypes | Mechanisms                                                                 | Ref. |
|-------------------------|---------------|--------------------------------|-----------------------------------------------------------------------------|------|
| Embryonic stem cell (ESC) | Upf1, knockdown in P19 cells (in vitro) | Upf1 promotes pluripotency of P19 cells; Upf1 knockdown promotes neuronal differentiation of P19 cells. | Upf1-NMD promotes pluripotency by destabilizing mRNAs of G1/S transition inhibitors, neuronal differentiation factors, and TGF-β inhibitors. | [78] |
|                         | Smg6, KO in mESC (in vitro + in vivo) | Smg6 KO mESCs have telomere defect, but no proliferation defect; Differentiation block is found in Smg6 KO mESCs. | Smg6-NMD destabilizes c-Myc mRNA and promotes mESC differentiation. | [20] |
|                         | Upf1/2 knockdown in mESC (in vitro) | NMD factor knockdowns render differentiation block of mESCs. | N.D. | [20] |
|                         | Upf1, knockdown in H9 human ESC (in vitro) | Upf1 knockdown promotes endoderm lineage differentiation of hESC. | NMD inhibits TGF-β signaling, while facilitates BMP/WNT signaling. | [98] |
| Neural stem cell (NSC)  | Upf3b KO in mNSCs (in vitro + in vivo) | Upf3b null promotes NSC proliferation, while inhibits differentiation. | RNA-Seq has been conducted; no specific target was found to be responsible for the phenotype. | [119] |
|                         | Upf3b KO in mNSCs (in vitro + in vivo) | Upf3b null promotes NSC proliferation, while inhibits differentiation and neuronal maturation. | RNA-Seq has been conducted; no specific target was found to be responsible for the phenotype. | [87] |
|                         | Upf1, knockdown in mNSCs (in vitro) | Upf1 knockdown promotes NSC differentiation and neuronal maturation. | UPF1-NMD inhibits neuronal differentiation by destabilizing the mRNAs of G1/S transition inhibitors, neuronal differentiation factors, and TGF-β inhibitors. | [78] |
|                         | Expression of truncated hUPF3B in rat hippocampal NSCs (in vitro) | Expression of truncated hUPF3B compromises neuronal differentiation and neurite outgrowth. | N.D. | [118] |
| Spermatogonial stem cell (SSC) | Upf2 cKO in spermatogonia (in vivo) | "Sertoli-only syndrome", testicular atrophy and dramatic germ cell depletion. | Accumulation of gene transcripts with long 3' UTRs; no specific target was found to be responsible for the phenotype. | [129] |
|                         | Upf2 cKO in spermatogonia (in vivo) | Testicular atrophy, delayed meiotic entry and massive depletion of spermatocytes and spermatids. | Accumulation of gene transcripts with long 3' UTRs; no specific target was found to be responsible for the phenotype. | [129] |
|                         | Upf3a cKO in spermatogonia (in vivo) | Defective spermatogenesis in Upf3a cKO males; dramatic reduction in sperm counts; a differentiation block of spermatocytes. | More that 10 Upf3a targets in pachytene spermatocytes have been identified with RNA-Seq. | [83] |
| Hematopoietic stem cell (HSC) | Upf2 cKO in adult HSCs (in vivo) | Upf2 deficiency causes the death of hematopoietic stem cells and progenitors. | N.D. | [82] |
| Hepatoblast              | Upf2, cKO in hepatoblasts (in vivo) | Upf2 deficiency causes mitotic arrest, and terminal differentiation failure of hepatoblasts. | N.D. | [137] |

Note: KO, knockout; cKO, conditional knockout; N.D., not defined.

step-wise process controlled with temporal gene expression regulation, NMD may play distinct roles at different stages of NSC differentiation through targeting specific RNA targets.

The biological roles of NMD factors in brain development are enlightened with mouse models for the EJC components Rbm8a, Magoh and Eif4a3 (84,85,122). Rbm8a is upregulated at the onset of neurogenesis in neocortex, regulates NSC proliferation and prevents premature neuronal differentiation (84). Haploinsufficiency of Rbm8a in mouse developing neocortex results in microcephaly with reduced cortex size, which is due to defective neurogenesis upon the loss of one Rbm8a allele in NSCs (84). Furthermore, Magoh haploinsufficient mice show microcephaly (85). The neocortex tissues from Magoh haploinsufficient mice display deletions of intermediate neural progenitors expressing Tbr2 and mature neurons due to high frequencies of apoptosis. Magoh haploinsufficient NSCs are prone to differentiate rather than self-renew at early stages of neocortex development. This phenomenon could be explained by the roles of Magoh in mitosis and genome integrity maintenance (85). Eif4a3 haploinsufficient mice phenotypically mimic Rbm8a and Magoh mutant mice, indicating that these three EJC factors regulate brain development in the same pathway. Interestingly, loss of p53 could partially rescue the microcephaly phenotype conferred by Eif4a3 haploinsufficiency.
suggesting that EJC haploinsufficiency in developing brain compromises neurogenesis through activating the p53 dependent cell death pathway. Of note, in all the studies conducted in mouse models with EJC component haploinsufficiencies, NMD activities in NSCs have not been assayed in the brain samples from mutants (122). Thus, a mechanistic link between NMD and neurological pathogenesis could not be established. Furthermore, EJC components play NMD independent roles in gene expression regulation (123–125), which potentially could contribute to the defective neurogenesis as seen in these EJC mutant mice. Since UPF2, UPF3A, SMG6, SMG9 and RBM8A are implicated in human neurodevelopmental diseases, establishments and analysis of respective mouse models for these NMD factors specifically deleted in embryonic and adults NSCs could advance our knowledge on NMD roles in mammalian brain development and adult brain function. Furthermore, identification of RNA targets of these NMD factors in NSCs will be helpful for elucidating NMD mediated post-transcriptional gene regulatory mechanisms in NSC self-renewal and differentiation.

NMD in spermatogonial stem cell and spermatogenesis

The emergence and development of spermatogonial stem cells (SSCs) and spermatogenesis are tightly regulated during embryonic and postnatal development of mammals (126). In mouse, the SSC development starts as early as embryonic day 6.25 (E6.25), when primordial germ cells (PGCs, the ancestors of SSCs) evolve within the proximal epiblast (126). During E10–11, PGCs migrate to the gonad ridge. At this development stage, the bi-potential gonad acquires sex specificity and forms the male germ cell niche, the embryonic sertoli cells (SCs). Once PGCs interact with embryonic SCs and other niche components, sex determination will be completed. The PGCs further proliferate to expand the PGC pool (namely, prospermatogonia) and finally develop into the spermatogonia or SSCs after birth. SSC is the only stem cell population which sustains the reproductive capacity of adult males. During puberty and adulthood, SSCs self-renew to maintain the stem cell pool and differentiate to progenitors, which undergo further meiosis to generate functional spermatids.

Large scale RNA-Seq analysis in human and mouse shows that testis tissues have the highest expression of NMD factors, such as Smg1, Upf1, Upf2, Smg5, Smg6 and Smg7, indicating that NMD is essential for spermatogenesis. Recently, conditional ablation of NMD factor Upf2 or Upf3a in developing germ cells and embryonic SCs suggested an indispensable role of NMD in regulating spermatogenesis (83,127–131) (Table 2). Bao et al. conducted several studies to investigate Upf2-NMD in spermatogenesis (128,129). SCs, together with Leydig cells, build up the developmental niche for spermatogenesis during embryonic stages and adulthood. By conditional deletion of Upf2 in embryonic SCs starting from E14.5, Bao et al. found that loss of Upf2 in developing SCs leads to severe testicular atrophy and mouse sterility (128). Upf2 deficient developing SCs cannot differentiate into mature SCs to support spermatogenesis. Specific deletion of Upf2 in prospermatogonia by the Ddx4-Cre mouse line (Upf2 deletion starts at E15) results in infertile mice with germ cell free seminiferous tubules in testis, phenotypically mimicking the ‘sertoli-only’ phenotype (129). The Upf2 deficient prospermatogonia fails to develop into spermatogonia. This finding indicates that Upf2-NMD is essential for the early male germ cell development (128,129). Furthermore, postnatal deletion of Upf2 in Type A spermatogonia with Stra8-Cre line (Upf2 deletion starts around postnatal day 3, P3) causes reduced testis weight and mice infertility in adult. Although, at postnatal day 12 (P12), zygote spermatocytes are present in wild-type and Upf2 deficient testis, Upf2 mutant mice, starting from P14, showed a dramatic reduction of spermatocytes and spermatids inside seminiferous tubules in testis (129).

These data suggest that Upf2-NMD is essential for cell fate transition during each developmental stage of testis. Transcriptomic analysis shows that Upf2-NMD has differential regulation modes in SCs and germ cells (128,129). In SCs, Upf2-NMD seems to degrade the PTC containing mRNAs, while in germ cells, Upf2-NMD specifically targets mRNAs with long 3′UTRs. This cell type specificity of NMD targets was further confirmed by the study on a mouse model with testis-specific knockout of Tdrd6, a component of the chromatoid body, where Upf2 executes its NMD function (131,132).

Loss of NMD compromises SSC development and spermatogenesis. On the other hand, hyper-activation of NMD results in spermatogenesis defect as well. Upf3a, a paralog of Upf3b in mice, has been recently identified as an NMD repressor through competing with NMD activator Upf3b for the binding to Upf2 (83). Overexpression of Upf3a represses Upf3b expression, and thus upregulates a subset of NMD targets; Upf3a deficiency could activate the NMD machinery. Deletion of Upf3a at postnatal day3 with Stra8-Cre mouse line causes spermatocyte progression defect and further depletion of spermatogonia, indicating that hyper-activation of NMD could partially block the differentiation and maturation of spermatids in mice (83). It seems that Upf3a also targets mRNA species with long 3′ UTRs, suggesting a cell type-specific requirement of NMD in degrading mRNAs with long 3′ UTRs in spermatogenesis and its progeny (83,129,132). It is interesting to note that testis tissues in wild type mice, mainly composed of mature spermatids, are enriched with gene transcripts with short 3′ UTRs (133), which are supposed to be resistant to mRNA decay. The short 3′ UTR setting in testis could preserve transcriptome stability of cells and maintain the cell identity.

These studies on NMD function in different developmental stages of testis indicate that fine-tuning of NMD activity is essential for the differentiation of embryonic SCs, spermatogonia and SSCs (83,128,129). Current studies employed whole testis tissues with the mixed cell populations, including SCs, spermatogonia as well as cells at different developmental stages of spermatogenesis, to conduct the RNA-Seq analysis and identified transcriptional changes in NMD factor deficient testis tissues (83,129,132). Further investigation on specific mRNAs or pathways targeted by NMD in purified SCs or spermatogonia will disclose more in-depth mechanisms of NMD in SSC maintenance, differentiation and spermatogenesis.

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Figure 4. ER stress, UPR and NMD in cell fate determination. ER is an organelle for protein-folding. Accumulations of unfolded/misfolded proteins in ER will trigger the activation of adaptive UPR. UPR-related transcriptional factors, such as ATF3, ATF4 and ATF6, are unregulated, and further bind to their target gene promoters and initiate cellular programs for ER homeostasis restoration and cellular adaptation to ER stress. Furthermore, NMD inhibition could induce ER stress and UPR activation possibly through productions of truncated and unfolded/misfolded proteins. At least 10 UPR components, including ATF3, ATF4 and ATF6, are direct targets of NMD. Thus, NMD, together with ER stress-UPR signaling, maintains stem cell identity and safeguards cell identity transition, such as stem cell self-renewal and differentiation.

NMD in hematopoietic stem cell and hepatoblast

The hematopoietic stem cell (HSC) is considered as a prototype to study the function of tissue specific stem cells in development, tissue homeostasis, ageing and regeneration (56,134,135). Many studies show that factors participating in transcription regulations, epigenetic modifications, metabolic pathways and genomic stability maintenance are essential for HSC self-renewal, differentiation and hematopoiesis (56,136). Weischenfeldt et al. conducted a pioneer study on whether NMD could affect adult hematopoiesis (82) (Table 2). Using an inducible Cre line (Mx1-cre), Weischenfeldt et al. showed that deletion of Upf2 in HSC and its progeny results in mice lethality due to severe bone marrow atrophy (82). Upf2 deficient HSCs fail to expand in vitro. Intriguingly, knocking out Upf2 specifically in the myeloid lineage with the LysM-Cre mouse line is compatible with cellular viability and terminal differentiation of myeloid lineage cells. In this regard, Upf2 is essential for the cellular fitness of highly proliferative cell populations such as HSCs, common lymphoid progenitors and B cell progenitors since Upf2 knockout results in fast depletion of these cells (82). However, the mechanism underlying the death of Upf2 deficient HSCs is unknown. Detailed analysis on the purified HSCs with deficiencies of Upf2 or other NMD factors could shed more light on the biological roles and mechanisms of NMD in HSC maintenance and differentiation.

In addition to aforementioned studies on functions of NMD in ESC, SSC and HSC, Upf2 conditional mice have been employed to study the role of NMD in embryonic liver development and adult liver regeneration (137). The development of liver begins around E8.5. Hepatoblasts proliferate during the embryonic stages, and further exit the cell cycle and form functional hepatocytes and cholangiocytes through the actions of multiple transcriptional factors, such as HNF4α (138). In adulthood, hepatocytes are quiescent, but will reenter cell cycle to proliferate and compensate for the loss of liver mass upon liver damages (139). Deletion of Upf2 in fetal liver around E10 with the Alfp-cre mouse line causes perinatal lethality of mutant mice (137) (Table 2). Upf2 deficiency does not alter the proliferation and viability of fetal liver cells, but confers a terminal differentiation defect of hepatoblasts toward mature hepatocytes (137). Furthermore, the liver regeneration assay (partial heptectomy) shows that Upf2 deficient hepatocytes fail to be activated, and reenter into cell cycle to proliferate and compensate the loss of liver mass (137). These data indicate that NMD, during development, may facilitate the cell identity transition, i.e. hepatoblasts toward differentiated hepatocytes, and during stress conditions, activate quiescent hepatocytes toward cycling hepatocytes (20).

Identification of NMD-mediated regulatory mechanisms in stem cell biology: a perspective

Through degrading PTC containing mRNAs and natural gene transcripts that play significant roles in cell functions, NMD serves as a general mRNA surveillance mechanism in regulating transcriptome stability and fine-tuning gene expression (1,5,7,9). Since the first description of the NMD phenomenon in eukaryotic cells in the late 1970s, there has been a booming of findings in understanding the biochemistry and structure of this conserved pathway (5,6,8,17). However, only until recently the biological roles of NMD in mammals start to emerge (5,36). Studies in mouse ESCs, NSCs, SSCs, HSCs and hepatoblasts indicate that NMD may not be required for the cellular viability of stem cells,
but essential for stem cell maintenance and differentiation (20,78,82,83,98,118,119,128,129,137).

How does NMD regulate the stem cell maintenance and differentiation programs and consequently safeguard embryonic development and maintain tissue homeostasis? The answer resides in the mRNA targets of the NMD machinery. NMD targets PTC containing mRNAs for elimination. Failure in degrading PTC containing mRNAs will allow the translational machinery to produce a large amount of truncated proteins, which may generate a stress condition in the endoplasmic reticulum (ER), where truncated proteins fold and mature (140). Defects in protein folding and maturate will generate ER stress and activate the unfolded protein response (UPR) (Figure 4). In line with this hypothesis, Sakaki et al. showed that knockdown of SMG6 in mammalian cells generates ER stress and UPR signaling activation (141). Thus, through degrading the aberrant mRNAs and reducing the accumulation of truncated or misfolded proteins, NMD could play an important role in protecting cells from the deleterious effect of ER stress and UPR activation. Surprisingly, Karam et al. found that gene transcripts from 10 UPR components, such as transcriptional factors ATF3, ATF4 and ATF6, are bona fide NMD targets (140). Thus, NMD plays an important role in shaping the UPR pathway to combat ER stress (36) (Figure 4). Of note, ER stress and its induced UPR signaling are implicated in the maintenance and differentiation of various types of stem cells (142–146). The magnitudes of ER stress and UPR signaling mediate distinct cell fates (147). Upon high ER stress, UPR activation will confer cell death (147). In low ER stress conditions, cells may adapt their transcriptional programs to sustain their viability in trade-off with other cellular functions, such as differentiation (147). It would be interesting to investigate the magnitudes of ER stress in NMD deficient cells and to test whether the genetic manipulation of UPR signaling could alleviate the defects as shown in NMD factor deficient stem cells and mouse models.

Since NMD targets natural gene transcripts with roles in cell functions, fine-tuning of mRNA stability and protein expression could be another NMD-mediated mechanism in regulating stem cell dynamics. For example, working with Smg6 deficient ESCs, Li et al. found that Smg6-NMD regulates the mRNA stability of a key pluripotency regulator (c-Myc) and safeguards ESC differentiation; Lout et al. demonstrated that Upf1-NMD destabilizes mRNA transcripts of TGF-β signaling repressors Smad6 and Smad7 to promote pluripotency (20,78,98,148). The NMD targets determining cell fates were mainly identified through analyzing the DEGs generated from RNA-Seq data. The integrated analysis of RNA-Seq and PAR-CLIP sequencing for direct determination of NMD factor binding RNAs will accelerate the findings of key genes in the maintenance and differentiation of distinct stem cell population.

**Manipulation of NMD for the treatment of human diseases**

A large proportion of human genetic diseases are caused by the in-frame PTCs generated by nonsense mutations or splicing abnormalities in genes (149). During mRNA translation, the recognition of a PTC will prematurely terminate translation process, consequentially generating a truncated polypeptide with functional abnormalities; Furthermore, a PTC in mRNA will trigger NMD activation and mRNA degradation. Thus, reduction of normal protein expression is considered as a major cause for nonsense mutation-generated human genetic diseases. The read-through chemicals, including Gentamicin, could simulate the insertion of an amino acid at the PTC site during mRNA translation and thus allow the translation machinery to produce full length polypeptide (150). Treatment of read-through chemicals shows certain improvements on cellular defects in human diseases models. Furthermore, NMD inhibition has been shown to be effective in increasing expression levels of functional truncated proteins in cellular models of human diseases, such as hereditary elliptocytosis and Ullrich’s disease (150–152). Consequently, NMD inhibition partially alleviates cellular defects in patients derived cell lines. Recently, combinational therapeutic strategies by applying read-through chemicals and NMD inhibitors show great promises in treatments of human diseases caused by nonsense mutations in genes (150,153–156). However, NMD inhibition in disease therapies should be applied with caution since knockdowns or knockouts of NMD factors in animal models indicate that NMD deficiency generates strong toxicities to cells as well as to tissues. Furthermore, deficiencies of different NMD factors in humans cause severe pathological symptoms in the central nervous system, as well as in the immune system (15,51). In this way, at current stage, a transcript-specific inhibition of NMD will have less adverse effects than general NMD inhibition (157). Furthermore, since NMD-mediated mechanisms contributing to these severe symptoms as shown in existing NMD factor mutants remain unsolved, understanding NMD biology and its mediated molecular pathways in mammalian stem cells is a prerequisite for future designing NMD inhibitors with minimal effects on disturbing stem cell maintenance and tissue homeostasis.

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