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Nonsense-Mediated RNA Decay Influences Human Embryonic Stem Cell Fate

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SUMMARY

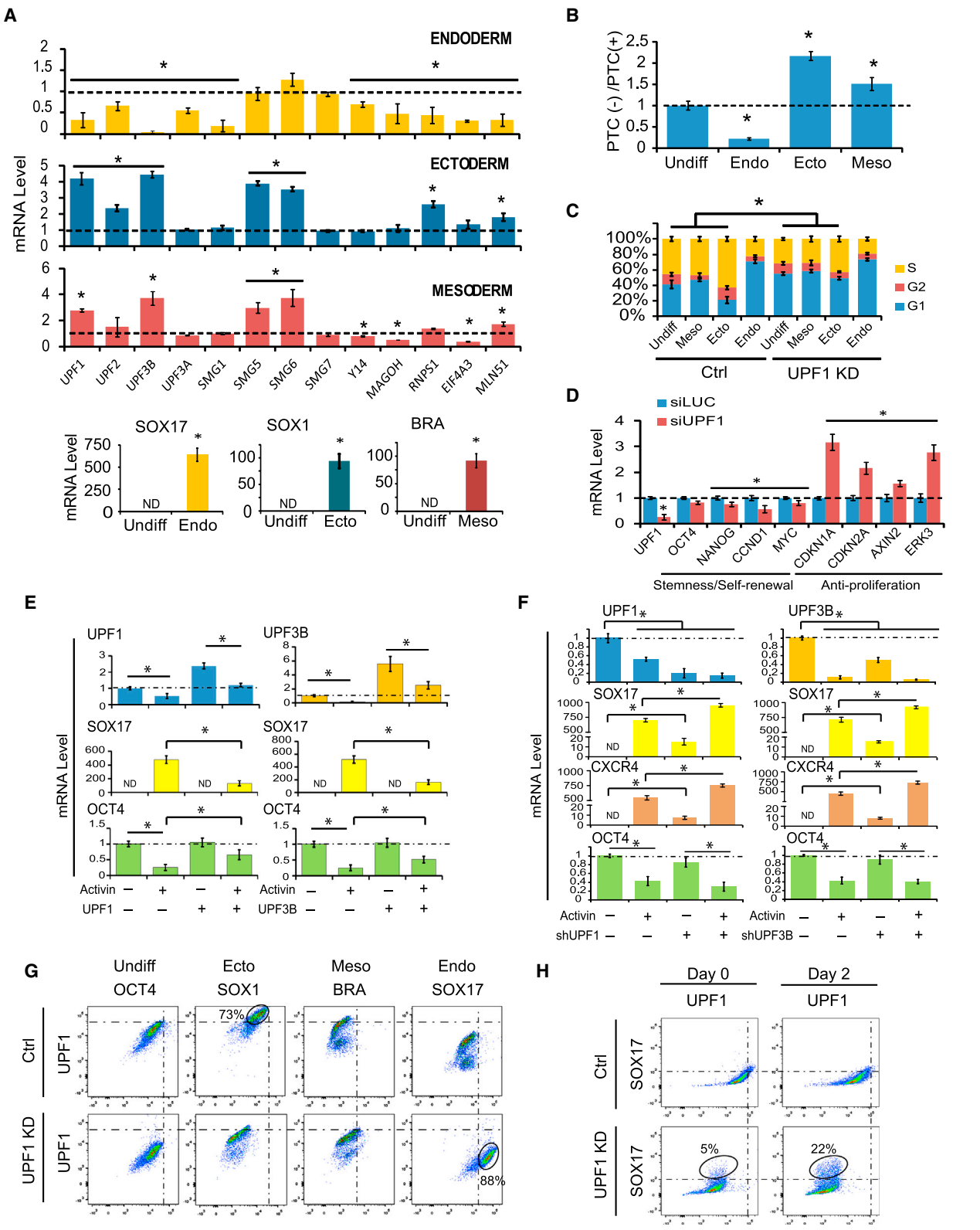
Nonsense-mediated RNA decay (NMD) is a highly conserved pathway that selectively degrades specific subsets of RNA transcripts. Here, we provide evidence that NMD regulates early human developmental cell fate. We found that NMD factors tend to be expressed at higher levels in human pluripotent cells than in differentiated cells, raising the possibility that NMD must be downregulated to permit differentiation. Loss- and gain-of-function experiments in human embryonic stem cells (hESCs) demonstrated that, indeed, NMD downregulation is essential for efficient generation of definitive endoderm. RNA-seq analysis identified NMD target transcripts induced when NMD is suppressed in hESCs, including many encoding signaling components. This led us to test the role of TGF-β and BMP signaling, which we found NMD acts through to influence definitive endoderm versus mesoderm fate. Our results suggest that selective RNA decay is critical for specifying the developmental fate of specific human embryonic cell lineages.

INTRODUCTION

Developmental processes depend on highly orchestrated shifts in the levels of specific mRNAs. While regulation of the synthesis of mRNAs (transcription) has traditionally been the focus of attention, increasing evidence suggests that developmentally regulated alterations in the rate of decay of specific transcripts also influences developmental decisions (Hwang and Maquat, 2011). The best-studied RNA degradation pathway is nonsense-mediated RNA decay (NMD). While originally identified as a quality control mechanism that rapidly degrades aberrant transcripts derived from mutant genes, NMD was subsequently found to also degrade many normal transcripts (Peccarelli and Kebaara, 2014). Between ~3% and 20% of the transcriptomes of eukaryotic organisms ranging from yeast to man are regulated (directly or indirectly) by NMD (Peccarelli and Kebaara, 2014). The specific transcripts targeted by NMD are those that harbor a stop codon in a "premature" context, as this leads to the formation of a complex of NMD proteins that subsequently recruits RNA decay factors (Schoenberg and Maquat, 2012).

The discovery that NMD regulates normal gene expression raised the possibility that NMD can influence normal biological events (Hwang and Maquat, 2011). By modulating the magnitude of NMD, batteries of transcripts can be stabilized or destabilized to achieve specific biological outcomes. Indeed, NMD has been found to be a highly regulated pathway, and mounting evidence supports the possibility that NMD is critical for many biological events, with loss of NMD resulting in developmental defects (Huang and Wilkinson, 2012; Hwang and Maquat, 2011; Karam et al., 2013). Most well-studied is the role of NMD in the neural cell lineage. Studies in *Drosophila melanogaster*, zebrafish, and mammalian cell lines have shown that NMD is critical for specific steps in neural development (Jolly et al., 2013; Lou et al., 2014; Metzstein and Krasnow, 2006; Wittkopp et al., 2009). In humans, mutations in the NMD gene, *UPF3B*, lead to intellectual disability (Nguyen et al., 2014). These cognitive disorders are likely to result from developmental defects; indeed patients with mutations in *UPF3B* and copy-number variants of other NMD genes commonly have neurodevelopmental disorders, including schizophrenia and autism (Nguyen et al., 2014). Less is known about the influence of NMD on non-neuronal cell lineages. Loss of the NMD factor UPF2 disrupts hematopoiesis and liver development in vivo (Thoren et al., 2010; Weischenfeldt et al., 2008), and evidence suggests that NMD cooperates with another RNA decay pathway to influence muscle cell differentiation (Gong et al., 2009). While these studies strongly suggest that NMD has roles in various developmental systems, the underlying mechanism is poorly understood.

Here we examine the role of NMD in the differentiation of human embryonic stem cells (hESCs). This was motivated by earlier work suggesting that NMD functions in early embryogenesis: null mutations in four NMD genes—*Upf1, Upf2, Smg1*, and *Smg6*—result in early embryonic lethality in mice (Li et al., 2015; McIlwain et al., 2010; Medghalchi et al., 2001; Weischenfeldt et al., 2008). These studies raised the possibility that NMD is critical for very early embryonic developmental events in mammals, a possibility we investigate in hESCs. Our studies reveal a role for NMD in definitive endoderm and mesoderm lineage



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segregation through the ability of NMD to regulate the levels of mRNAs encoding signaling pathway factors.

RESULTS

NMD Regulation in Stem and Differentiated Cells

We previously reported that mouse neural stem cells have a high magnitude of NMD and that this must be downregulated to permit neural differentiation (Lou et al., 2014). To explore whether this is a general property of NMD, we investigated other differentiation systems, with a focus on human development. We identified three scenarios in which NMD-factor genes were downregulated: (1) human neural progenitor cell differentiation, (2) human epidermal keratinocyte differentiation, and (3) human pancreatic progenitor cells differentiated into β-islet cells in vivo (Figure S1A). As evidence that the downregulation of these NMD factors is functionally relevant, the well-established NMD direct target transcripts, *GADD45B* and *ATF3* (Chan et al., 2007; Mendell et al., 2004), were upregulated, indicative of decreased NMD activity during differentiation (Figure S1A).

To further test whether NMD downregulation is a general property associated with differentiation, we mined an RNA-seq database from 452 human pluripotent and 254 non-pluripotent cell lines and found that three NMD factors are highly significantly ($p < 2.5 \times 10^{-123}$) downregulated in non-pluripotent human cell lines relative to pluripotent human cell lines (Figure S1B). Because this represents a very large number of cell lines, we regard this as strong evidence that NMD factors are highly expressed in human pluripotent stem cells and that their level decreases upon loss of pluripotency.

We next investigated NMD regulation during early differentiation using hESCs and found that culturing H9 hESCs under conditions that favor their differentiation into definitive endoderm led to reduced expression of most NMD-factor genes (Figure 1A). In contrast, directed differentiation toward ectoderm and mesoderm led to significantly increased expression of several NMD-factor genes (Figure 1A). To determine whether the divergent expression of NMD-factor genes during the differentiation of the three primary germ layers is a peculiarity of the H9 hESC line, we examined the Hue6 and Cyt49 hESC lines and observed similar results (Figure S1C). As evidence for conservation, we found that the pluripotent mouse P19 cell line displayed similarly divergent regulation when differentiated into the three primary germ layers (Figure S1D).

In agreement with the analysis described above, immunofluorescence showed that UPF1 protein expression was significantly reduced in the endoderm lineage compared with undifferentiated H9 cells (Figure S1E), while UPF1 protein levels were relatively higher in mesoderm- and ectoderm-differentiated H9 cells (data not shown). As evidence

Figure 1. NMD Downregulation Promotes hESC Endoderm Differentiation

(A) qPCR analysis of the indicated NMD-factor mRNAs in H9 hESCs differentiated into the three germ-layer cells. Bottom panel: lineage-specific markers. All transcript levels were normalized to the level of *L19* RNA. Error bars depict SEM. *$p < 0.05$ (Student's t test; n = 3 independent experiments).

(B) NMD activity in H9 hESCs differentiated into the three primary germ-layer cells. NMD activity is reflected by the ratio of PTC^+ and PTC^- mRNA (determined by qPCR analysis) expressed from transiently transfected NMD reporter plasmids. Statistical analysis was performed as indicated in (A) (n = 3 independent experiments).

(C) Cell-cycle analysis of undifferentiated and differentiated hESCs depleted of NMD factors. The cells were cultured under "non-differentiation" conditions or differentiated into the specific lineages indicated. Ctrl, H9 hESCs; UPF1 KD, A6-shUPF1 H9 cell clone (stably depleted of UPF1, see Figure S1F). Error bars represent SD. *$p < 0.05$ (Student's t test; n = 3 independent experiments).

(D) qPCR analysis of transcripts encoding cell cycle factors in hESCs depleted of the NMD factor UPF1. siLUC, H9 cells transiently transfected with the siLUC negative control small interfering RNA (siRNA); siUPF1, H9 cells transiently transfected with a UPF1 siRNA. All cells were cultured under "non-differentiation" conditions. Statistical analysis was performed as indicated in (A) (n = 3 independent experiments).

(E) The effect of sustained NMD on endoderm differentiation. qPCR analysis of H9 hESCs transfected with the indicated expression vectors and incubated with activin or diluent alone. Statistical analysis was performed as indicated in (A) (n = 3 independent experiments). ND, not detectable.

(F) The effect of NMD perturbation on endoderm differentiation. Left: qPCR analysis of the A6-shUPF1 H9 cell clone (panel C) versus control H9 cells. Right: qPCR analysis of H9 cells depleted of UPF3B (shUPF3B; DOX-treated Tet-shUPF3B cell clone) versus control cells (EtOH-treated H9 Tet-shUPF3B cell clone) (see Figure S1F). Cells were incubated with activin or diluent alone. Statistical analysis was performed as indicated in (A) (n = 3 independent experiments).

(G) FACS analysis of UPF1 protein expression and lineage-specific markers in hESCs differentiated into the indicated cell lineages. Ctrl, H9 hESCs; UPF1 KD, the A6-shUPF1 H9 cell clone (panel C). The cells were cultured either under "non-differentiation" conditions or differentiated down the lineages shown using the 4-day protocol described in Experimental Procedures (n = 3 independent experiments).

(H) FACS analysis of UPF1 protein expression and the endoderm marker, *SOX17*, in the hESCs described in (G) cultured either under "non-differentiation" conditions or for 2 days of the 4-day definitive endoderm differentiation protocol used in (G) (n = 3 independent experiments).

for the functional significance of modulation of the NMD factors, a luciferase-based NMD reporter demonstrated that hESCs cultured under conditions that favor endoderm differentiation had strongly reduced NMD activity, while hESCs cultured under conditions that favored mesoderm or ectoderm differentiation had increased NMD activity (Figure 1B). We conclude that the differentiation of human pluripotent cells tends to be accompanied by reduced magnitude of NMD, but differentiation of hESCs into the primary germ layers leads to divergent regulation of NMD, with dramatic NMD downregulation exhibited by endoderm, and increased NMD magnitude exhibited by mesoderm and ectoderm.

NMD Promotes hESC Proliferation

We next considered whether the regulation of NMD activity during hESC differentiation was linked with cellular proliferation. We previously showed that NMD stimulates neural cell proliferation by promoting progression through the G_1/S phase of the cell cycle (Lou et al., 2014). We examined whether this G_1/S transition-promoting activity extends to the primary germ layers. In support of this hypothesis, we found that hESCs cultured under conditions that favor ectoderm differentiation had both a high level of NMD activity (Figure 1B) and a high S/G_1 ratio (Figure 1C), indicative of high proliferative activity (Kapinas et al., 2013). In contrast, hESCs cultured to generate endoderm had low NMD activity (Figure 1B) and low S/G_1 ratio (Figure 1C), while hESCs cultured to form mesoderm had intermediate levels of both (Figures 1B and 1C). We conclude that there is a correlation between the extent of the S phase of the cell cycle and NMD activity.

To determine whether NMD has a causal role in the cell cycle of hESC-derivative lineages, we examined the effect of NMD perturbation. Consistent with our previous results in mouse neural stem cells (Lou et al., 2014), we found that knockdown of UPF1 or UPF3B (Figure S1F) in hESCs cultured to form mesoderm or ectoderm led to accumulation in G1 (Figures 1C and S1G). In contrast, cells differentiated toward endoderm exhibited little or no cell-cycle shift in response to NMD-factor knockdown, consistent with the fact that these cells already have low NMD magnitude (Figure 1B). In support of NMD stimulating G_1-to-S progression by destabilizing mRNAs encoding G_1/S inhibitors, we found that the mRNAs encoding several cell-cycle inhibitors were upregulated in UPF1-depleted hESCs (Figure 1D), two of which were also upregulated by depletion of UPF3B (Figure S1H). To investigate whether NMD acts to promote proliferation by promoting "stemness," we examined mRNAs encoding pro-stem factors. UPF1-depleted hESCs had only modestly reduced levels of pro-stem factor mRNAs (Figure 1D), while UPF3B-depleted cells did not have significantly altered levels of these mRNAs (Figure S1H), suggesting that NMD is unlikely to act by promoting the stem-like state of hESCs. We conclude that NMD stimulates the proliferation of hESC-derivative cells primarily by promoting the G_1-to-S cell-cycle transition.

The NMD Downregulatory Response Drives Endoderm Differentiation

As demonstrated above, NMD factor downregulation is a general property of many differentiation systems (Figures 1A, S1A, and S1B). To determine whether this phenomenon has a functional role, we elected to focus most of our remaining studies on endoderm differentiation, which is accompanied by strong NMD repression (Figure 1B). As one approach, we force-expressed a sufficient level of NMD factors to prevent their downregulation to determine whether this inhibited endoderm-directed differentiation and maintained stem cell markers, as predicted if NMD downregulation is required for this differentiation event. We found that maintenance of *UPF1* expression in this manner inhibited the upregulation of the endoderm marker, *SOX17*, and largely maintained expression of the stem cell marker, *OCT4* (Figure 1E, left). Forced expression of modest levels of *UPF3B* led to similar results (Figure 1E, right). Forced expression of either *UPF1* or *UPF3B* also inhibited *SOX17* expression in hESCs differentiated into embryoid bodies (data not shown). While maintenance of neither *UPF3B* nor *UPF1* expression was sufficient to completely suppress endoderm differentiation, we note that, in these experiments, we manipulated the level of only one of the many NMD factors downregulated during endoderm differentiation (Figure 1A, top; Figures S1C and D).

To determine whether NMD downregulation is sufficient to elicit the endoderm program in hESCs grown under "non-differentiation" conditions, we performed loss-of-function experiments. We found that depletion of UPF1 was sufficient to increase *SOX17* expression in hESCs under these conditions (Figure 1F, left). While this induction was much less than elicited by the potent endoderm differentiation inducer, activin, it was still >10-fold, indicating that UPF1 depletion is sufficient to trigger at least the initial stages of the endoderm program. As further evidence for this, another endoderm marker, *CXCR4*, exhibited the same pattern of expression. Two additional lines of evidence supported the notion that NMD downregulation promotes endoderm differentiation. First, depletion of UPF1 in combination with activin treatment elicited higher *SOX17* expression than activin treatment alone (Figure 1F, left). Second, depletion of another NMD factor, UPF3B, caused virtually the same effects as UPF1 depletion (Figure 1F, right).

As a final test of the role of NMD downregulation in endoderm differentiation, we examined the effect of

NMD-factor knockdown on hESCs grown under pro-endoderm differentiation conditions. Using fluorescence-activated cell sorting (FACS) analysis, we found that depletion of UPF1 coupled with activin treatment for 4 days led to a dramatically increased SOX17 protein level in most hESCs relative to cells treated with activin alone (Figure 1G). Furthermore, UPF1 depletion coupled with activin induced SOX17 protein expression after only 2 days of activin culture; whereas activin treatment alone did not significantly induce SOX17 protein expression at this early time point (Figure 1H). Together, these gain- and loss-of-function experiments provided strong evidence that NMD downregulation is a key driver of the endoderm differentiation program.

Identification of NMD Substrates in hESCs

To understand the underlying mechanism by which NMD influences hESC differentiation, it is critical to define NMD-regulated transcripts. To this end, we performed RNA-seq analysis on hESCs depleted of UPF1 and control hESCs. We found that ~90% depletion of *UPF1* resulted in significant dysregulation of 1,497 genes (Figure 2A and Table S1). As evidence that a large proportion of genes upregulated by *UPF1* depletion encode direct target transcripts, we found that ~3.5 times more genes were upregulated (1,163) than downregulated (334) ($q < 0.05$; >1.5-fold change) (Figure 2A and Table S1). In addition, we assembled a list of "core NMD substrates," defined by high phospho-UPF1 occupancy and other assays in previous studies. The majority of these RNAs (28 of 34 examined) were upregulated in *UPF1*-depleted hESCs (Figure 2B), validating our RNA-seq analysis and providing strong evidence that NMD is disrupted in *UPF1*-depleted hESCs.

To compile a list of direct NMD targets in hESCs, we first generated a database of high-confidence NMD substrates previously identified in the literature. To qualify as an NMD target, the RNA must have been shown to be an NMD substrate on the basis of more than one assay (e.g., upregulation and/or stabilization in response to NMD-factor depletion or high UPF1 or phospho-UPF1 occupancy). We found that ~200 of these high-confidence NMD substrate genes were upregulated in *UPF1*-depleted hESCs (Figure S2A and Table S5). As another means to identify direct NMD targets in hESCs, we performed RNA-seq half-life analysis on *UPF1*-depleted and control hESCs treated with the transcriptional inhibitor actinomycin D. This identified RNAs from 389 genes that were stabilized by depletion of *UPF1*, all of which are therefore strong candidates to be direct NMD targets (Table S3 and Figure S2B). A large percentage (~30%) of the genes corresponding to RNAs stabilized by depletion of *UPF1* overlapped with previously defined high-confidence NMD substrate genes (Figure 2C and Table S5).

Several features in mRNAs that are responsible for triggering decay by NMD have been defined. Two features that elicit NMD in some circumstances are an exon-exon junction downstream of the stop codon defining the main open reading frame and a long 3′ UTR (Lykke-Andersen and Jensen, 2015). When we compared RNA transcripts upregulated versus unaltered by *UPF1* depletion, we did not observe a statistical difference in either downstream exon junction (dEJ) frequency or 3′ UTR length (Figure S2C and Table S2). Likewise, the RNAs stabilized by *UPF1* depletion did not have a shift in these two parameters compared with unchanged RNAs (Figure S2C and Table S4). While there was a statistical difference in both parameters between upregulated and downregulated transcripts, this was not observed between stabilized and destabilized transcripts (Figure S2C). We conclude that neither 3′ UTR length nor dEJ content is sufficiently enriched in RNAs expressed in NMD-depleted hESCs to be detected at the genome-wide level, probably due to indirect effects of *UPF1* depletion. These findings are also consistent with the emerging evidence that context determines whether or not an "NMD-inducing feature" elicits RNA decay (Fatscher et al., 2015; Huang and Wilkinson, 2012; Zetoune et al., 2008).

NMD Targets Signaling Pathway mRNAs in hESCs

Gene ontology (GO) analysis revealed that mRNAs stabilized or upregulated by UPF1 depletion correspond to genes enriched in many functional categories (Figure 2D). Several subcategories of "differentiation" and "development" were significantly enriched (colored in purple in Figure 2D), including components of the nuclear factor κB signaling pathway (Figures 2D and S2D), which prompted us to investigate other signaling pathways, including those critical for hESC differentiation (Sui et al., 2013). Manual inspection of our RNA-seq datasets revealed that *UPF1* depletion altered the expression of genes involved in several major signaling cascades (Figures 2E and S2D). We used qPCR to verify regulation by UPF1 in a subset of these signaling genes (Figure 3A). Many of the mRNAs encoded by these genes appeared to be direct NMD substrates, as they were also stabilized in *UPF1*-depleted hESCs (Figure S3 and Table S6).

The known actions of many of these signaling pathways raised the possibility that one or more of them act downstream of NMD to repress definitive endoderm differentiation. For example, we hypothesized that the transforming growth factor β (TGF-β) and fibroblast growth factor (FGF) pathways are inhibited by NMD, as both of these signaling mechanisms are known to promote definitive endoderm differentiation (Loh et al., 2014; Sui et al., 2013; Wang and Chen, 2015). Consistent with this hypothesis, we found that the majority of positive TGF-β signaling factors were negatively regulated by UPF1 (Figure 2E). Conversely, most negative regulators of TGF-β signaling

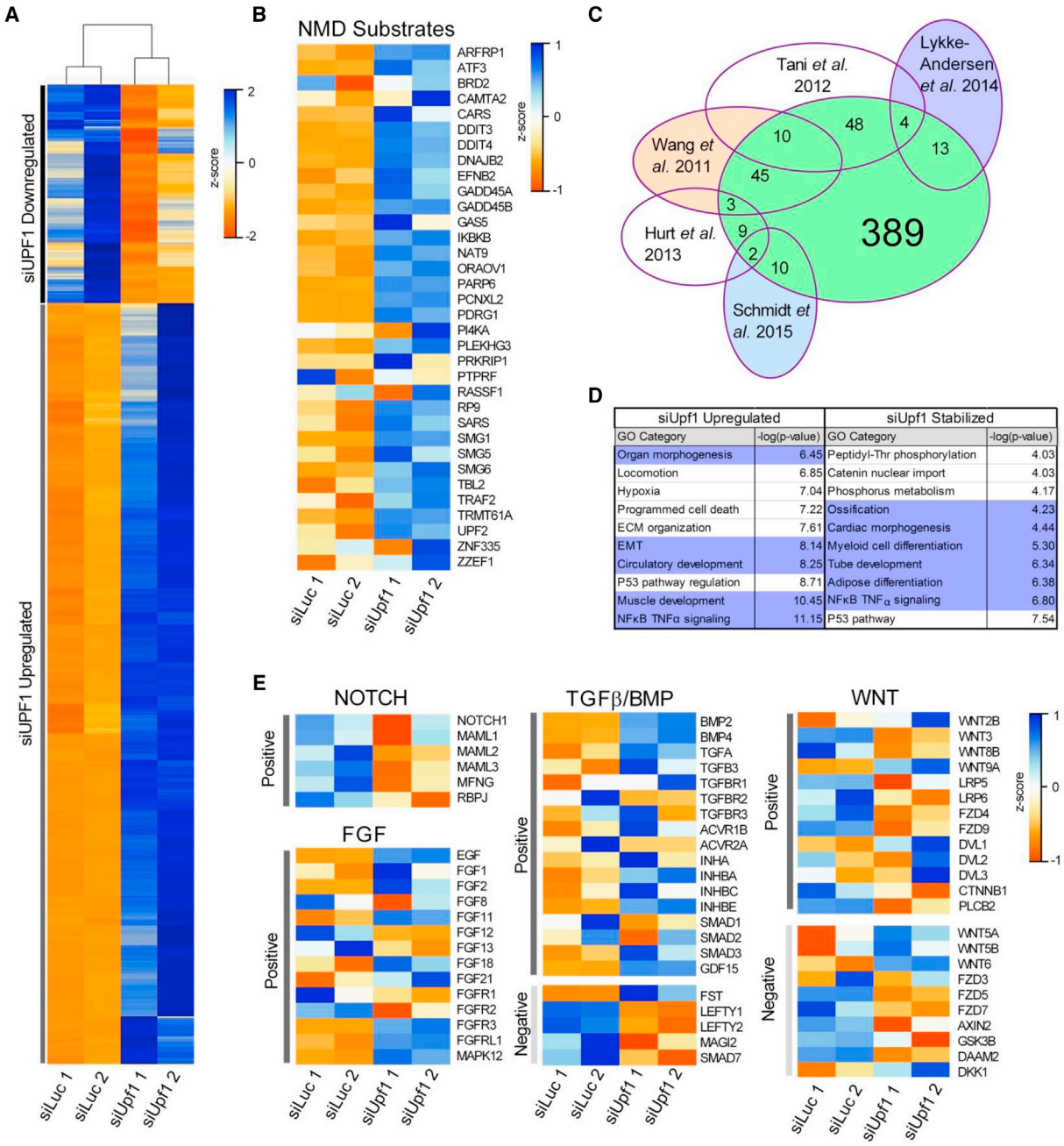

Figure 2. Genome-wide Analysis of UPF1-Regulated Transcripts in hESCs

(A) Gene expression of *UPF1*-depleted hESCs compared with control hESCs. *UPF1* was depleted by transfecting *UPF1* siRNA (20 nM) in H9 cells (siUPF1 1 and siUPF1 2) as described in Figure 1D; cells transiently transfected with a siRNA against *Luciferase* (siLuc 1 and siLuc 2) serve as the negative control. $q < 0.05$; >1.5-fold differentially expressed ($n = 2$ independent experiments).

(B) NMD substrates are upregulated in *UPF1*-depleted hESCs. RNA-seq analysis, described in (A), of 28 "core NMD substrates" (previously identified NMD substrates enriched for phospho-UPF1 binding) (Kurosaki et al., 2014 and Lynne Maquat, personal communication) and six transcripts previously identified as NMD substrates in at least two literature sources (Table S5).

(C) Overlap of genes encoding mRNAs stabilized by *UPF1* depletion with genes encoding NMD targets identified by previous papers. References can be found in Supplemental Experimental Procedures.

(D) GO analysis of functional categories overrepresented ($p < 0.01$) among genes significantly upregulated or significantly stabilized in hESCs depleted of *UPF1*. Development/differentiation-related and signaling categories are highlighted in purple.

(E) Transcripts encoding signaling proteins dysregulated in *UPF1*-depleted hESCs. RNA-seq analysis is described in (A).

were upregulated by UPF1. As evidence that NMD acts through FGF signaling, we found that most positive FGF signaling genes were negatively regulated by UPF1 (Figure 2E). We next examined two signaling pathways that are known to inhibit definitive endoderm differentiation: the WNT and NOTCH pathways (Loh et al., 2014; Ogaki et al., 2013; Sui et al., 2013; Wang and Chen, 2015). These pathways would be predicted to be stimulated by NMD, since NMD inhibits endoderm differentiation. Consistent with this, we found that most of the WNT and NOTCH pathway genes exhibiting consistently altered expression were positively regulated by UPF1 (Figure 2E). Finally, we examined the HIPPO pathway, as it is also known to influence definitive endoderm differentiation (Estarás et al., 2015), but we did not observe a clear trend in UPF1-mediated regulation (Figure S2D).

Because of the large body of literature demonstrating that the WNT and TGF-β pathways direct and regulate definitive endoderm differentiation (Loh et al., 2014; Sui et al., 2013; Wang and Chen, 2015), we performed follow-up experiments to examine more directly whether NMD acts through these two pathways to influence endoderm differentiation. As further support that NMD augments WNT signaling, qPCR analysis verified that most positive regulators of the canonical WNT pathway were downregulated by UPF1 depletion (Figure 3A). This notion was further supported by the finding from RNA-seq analysis that negative regulators of canonical WNT signaling were upregulated by UPF1 depletion (Figure 2E). As direct evidence that NMD promotes canonical WNT signaling, we found that UPF1-depleted hESCs had reduced upregulation of canonical WNT target genes in response to treatment with WNT3A ligand (Figure 3B). Conversely, *UPF1* overexpression increased canonical WNT target gene expression (Figure 3B). These data, coupled with the well-established ability of WNT signaling to inhibit endoderm differentiation (Loh et al., 2014; Sui et al., 2013; Wang and Chen, 2015), support a model in which NMD represses endoderm differentiation by promoting WNT signaling.

We next performed further analysis to confirm whether NMD represses TGF-β signaling. As described above, RNA-seq analysis demonstrated that positive regulators of this pathway were generally increased in expression following *UPF1* depletion, while most negative regulators exhibited decreased expression (Figure 2E), a finding we confirmed by qPCR analysis (Figure 3A). As direct evidence that UPF1 represses TGF-β signaling in hESCs, we found that UPF1-depleted hESCs had elevated levels of SMAD2 and SMAD3 protein, as well as phosphorylated SMAD2 and SMAD3 (Figure 3C). Together, these data support a model in which NMD downregulation drives endoderm differentiation by stimulating TGF-β signaling and repressing WNT signaling (Figure 3D).

Because it is well established that the ligands stimulating TGF-β signaling in vivo often operate in a dose-dependent manner to influence developmental fate (Guo and Wang, 2009; Mehlen et al., 2005), we analyzed the dose-dependent effects of the TGF-β ligand, activin. We found that modest overexpression of *UPF1* was sufficient to dampen the upregulation of the TGF-β-signaling component genes, *MIXL1* and *SOX17*, in response to a range of activin concentrations (Figure 4A). Similar results were obtained in response to forced expression of modest levels of the NMD factor *UPF3B* (Figure 4B), providing strong evidence that NMD itself confers repression of this pathway. We also tested the converse—the effect of repressed UPF1 on TGF-β signaling—and found a dose-dependent increase in TGF-β signaling (Figures 4C and D). Intriguingly, a threshold was observed in which suppressed UPF1 enhanced TGF-β signaling in response to high doses, but not low doses, of activin (Figure 4C). This supports the possibility that NMD regulation specifically influences TGF-β-signaling-induced events when cells are bathed in high concentrations of activin, such as when they are near the source cells that generate this morphogen.

NMD Modulates the Balance of Two Morphogenetic Signals Driving hESC Differentiation

A delicate balance of bone morphogenetic protein (BMP) and TGF-β signaling dictates hESC lineage specification toward endoderm or mesoderm. Both BMP and TGF-β signaling drive formation of mesendoderm, an intermediate lineage between hESCs and definitive endoderm and mesoderm (Loh et al., 2014). After mesendoderm formation, TGF-β signaling triggers endoderm differentiation, while BMP signaling elicits mesoderm differentiation (Guo and Wang, 2009; Wang and Chen, 2015). If NMD were to serve as a switch between these lineages, it would need to differentially regulate TGF-β and BMP signaling. To test this hypothesis, we examined the effect of NMD manipulation on the response of hESCs to BMP4. We found that *UPF1* overexpression enhanced BMP signaling, as measured using the BMP pathway target genes *Brachyury* and *Hand1* (Figure 5A), while UPF1 depletion reduced BMP signaling (Figure 5B). Coupled with our finding that NMD inhibits TGF-β signaling (Figures 2 and 3), this supports the notion that NMD inversely regulates the TGF-β and BMP signaling pathways and thereby is a good candidate to influence endoderm versus mesoderm cell fate.

Because definitive endoderm formation is most efficiently initiated by a combination of TGF-β and BMP signaling (Guo and Wang, 2009), we tested whether NMD influences endoderm differentiation when both of these pathways are activated simultaneously. In control hESCs, endoderm differentiation was elicited by activin alone but not BMP4 alone (Figures 5C, S4A, and S4B),

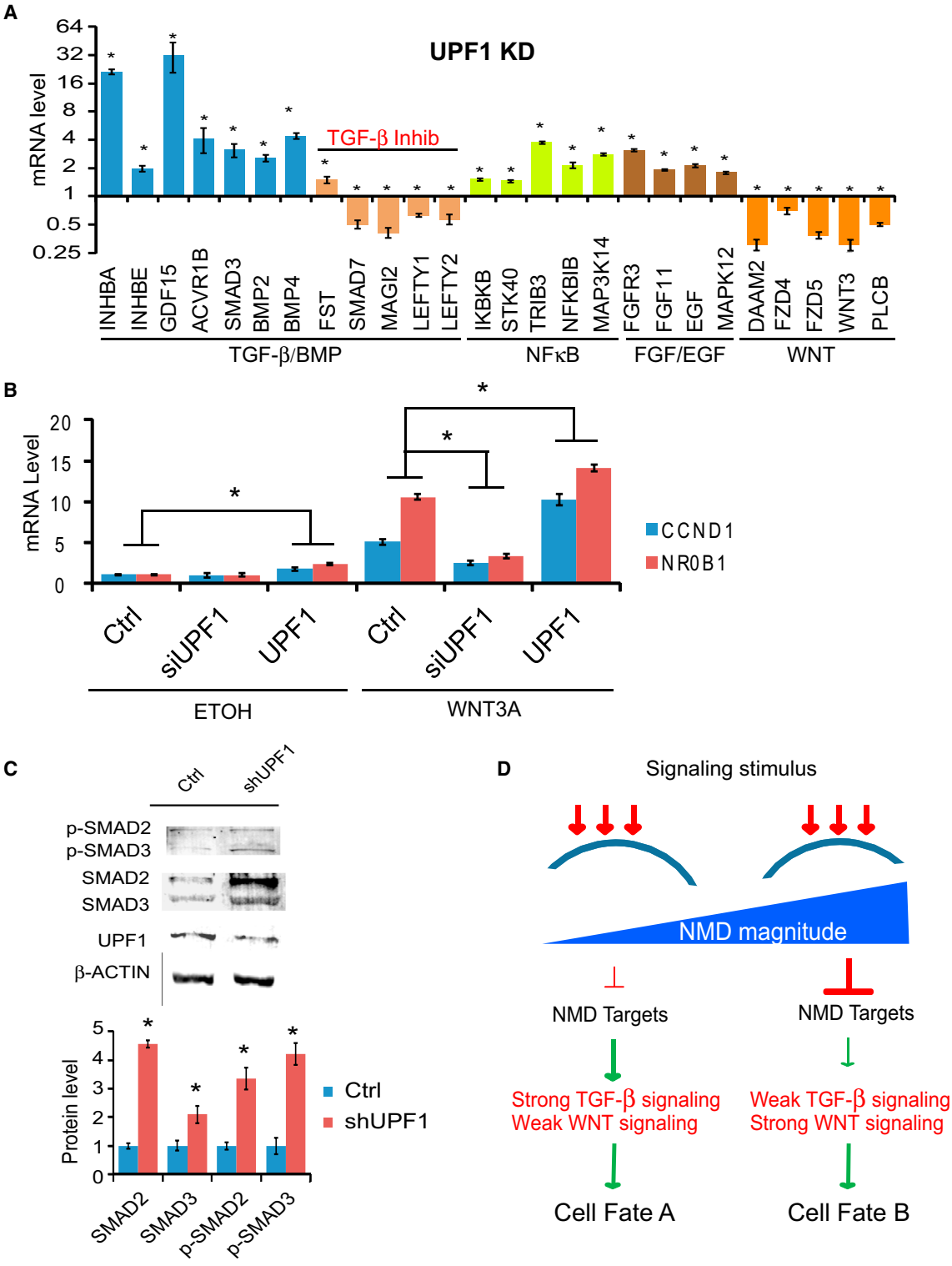

Figure 3. NMD Regulates TGF-β and BMP Signaling

(A) NMD regulates signaling factor mRNAs. qPCR analysis of the A6-shUPF1 clone (see Figure 1C) relative to that of control H9 cells, that latter of which was normalized to 1. TGF-β signaling inhibitors are highlighted. All other factors are positive regulators of their respective signaling pathways. Statistical analysis was performed as in Figure 1A (n = 3 independent experiments).

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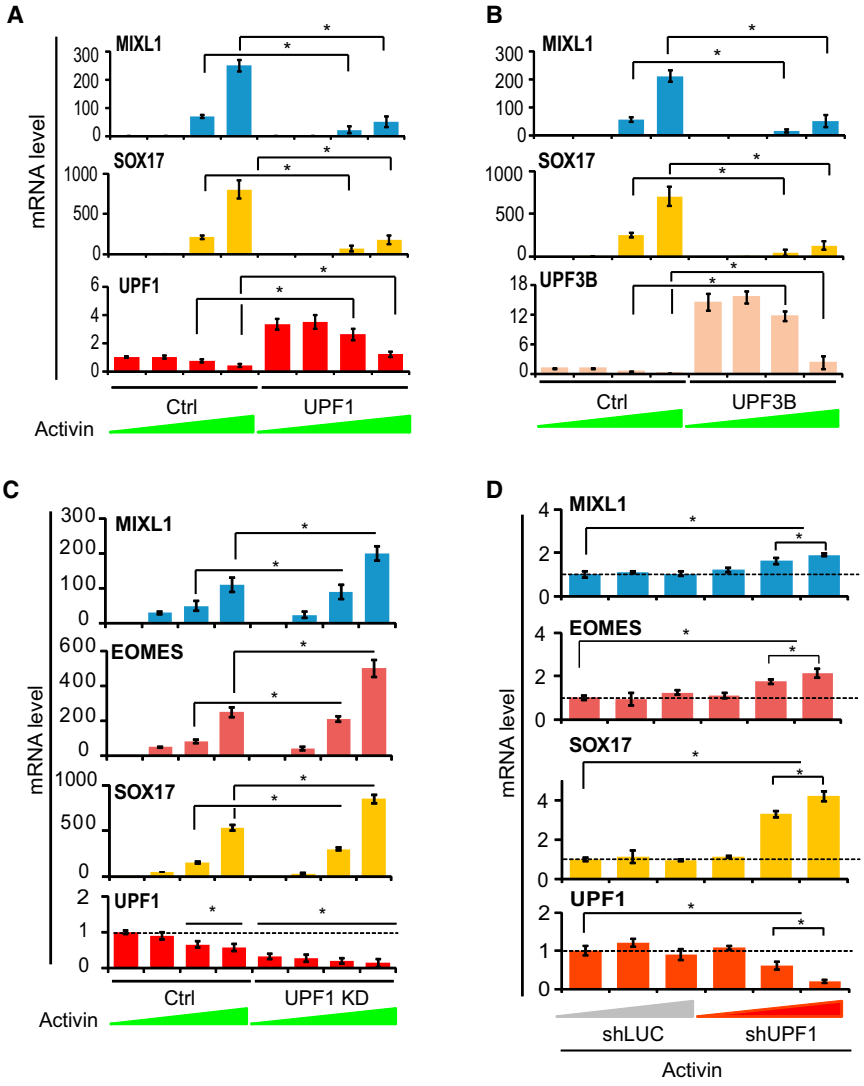

Figure 4. UPF1 Represses Endoderm Differentiation

(A) Forced maintenance of UPF1 expression represses TGF-β signaling and endoderm differentiation. qPCR analysis of H9 hESCs transiently transfected with a *UPF1* expression vector or empty vector and differentiated into the endoderm lineage with different doses of activin (0, 25, 50, and 100 ng/ml). Statistical analysis was performed as in Figure 1A (n = 3 independent experiments).

(B) Forced maintenance of *UPF3B* expression represses TGF-β signaling and endoderm differentiation. The experiment was performed analogous to (A) except with a *UPF3B* expression vector instead of a *UPF1* expression vector. Statistical analysis was performed as in Figure 1A (n = 3 independent experiments).

(C) UPF1 depletion stimulates activin-induced TGF-β signaling and endoderm differentiation. qPCR analysis of the A6-shUPF1 clone (Figure 1C) or control hESCs differentiated into the endoderm lineage with different doses of activin. Statistical analysis was performed as in Figure 1A (n = 3 independent experiments).

(D) Dose-dependent effect of UPF1 on TGF-β signaling and endoderm differentiation. qPCR analysis of H9 cells transfected with different doses of *LUC* or *UPF1* small hairpin RNA (shRNA) expression vectors and then incubated for 3 days with 100 ng/ml activin. Statistical analysis was performed as in Figure 1A (n = 3 independent experiments).

confirming previous studies (Loh et al., 2014). Also validating published findings (Teo et al., 2012), we found that optimal endoderm differentiation was triggered by activin and a low concentration of BMP4 (Figures 5C, S4A, and S4B). To test the role of NMD in this process, we depleted UPF1 and found that this triggered increased *SOX17* and *CXCR4* expression in response to most concentrations of activin and BMP4 (Figures 5C and S4A). Similar results were observed in response to depletion of the NMD factor UPF3B (Figure S4B). Together, these data demonstrate that NMD downregulation is critical for endoderm differentiation, not only under suboptimal conditions (e.g., activin alone) but also under optimal (activin and BMP signaling) conditions.

(B) NMD modulates the WNT signaling pathway. qPCR analysis of mRNAs encoding the WNT downstream targets, CCND1 and NR0B1, in H9 hESCs transfected with the vectors described in Figure 1 for 48 hr, and then incubated with WNT3A (100 ng/ml) for 24 hr. Treatment with siUPF1 depleted UPF1 levels by 90% ± 4% and UPF1 overexpression increased UPF1 levels by 2.1 ± 0.2 fold (Table S6). Statistical analysis was performed as in Figure 1A (n = 3 independent experiments).

(C) UPF1 inhibits TGF-β signaling in hESCs. Western blot analysis of the A6-shUPF1 H9 cell clone (Figure. 1C) and H9 cells as a control. The right panel shows the mean of protein quantification; values were normalized to β-ACTIN as the internal control. Error bars represent SEM. *$p < 0.05$ (n = 3 independent experiments).

(D) Model: NMD magnitude shifts cell signaling competence. Two cells with different levels of NMD activity respond differently to the same milieu of signaling ligands.

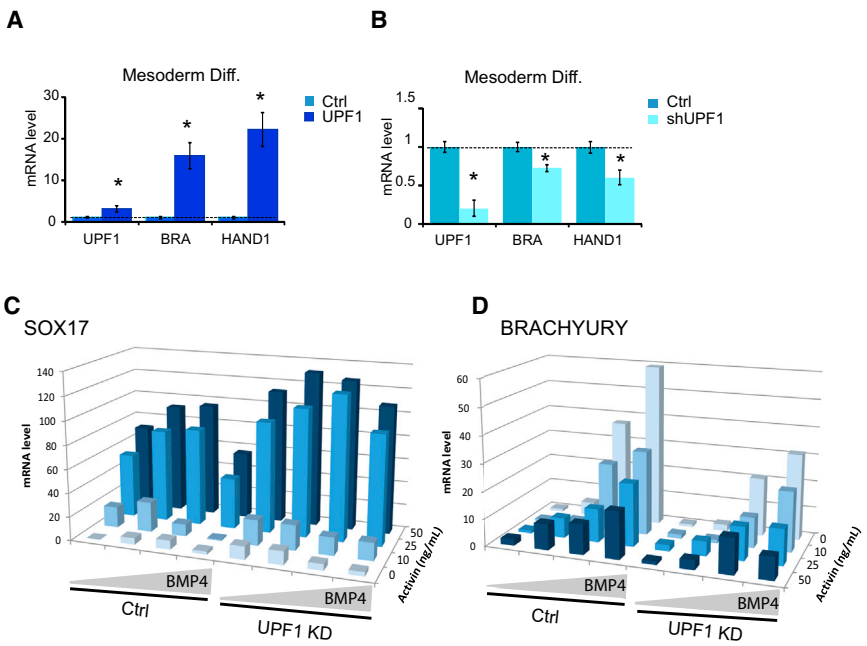

Figure 5. Evidence that UPF1 Influences Mesoderm vs. Primitive Endoderm Fate

(A) UPF1 upregulates BMP signaling and mesoderm differentiation markers. qPCR analysis of H9 cells transiently transfected with a *UPF1* expression vector or empty expression vector (Ctrl) and cultured for 3 days in mesoderm induction medium. Statistical analysis was performed as in Figure 1A (n = 3 independent experiments).

(B) UPF1 downregulation inhibits BMP signaling and mesoderm differentiation markers. qPCR analysis of H9 cells transiently transfected with a *UPF1* shRNA or *LUC* shRNA (control) expression vector. Cell-culture conditions and statistical analysis are as described in (A) (n = 3 independent experiments).

(C) UPF1 downregulation stimulates endoderm differentiation markers. qPCR analysis of the endoderm marker, *SOX17*, performed on the A6-shUPF1 clone (UPF1 KD) and control (Ctrl) H9 hESCs described in Figure 1C. The cells were differentiated into the mesendoderm lineage (for 1 day) and then incubated for 3 days with different concentrations of BMP4 (0, 5, 10, and 50 ng/ml) and activin (0, 25, 50, and 100 ng/ml). Statistical analysis was performed as in Figure 1A (n = 3 independent experiments).

(D) UPF1 downregulation inhibits BMP signaling and mesoderm differentiation markers. qPCR analysis of the mesoderm marker, *BRACHYURY*, performed on A6-shUPF1 clone and control cells described in (C). Statistical analysis was performed as in Figure 1A (n = 3 independent experiments).

We next tested the role of NMD in hESCs grown under mesoderm differentiation conditions. As previously demonstrated (Wang and Chen, 2015), we found that BMP4 alone elicited mesoderm differentiation, whereas activin alone did not, as measured using the mesoderm marker gene, *Brachyury* (Figures 5D and S4C). In striking contrast to what we observed for endoderm differentiation, depletion of the NMD factor, UPF1, caused decreased *Brachyury* expression in response to most concentrations of BMP4 and activin (Figure 5D). Similar results were observed in response to depletion of the NMD factor, UPF3B (Figure S4C), reinforcing our hypothesis that NMD serves to stimulate mesoderm induction. As further evidence that NMD influences mesoderm versus endoderm fate, we found that NMD perturbation (by UPF1 or UPF3B depletion) eliminated the repression of endoderm differentiation caused by high BMP4 concentrations (Figures 5C, S4A, and S4B). Together, these data provide strong evidence that NMD acts through well-established signaling pathways to control hESC fate.

DISCUSSION

In this study, we provide several lines of evidence that the highly conserved RNA degradation pathway, NMD, strongly influences hESC differentiation. Through marker analysis, we found that NMD promotes the differentiation of hESCs into mesoderm and inhibits their differentiation into definitive endoderm (Figures 1E–1H, 3, 4, and 5). Our results suggest that NMD magnitude is critical for dictating the proportion of cells that progress down these two cell lineages by regulating the balance between TGF-β and BMP signaling (Figure 6). We obtained several lines of evidence that NMD inhibits TGF-β signaling and activates BMP signaling (Figures 3, 4, 5, S3, and S4), which are known to promote endoderm and mesoderm differentiation, respectively (Loh et al., 2014). Thus, by altering NMD magnitude, the relative strength of these two signaling pathways is shifted, thereby influencing cell fate (Figure 6). Recent studies have identified an array of modulatory factors that shift NMD magnitude, including microRNAs, eIF2α phosphorylation, RNA-binding proteins, and the level of specific NMD factors (Ge et al., 2016; Huang and Wilkinson, 2012; Karam et al., 2013; Shum et al., 2016). We suggest that one or more of these mechanisms may be responsible for altering the magnitude of NMD in hESCs to influence cell fate.

We obtained some evidence that NMD not only influences hESC fate through TGF-β and BMP signaling, but also through the WNT, FGF, and NOTCH pathways

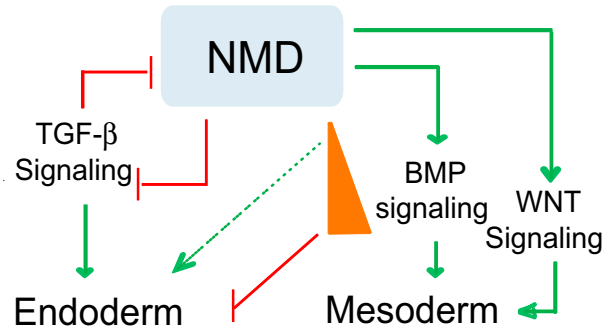

Figure 6. Model
Endoderm versus mesoderm fate is dictated by NMD through regulation of TGF-β and BMP signaling.

(Figure 2E). This raises the possibility that the ability of NMD to influence endoderm and mesoderm differentiation is a combinatorial process amplified by its ability to act through several signaling pathways. As a further level of complication, NMD may act at several differentiation steps. The differentiation of definitive endoderm and mesoderm from hESCs requires an initial commitment to the mesendodermal cell lineage (Sui et al., 2013). Commitment to this intermediate cell fate is promoted by FGF, WNT, BMP, and TGF-β signaling (Loh et al., 2014). After hESCs reach the mesendoderm stage, their subsequent development into mesoderm or endoderm further depends on the local signaling milieu. TGF-β and FGF signaling promote differentiation into definitive endoderm, while BMP and WNT signaling elicit differentiation into mesoderm (Loh et al., 2014). Given our evidence that NMD influences all these signaling pathways, it would not be surprising if NMD magnitude affects several of these differentiation steps, including the initial generation of mesendoderm. We note that while some definitive endoderm markers regulated by NMD (*MIXL1* and *EOMES*) are also expressed by mesendoderm, other markers regulated by NMD are considered specific for definitive endoderm or mesoderm (*SOX17/CXCR4* and *BRACHYURY/HAND1*, respectively) (Izumi et al., 2007).

NMD may act in two non-mutually exclusive ways to influence hESC cell fate. First, NMD may serve as a switch that cooperates with transcriptional mechanisms to drive hESC differentiation. This follows from the fact that simultaneous transcriptional activation and mRNA stabilization elicit more dramatic increases in steady-state mRNA level than either of these processes alone. For example, our results suggest that activin-triggered transcriptional induction coupled with NMD suppression would increase not only the synthesis but also the stability of mRNAs encoding pro-endoderm differentiation proteins, thereby driving robust endoderm differentiation. Second, NMD may reinforce cell-fate decisions in the face of genetic or environmental perturbation. This follows from our finding that extracellular signals triggering a given cell fate alter the magnitude of NMD to reinforce that particular cell fate. For example, we found that activin treatment suppresses NMD magnitude (Figure 1B) which, in turn, leads to upregulation of mRNAs encoding pro-TGF-β factors (Figure 3A). This would further stimulate TGF-β signaling and strongly drive endoderm differentiation. Our results suggest that a similar feedback mechanism involving the WNT signaling pathway may exist (Figure 6). Finally, our results imply that NMD allows for subtle responses to morphogen gradients. For example, NMD specifically modulates hESC fate in response to high activin doses but not low activin doses (Figure 4). If confirmed in vivo, this indicates an extra layer of developmental control whereby the magnitude of NMD can trigger different cell fates that depend on the local morphogen milieu.

Our finding that NMD has inverse effects on hESC endoderm and mesoderm differentiation differs from what was recently reported for mouse ESCs (mESCs). Li et al. (2015) found that loss or depletion of NMD factors in mESC blocks their differentiation into all three primary germ layers. Because these authors depleted several different NMD factors, including UPF1, which we also depleted, we regard it as unlikely that the different findings of our two studies are due to NMD branch-specific effects or non-NMD functions of NMD factors. Instead, a more likely explanation is that mESCs and hESCs differ with regard to their responses to NMD. This may stem from the fact that mESCs and hESCs exhibit intrinsic species-specific differences in factor requirements and expression patterns (Blair et al., 2011; Moon et al., 2006). In addition, recent studies have identified differences in lineage-specific markers originally thought to be conserved between mouse and human (Blakeley et al., 2015), as well as differences between mESC and hESC differentiation programs (Moon et al., 2006). As an example of the latter, while both the FGF and WNT pathways govern primate primitive endoderm versus epiblast cell fate, only FGF acts in the equivalent pathway in rodents (Boroviak et al., 2015). Our evidence that NMD regulates both WNT and FGF signaling (Figures 2E, 3A, and 3B) may partly explain NMD's differential effects on hESCs and mESCs. These studies highlight the need to perform research on human pluripotent cells, which often behave differently from mouse pluripotent cells.

Using both standard RNA-seq and RNA-seq half-life analysis, we identified UPF1-regulated transcripts that are good candidates to be direct NMD targets in hESCs. There was considerable overlap between these candidate NMD target transcripts expressed in hESCs with previously identified high-confidence NMD substrates (Figures 2C and S2A;

Table S5). We regarded this overlap as remarkable, given that few high-confidence NMD substrates have thus far been defined in the field and most have been identified in cell lines that bear little resemblance to hESCs (e.g., HeLa and HEK). The overlap was particularly surprising given that NMD has been shown to exhibit tissue specificity (Huang et al., 2011; Karam et al., 2013). We suggest that the high-confidence NMD substrate database we compiled (Figure 2B and Table S5) is a valuable resource for the field.

The mRNAs identified as significantly upregulated or significantly stabilized upon UPF1 depletion in hESCs were enriched in several GO categories related to differentiation and morphogenesis (Figure 2D). We note, however, that many of these putative NMD substrates require further assays to determine whether they are bona fide direct NMD targets. The identification of direct NMD targets is clouded by the fact that known "NMD-inducing features" do not necessarily trigger NMD. For example, while long 3′ UTRs are a well-established NMD-inducing feature, many long 3′ UTRs do not trigger NMD (Lykke-Andersen and Jensen, 2015). Indeed, elements have been identified in long 3′ UTRs that permit NMD evasion (Toma et al., 2015), and short 3′ UTRs (<1 kb) can also trigger NMD (Eberle et al., 2008; Hogg and Goff, 2010; Singh et al., 2008; Yepiskoposyan et al., 2011), consistent with the recent finding that candidate NMD substrates in mESCs have 3′ UTRs with shorter average length than control RNAs (Hurt et al., 2013). Further empirical studies will be required before algorithms can be created to identify high-confidence NMD substrates in silico.

The ability of NMD to influence cell fate is not restricted to ESCs. For example, we previously showed that high NMD magnitude promotes epidermal cell fate and represses neural cell fate (Lou et al., 2014). NMD represses neural fate by promoting the decay of the mRNA encoding the pro-neural differentiation factor SMAD7 (Lou et al., 2014). How NMD stimulates epidermal cell fate is not known. One possibility is that NMD achieves this through its ability to stimulate BMP signaling (Figures 5 and S4C), as epidermal differentiation requires BMP signaling (Bier and De Robertis, 2015). Our evidence that NMD degrades transcripts encoding a wide array of signaling factors (Figures 2E, 3A, S2B, S2D, and S3) raises the possibility that NMD serves as a regulator of many other cell-fate decisions during development. One subject of future investigation is to examine the role of NMD in the differentiation of primary germ layers at stages following those we examined in this report. Given the diverse roles of NMD in differentiation, it is not surprising that recent evidence suggests that NMD is also involved in malignancy (Chang et al., 2016; Liu et al., 2014; Wang et al., 2011), including through altered TGF-β signaling (Chang et al., 2016). Future studies will be required to elucidate the full complement of developmental events and diseases influenced by NMD.

EXPERIMENTAL PROCEDURES

RNA and Protein Analysis

Total cellular RNA was isolated from cells and tissues using Trizol (Invitrogen), as described by Chan et al. (2007, 2009). qPCR analysis was done in triplicate on each sample using iScript reverse transcriptase (Bio-Rad) and a SYBR-Green PCR kit (Bio-Rad), as described by Chan et al. (2007, 2009). Primer sequences are provided in Supplemental Experimental Procedures. Western blot analysis was performed on protein lysates resolved in 8% and 10% SDS-PAGE gels and blotted using standard procedures, as described by Chan et al. (2007, 2009). The membranes were probed with the following primary antibodies: rabbit anti-human UPF1 (from Jens Lykke-Andersen; 1:1,000 dilution), rabbit anti-human UPF3B (from Jens Lykke-Andersen; 1:1,000 dilution), rabbit anti-mouse SMAD2/3 (Cell Signaling, catalog #3106; 1:500 dilution), rabbit anti-mouse phospho-SMAD2/3 (Cell Signaling, cat: #3108; 1:250 dilution), and mouse anti-β-actin (Sigma A5441; 1:2,000 dilution). The secondary antibodies used were fluorescein isothiocyanate- or PG-YA-conjugated anti-goat, anti-mouse or anti-rabbit immunoglobulin G (Thermo Fisher Scientific). Quantification was done by densitometry using Odyssey immunofluorescence software.

Immunofluorescence and FACS Analysis

Immunofluorescence and FACS analysis were performed as conventional procedures. See Supplemental Experimental Procedures for details and antibodies used.

Cell-Cycle Analyses

Cells were trypsinized and resuspended in 0.5 ml PBS followed by the addition of 0.5 ml of 100% ice-cold ethanol in a dropwise manner while vortexing. After incubation for 20 min on ice, the cells were harvested and washed with PBS, incubated in 25 mg/ml RNase A in PBS at 37°C for 30 min, and stained with 50 mg/ml propidium iodide in PBS for 10 min at room temperature and analyzed by flow cytometry.

hESC Culture

All hESC cultures were maintained at 37°C with 5% CO_2. The hESC lines H9, CyT49, and Hue6 (WiCell) were propagated on Matrigel (BD Sciences)-coated six-well plates in E8 medium (StemCell Technologies). See Supplemental Experimental Procedures for specific culture conditions for in vitro differentiation into the three germ layers.

ACCESSION NUMBERS

The accession number for the RNA-seq data reported in this paper is GEO: GSE81214. Additional files associated with this study are available in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.05.008.

AUTHOR CONTRIBUTIONS

C.H.L. and M.F.W. conceived most of the experiments. The majority of the experiments were performed and analyzed by C.H.L. RNA-seq was analyzed by J.D. and E.Y.S. Additional experiments were performed by A.G., E.Y.S., and M.R. Pluripotent cell analysis was performed by X.L. and S.M.C. with lines from D.B. The manuscript was written by C.H.L., A.G., J.D., and M.F.W.

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