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SMG5–PNRC2 is functionally dominant compared with SMG5–SMG7 in mammalian nonsense-mediated mRNA decay

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ABSTRACT

In mammals, nonsense-mediated mRNA decay (NMD) functions in post-transcriptional gene regulation as well as mRNA surveillance. A key NMD factor, Upf1, becomes hyperphosphorylated by SMG1 kinase during the recognition of NMD substrates. Hyperphosphorylated Upf1 interacts with several factors including SMG5, SMG6, SMG7 and PNRC2 to trigger rapid mRNA degradation. However, the possible cross-talk among these factors and their selective use during NMD remain unknown. Here, we show that PNRC2 is preferentially complexed with SMG5, but not with SMG6 or SMG7, and that downregulation of PNRC2 abolishes the interaction between SMG5 and Dcp1a, a component of the decapping complex. In addition, tethering experiments reveal the function of Upf1, SMG5 and PNRC2 at the same step of NMD and the requirement of SMG6 for Upf1 for efficient mRNA degradation. Intriguingly, microarray results reveal the significant overlap of SMG5-dependent NMD substrates more with PNRC2-dependent NMD substrates than with SMG7-dependent NMD substrates, suggesting the functional dominance of SMG5–PNRC2, rather than SMG5–SMG7, under normal conditions. The results provide evidence that, to some extent, endogenous NMD substrates have their own binding preference for Upf1-interacting adaptors or effectors.

INTRODUCTION

Nonsense-mediated mRNA decay (NMD) targets normal cellular transcripts as well as aberrant transcript harboring premature translation termination codons (PTCs), which functions as a post-transcriptional regulatory mechanism and mRNA surveillance mechanism (1–4). In mammals, NMD substrates are recognized during translation, which is mediated by the nuclear cap-binding protein complex (4–8). At the termination step of this round of translation, the fate of mRNAs is determined by a competition between NMD-stimulating factors, such as the exon–junction complex (EJC), which is deposited onto mRNA as a consequence of splicing and NMD-antagonizing factors, such as cytoplasmic poly(A)-binding protein (PABPC) and microRNAs, both of which reside downstream of the termination codon (9–12). If an EJC prevails, Upf1 is easily recruited into the terminating ribosome and is hyperphosphorylated by SMG1 kinase (13–15), which results in degradation of mRNAs by NMD.

When mRNAs are recognized as NMD substrates, the hyperphosphorylated (hp) Upf1 can interact with several proteins—SMG5, SMG6, SMG7 and with proline-rich nuclear receptor coregulatory protein 2 (PNRC2)—which function as adaptor or effector proteins for rapid mRNA degradation (1,16–21). However, any preferential interactions and possible cross-talks among different Upf1-interacting factors have not yet been well characterized.

Mammalian NMD degrades mRNAs from both the 5′-and 3′-ends by recruiting a decapping complex followed by 5′-to-3′-exonuclease, as well as by recruiting deadenylation complex followed by 3′-to-5′-exonuclease (22–24). Furthermore, recent reports have proposed endonucleolytic degradation of NMD substrates (17,25). A diverse range of mechanisms to trigger mRNA degradation may be due to the actions of different Upf1-interacting factors. First, when hp-Upf1 binds to SMG5/7, NMD substrates are subject to decapping followed by 5′-to-3′-exonucleolytic cleavage by an unknown mechanism (18,19). Second, when hp-Upf1 binds to PNRC2, PNRC2 links hp-Upf1 and Dcp1a, which is a component of the decapping complex, triggering decapping followed

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by 5′-to-3′-exonucleolytic cleavage (16,21). In particular, PNRC2 triggers the movement of hp-Upf1 into processing bodies (16), where translationally silenced mRNAs are stored or degraded (26,27). Third, when hp-Upf1 binds to SMG6, the NMD substrates are subject to endoribonucleolytic cleavage (1,17,25). However, it is unknown whether the decay of each NMD substrate requires all of these Upf1-interacting factors at the same time or sequentially, or whether each NMD substrate preferentially uses different Upf1-interacting factors.

Here we show that SMG5–PNRC2 is a functionally predominant complex under normal conditions, rather than SMG5–SMG7, and that, to some extent, endogenous NMD substrates have their own preferences for different Upf1-interacting adaptors or effectors.

MATERIALS AND METHODS

Plasmid constructions

Details are provided in Supplementary 'Materials and Methods' section.

Immunoprecipitation

HEK293T cells or Cos-7 cells were transiently transfected by calcium phosphate precipitation with the indicated plasmids. Immunoprecipitation (IP) was performed as described previously (6,16).

Western blotting

Immunopurified proteins or cell extracts were separated by 8–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to HyBond ECL nitrocellulose (Amersham). The following antibodies were used: FLAG (Sigma-Aldrich), HA (Roche), Myc (Calbiochem), SMG5 (Abcam), SMG6 (28), SMG7 (Bethyl), Upf1 (a gift from Lynne E. Maquat), phospho-S1078-Upf1 (21), phospho-S1096-Upf1 (21), phospho-S/TQ (Cell Signaling Technology), PNRC2 (16), Dcp1a (21) and β-actin (Sigma-Aldrich).

Downregulation-coupled tethering assay

HeLa cells were grown in Dulbecco's Modified Eagle's Medium (Hyclone) containing 10% fetal bovine serum (Hyclone). Cells were transfected with 100 nM *in vitro*-synthesized small interfering RNA (siRNA) (Invitrogen) using Oligofectamine (Invitrogen). The following sequences for siRNA were used: control siRNA (29), Upf1 siRNA (29), PNRC2 siRNA (16), SMG5 siRNA [5′-r(GCCAGAAAGAGGUGGGAAA)d(TT)-3′], SMG6 siRNA (19,30) and SMG7 siRNA [5′-r(GCAAGAAAC AUCUGUGAUA)d(TT)-3′]. Two days after siRNA transfection, cells were retransfected with 0.15 μg of pcβ-6bs, 0.05 μg of phCMV-MUP and 0.5 μg of effector plasmid using Lipofectamine 2000 (Invitrogen) or Turbofect (Fermentas) as previously described (6,29). One day later, the cells were harvested and then total-cell RNA and protein were purified using TRIzol Reagent (Invitrogen).

Quantitative real-time PCR and RT–PCR using α-[^{32}P]-dATP

Details are provided in Supplementary 'Materials and Methods' section. The oligonucleotides are listed in Supplementary Table S4.

Microarray analysis

Microarray analysis was performed by Macrogen, Korea. Total RNA was extracted using Trizol (Invitrogen). Total RNA was amplified and purified using an Illumina RNA amplification kit (Ambion). The biotin-labeled cRNA was hybridized to each humanHT-12 expression v.4 bead array (48K human gene chips; Illumina). Detection of array signals was carried out using an Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences). Hybridized chips were scanned using an Illumina bead array Reader confocal scanner (Illumina). The scanned images were analyzed with an Illumina GenomeStudio v2009.2 (Gene Expression Module v1.5.4). Transcripts that were commonly downregulated or upregulated by at least 1.5-fold (Figure 5B, left panel and Figure 5C) or 2-fold (Figure 5B, right panel and Figure 5D) in two independent microarray analyses were considered differentially expressed. Cluster analysis was performed using PermutMatrix. The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus web-based data repository (series ID: GSE34876).

RESULTS

PNRC2 is preferentially complexed with SMG5

Specific downregulation of each factor that interacts with hp-Upf1 using siRNA produced different effects on the NMDs of globin (Gl) mRNAs and glutathione peroxidase 1 (GPx1) mRNAs harboring PTC (Figure 1). Downregulation of SMG5, SMG6 or PNRC2 abrogated NMDs of PTC-containing Gl and GPx1 mRNA. However, although SMG7 is known to form a heterodimer with SMG5 (31), downregulation of SMG7 had no significant effect on NMDs of Gl and GPx1 mRNA. These results imply that selective or combination of each Upf1-interacting factor is needed to degrade different NMD substrates. In particular, considering the previous reports that decapping activity is commonly involved in SMG5/7-dependent and PNRC2-dependent NMD (16,18,19), a PNRC2-dependent NMD pathway may mechanistically overlap with a SMG5 (and/or SMG7)-dependent NMD pathway.

To test this hypothesis, we first investigated the possible complex formation among different Upf1-interacting factors using IP. The results showed that endogenous PNRC2 and Dcp1a detectably co-immunopurified only with FLAG-SMG5, but not with FLAG-SMG6 or FLAG-SMG7 in a RNase A-resistant manner (Figure 2A). The reciprocal IPs also showed that only FLAG-SMG5 was enriched in the IP of Myc-PNRC2 (Figure 2B), confirming the preferential interaction between SMG5 and PNRC2. Consistent with the direct

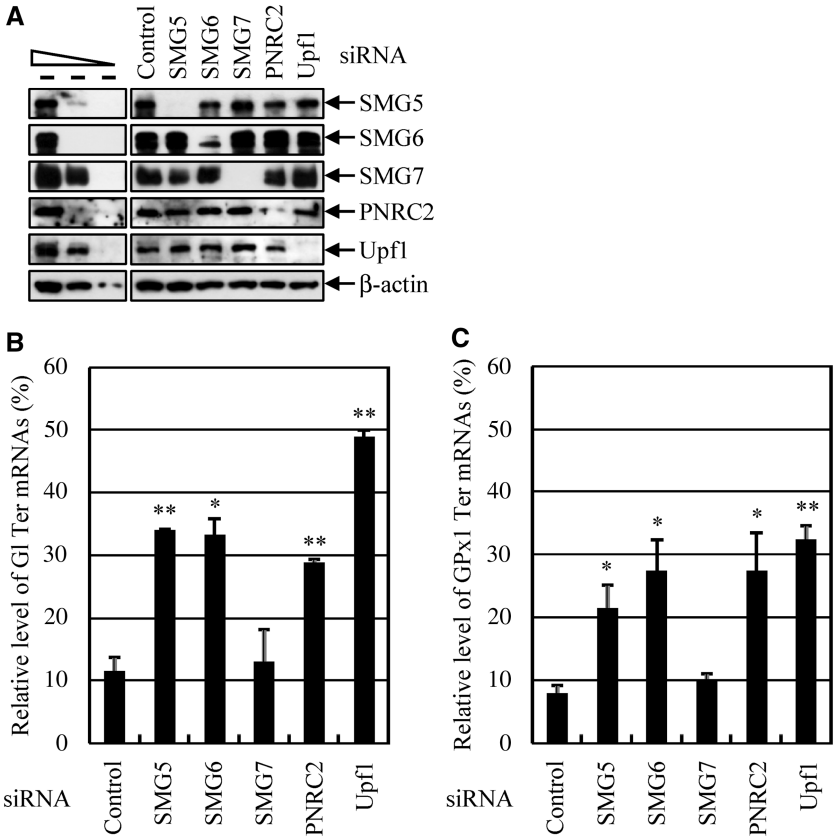

Figure 1. Different effects of Upf1-interacting factors on NMDs of Gl mRNAs and GPx1 mRNAs. HeLa cells were transiently transfected with the indicated siRNAs. Two days later, cells were transfected with NMD reporters (i) either Gl norm or Ter and (ii) either GPx1 norm or Ter, and reference plasmid phCMV-MUP. One day later, total-cell protein and RNA were prepared and analyzed by western blotting and qRT–PCR, respectively. (**A**) Western blotting to demonstrate specific downregulation by siRNAs. To demonstrate that the western blotting was sufficiently quantitative, 3-fold serial dilutions of total-cell extracts were loaded in the three left-most lanes. (**B**) qRT–PCRs of Gl mRNA. The level of Gl mRNA was normalized to the level of MUP mRNA. The levels of normalized Gl Norm mRNA in each siRNA were set to 100%. The relative levels of Gl Ter mRNA, compared with normalized level of Gl Norm mRNA in each siRNA, were shown and compared. The columns and bars represent the mean and standard deviation of three independently performed transfections and qRT–PCRs. $^{**}P < 0.01$; $^{*}P < 0.05$ as determined by Student's *t*-tests. (**C**) qRT–PCRs of GPx1 mRNA. As in (B), except that the relative levels of GPx1 Ter mRNA were shown and compared.

interaction between PNRC2 and Dcp1a (16), endogenous Dcp1a was observed in all IPs of Myc-PNRC2 (Figure 2B). More intriguingly, downregulation of PNRC2 abolished the association between SMG5 and Dcp1a (Figure 2C and D). The collective data suggest that PNRC2 and Dcp1a are preferentially complexed with SMG5 rather than with SMG6 or SMG7, and that an interaction between SMG5 and Dcp1a is dependent on PNRC2.

SMG5 and PNRC2 coordinately function together with Upf1 to trigger mRNA degradation

To determine the hierarchy of Upf1-interacting factors during NMD, a downregulation-coupled tethering assay was employed using bacteriophage MS2 coat protein (MS2) system and specific siRNAs (16,29,32).

First, HeLa cells were depleted of endogenous SMG5, SMG6 or SMG7, and then MS2-HA, MS2-HA-PNRC2 or MS2-HA-Upf1 was tethered to the 3′-untranslated region of β-6bs mRNAs. Western blotting revealed that endogenous SMG5, SMG6 and SMG7 were downregulated to 4, 29 and 24% of normal levels, respectively (Figure 3A), which demonstrated the specific siRNA-mediated downregulation of each protein. The relative expression levels of effector proteins were also measured by western blotting (Figure 3B). Quantitative real-time RT–PCR (qRT–PCR) of β-6bs mRNA revealed that tethering of MS2-HA-PNRC2 and MS2-HA-Upf1 reduced the level of β-6bs mRNA to 18% and 31%, respectively, compared with tethering of MS2-HA (Figure 3C), consistent with previous reports (16,29). Intriguingly, the downregulation of SMG5, but not SMG6 or SMG7, significantly inhibited the β-6bs mRNA degradation elicited by tethered PNRC2 and Upf1 (Figure 3C).

Next, the reciprocal experiments were performed. Western blotting results demonstrated the specific downregulation of endogenous proteins by siRNAs (Figure 3D) and specific expression of effector proteins (Figure 3E). Endogenous PNRC2 and Upf1 were downregulated to 14% and 5% of normal levels, respectively (Figure 3D). In the qRT–PCR analysis, tethering of MS2-HA-SMG5, -SMG6, -SMG7, -PNRC2 and -Upf1 reduced the level of β-6bs mRNA to 44, 20, 6, 18 and 23%, respectively, compared with tethering of MS2-HA (Figure 3F). Efficient mRNA degradation by tethered

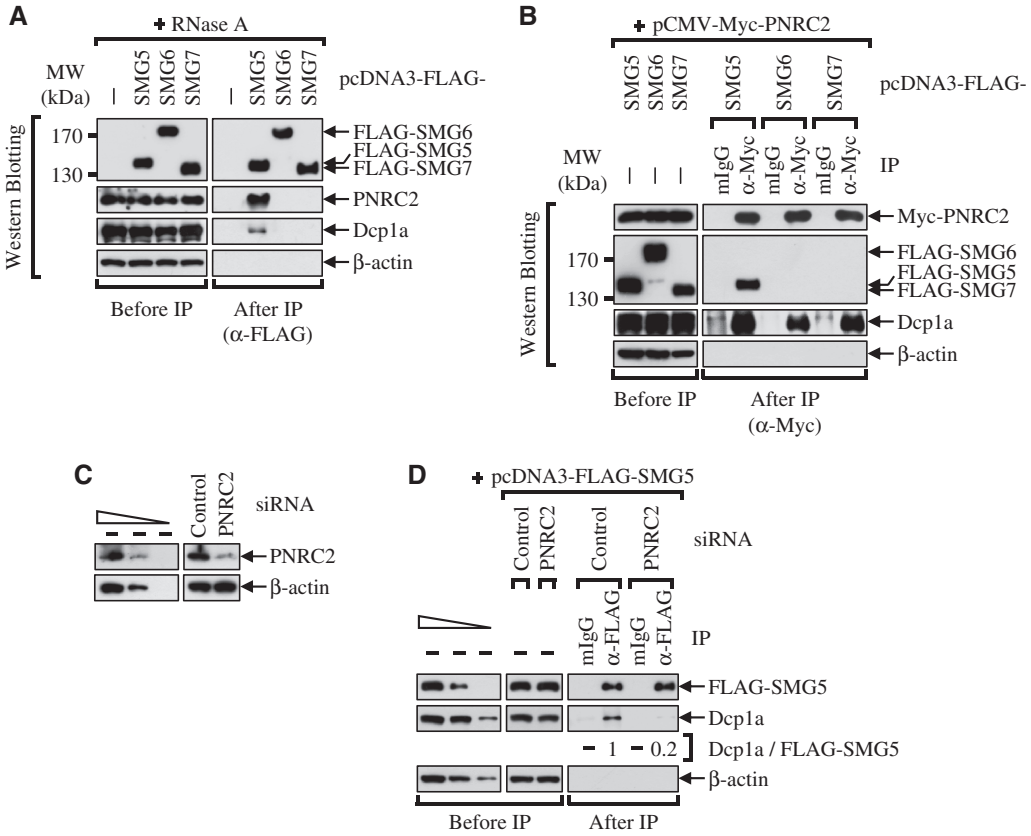

Figure 2. PNRC2 preferentially associates with SMG5. (**A**) IPs of SMG5, 6 and 7. HEK293T cells were transiently transfected with plasmid expressing FLAG, FLAG-SMG5, FLAG-SMG6 or FLAG-SMG7. Two days after transfection, cell lysates were obtained and treated with RNase A. And then IPs were performed using the α-FLAG antibody. (**B**) IPs of Myc-PNRC2. HEK293T cells were transiently co-transfected with the indicated plasmids. IPs were performed using the α-Myc antibody or mouse (m) IgG as a non-specific control. (**C** and **D**) IPs of FLAG-SMG5 using the extracts of cells depleted of PNRC2. (**C**) The selective downregulation of endogenous PNRC2 by siRNA transfection was demonstrated by western blotting. To demonstrate the quantitativity of western blotting, 3-fold serial dilutions of total-cell extracts were loaded in the three left-most lanes. (**D**) IPs were performed using the α-FLAG antibody or mIgG. The level of co-immunoprecipitated Dcp1a was normalized to the level of immunoprecipitated FLAG-SMG5. The normalized level of Dcp1a in the IP using the α-FLAG antibody and the undepleted cell extracts was set to 1.

SMG7, PNRC2 or Upf1 was consistent with previous reports (16,19,29,32). However, efficient mRNA degradation by tethered SMG5 or SMG6 was not consistent with a previous report (19). This discrepancy was probably due to the difference in the tethering system. Indeed, it has been reported that Magoh is more functional in the λN tethering system, whereas RNPS1 and eIF4AIII are more functional in the MS2 tethering system (33,34). Together with the finding that downregulation of endogenous SMG5 or SMG6 inhibits NMD (Figure 1) (30), it was not surprising that tethered SMG5 and SMG6 were functional in our system.

The results of the downregulation-coupled tethering experiments showed that downregulation of Upf1 inhibited the β-6bs mRNA degradation elicited by tethered SMG5, SMG6 and PNRC2 by 2.3-, 4.6- and 2.9-fold, respectively, without affecting mRNA degradation by tethered SMG7 (Figure 3F). On the other hand, downregulation of PNRC2 inhibited the β-6bs mRNA degradation elicited by tethered SMG5 and Upf1 by 2.1- and 2.5-fold, respectively, without affecting mRNA degradation by tethered SMG6 and SMG7 (Figure 3F). These observations agreed with the IP results showing the preferential interaction of PNRC2 with SMG5 (Figure 2) and with our previous report showing that PNRC2 and Upf1 function together to trigger mRNA degradation (16). The collective tethering results suggest that Upf1, SMG5 and PNRC2 function together at the same step to trigger rapid mRNA degradation.

SMG6 requires Upf1 for efficient mRNA degradation

Consistent with the previous observation that SMG7 functions downstream of PTC recognition (19), the mRNA degradation elicited by tethered SMG7 occurred independently of Upf1 and PNRC2 (Figure 3F). Unexpectedly, mRNA degradation elicited by tethered SMG6 was dependent on Upf1 (Figure 3F). Thus, it may be possible that Upf1 regulates the endonucleolytic activity of SMG6, in addition to functioning in the recruitment of SMG6 to NMD substrates.

To test the above idea, we employed an Upf1 (T28A) mutant, which contains a single amino acid substitution from threonine to alanine at position 28 and consequently lacks SMG6-binding activity (28). Using the SMG6 tethering system and siRNA-resistant (R) Upf1^{R}

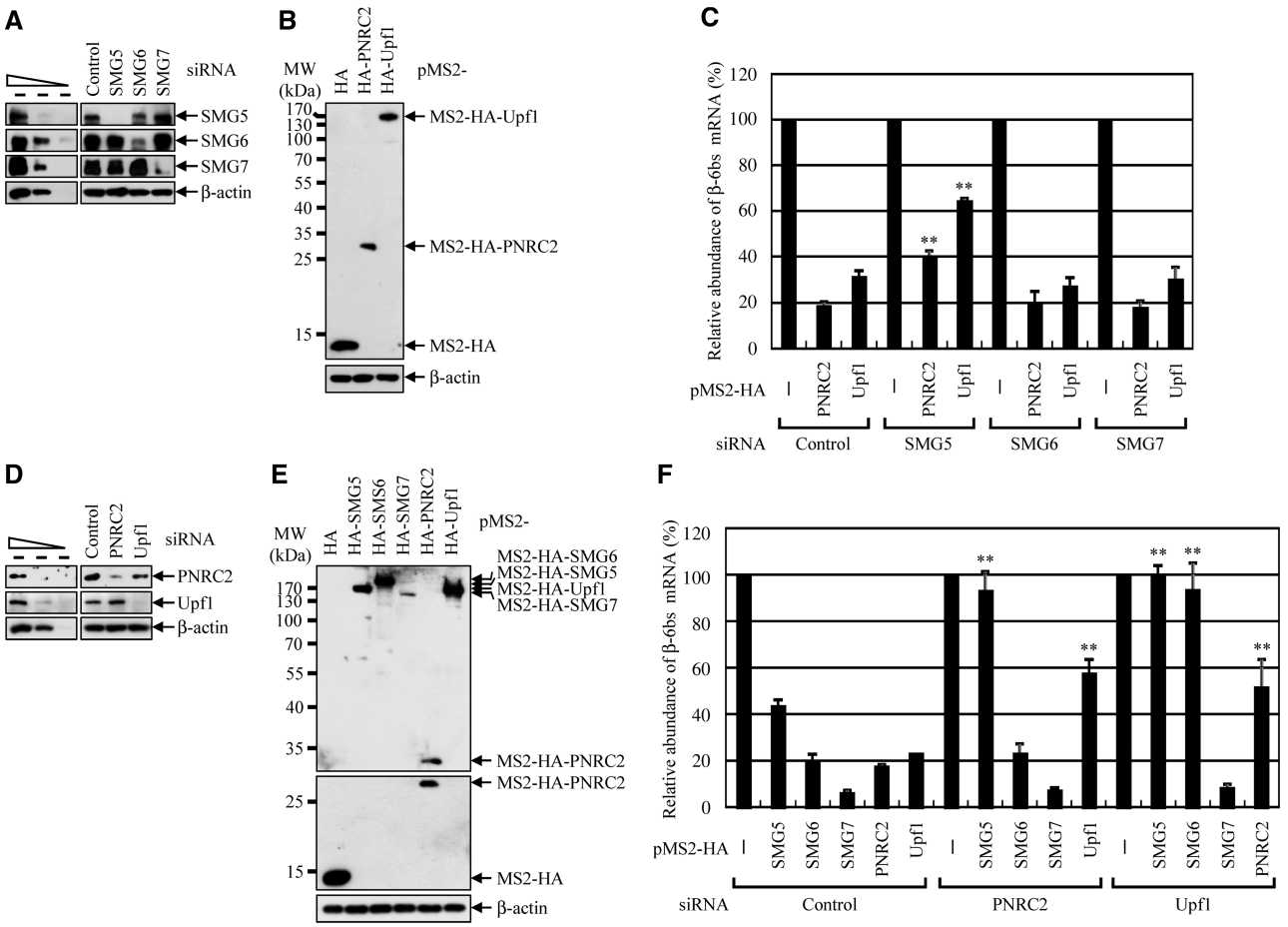

Figure 3. Upf1, PNRC2 and SMG5 coordinately function at the same step to trigger rapid mRNA degradation. (**A–C**) HeLa cells were transiently transfected with the SMG5, 6, 7, Upf1 or non-specific control siRNA. Two days after transfection, cells were retransfected with three plasmids: (i) a tethering reporter pcβ-6bs test plasmid, which encodes the β-Gl sequence followed by six tandem repeats of the MS2-binding site within the 3′-UTR, (ii) an effector plasmid expressing MS2-HA or MS2-HA-fused effector protein and (iii) the reference plasmid phCMV-MUP, which was used to control for variations in the transfection, RNA purification and gel loading. (**A**) Western blotting demonstrating specific downregulation by siRNAs. (**B**) Western blotting showing relative expressions of tethered proteins. (**C**) qRT–PCR of β-6bs mRNAs. The level of β-6bs mRNA was normalized to the level of MUP mRNA. Normalized levels of β-6bs mRNA obtained from cells expressing MS2-HA were set to 1.0. $^{**}P < 0.01$ as determined by Student's *t*-tests. (**D–F**) As in Figure 4A–C, except that HeLa cells were depleted of PNRC2 or Upf1, and SMG5, 6, 7, PNRC2 or Upf1 was tethered. All expressed proteins could not be represented in a single gel because of the large differences in protein sizes. Therefore, total-cell proteins were analyzed by 10% (E, upper) and 15% (E, middle) SDS–PAGE, respectively.

constructs, complementation experiments were done (Figure 4). To this end, HeLa cells were depleted of endogenous Upf1 and then transiently co-transfected with (i) an effector plasmid expressing MS2-HA or MS2-HA-SMG6, (ii) reporter plasmid expressing β-6bs mRNAs, (iii) plasmid encoding siRNA-resistant Myc-Upf1^{R} either wild-type (WT) or T28A and (iv) reference plasmid phCMV-MUP. Western blotting showed that endogenous Upf1 were downregulated to 5% of the normal level and that Myc-Upf1(WT) and Myc-Upf1(T28A) were expressed to 91% and 100%, respectively, of the endogenous level (Figure 4A). The relative expression levels of MS2-HA and MS2-HA-SMG6 were also compared by western blotting (Figure 4B). qRT–PCR of β-6bs mRNA revealed that tethering of MS2-HA-SMG6 reduced the level of β-6bs mRNA to 33% (Figure 4C). Consistent with the results in Figure 3F, downregulation of Upf1 inhibited β-6bs mRNA degradation elicited by tethered SMG6 (Figure 4C). Intriguingly, whereas the expression of siRNA-resistant Myc-Upf1 (WT) completely restored the β-6bs mRNA degradation elicited by tethered SMG6, the expression of siRNA-resistant Upf1 (T28A) slightly restored the β-6bs mRNA degradation elicited by tethered SMG6. All these results support the idea that the tethered SMG6 degrades mRNAs in an Upf1-dependent manner.

Endogenous NMD substrates have their own preferences for Upf1-interacting factors

The results in Figure 3 suggested that SMG5 requires PNRC2 to trigger the decay of NMD substrates and vice versa. To generalize this idea, we carried out microarray analyses using total transcripts purified from HeLa cells depleted of Upf1, SMG5, SMG7 or PNRC2. We also included a sample where SMG7 was downregulated, since it is known to form a heterodimeric complex with SMG5 (31). The specific downregulation by siRNAs was

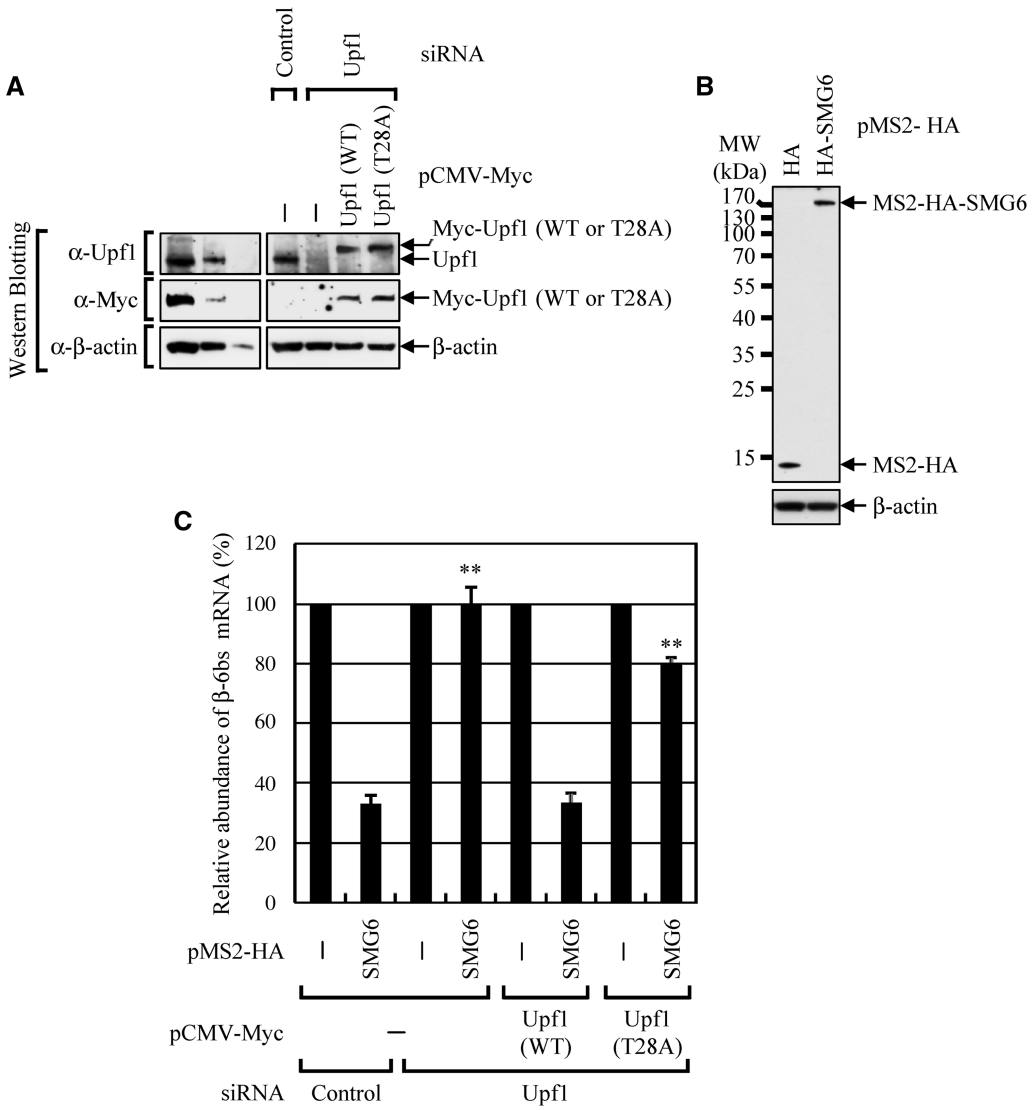

Figure 4. Rapid mRNA degradation by tethered SMG6 requires Upf1. HeLa cells were transiently transfected with either Upf1 siRNA or non-specific Control siRNA. Two days after transfection, cells were retransfected with four plasmids: (i) a tethering reporter pcβ-6bs test plasmid, (ii) an effector plasmid expressing MS2-HA or MS2-HA-SMG6, (iii) plasmid encoding siRNA-resistant (R) Myc-Upf1^{R} either WT or T28A and (iv) the reference plasmid phCMV-MUP. (**A**) Western blotting showing specific downregulation by siRNAs and relative expressions of transiently expressed Myc-Upf1 WT and T28A. (**B**) Western blotting showing relative expressions of tethered proteins. (**C**) qRT–PCR of β-6bs mRNAs. The level of β-6bs mRNA was normalized to the level of MUP mRNA. Normalized levels of β-6bs mRNA obtained from cells expressing MS2-HA were set to 1.0. $^{**}P < 0.01$.

confirmed by western blotting (Figure 5A). Microarray analysis using 48K human gene chips revealed that 503, 294, 291 and 335 transcripts were upregulated and 372, 286, 203 and 268 transcripts were downregulated by at least 1.5-fold upon Upf1, SMG5, SMG7 and PNRC2 downregulation, respectively (Figure 5B, left panel and Supplementary Table S1). Among the 503 transcripts that were upregulated by Upf1 dowregulation, 106 (21%), 73 (15%) and 116 (23%) transcripts were commonly upregulated by at least 1.5-fold upon SMG5, SMG7 and PNRC2 downregulation, respectively (Figure 5C and Supplementary Table S2).

To determine how many SMG5-dependent NMD substrates overlapped with PNRC2- or SMG7-dependent NMD substrates, the commonly upregulated transcripts were further compared. Among the 106 transcripts commonly upregulated by SMG5 and Upf1 downregulations (SMG5&Upf1), 68 (64%) and 37 (35%) transcripts were commonly upregulated in PNRC2&Upf1 and SMG7&Upf1 (Figure 5C), respectively, suggesting a 1.8-fold more preference for SMG5–PNRC2, compared with SMG5–SMG7.

To increase the stringency of data analyses, we compared the transcripts that were upregulated by at least 2-fold, instead of 1.5-fold. In this analysis, 115, 45, 30 and 49 transcripts were upregulated by at least 2-fold upon Upf1, SMG5, SMG7 and PNRC2 downregulation, respectively (Figure 5B, right panel and Supplementary Table S1). Among the 115 transcripts upregulated by Upf1 downregulation, 17 (15%), 2 (2%) and 13 (11%)

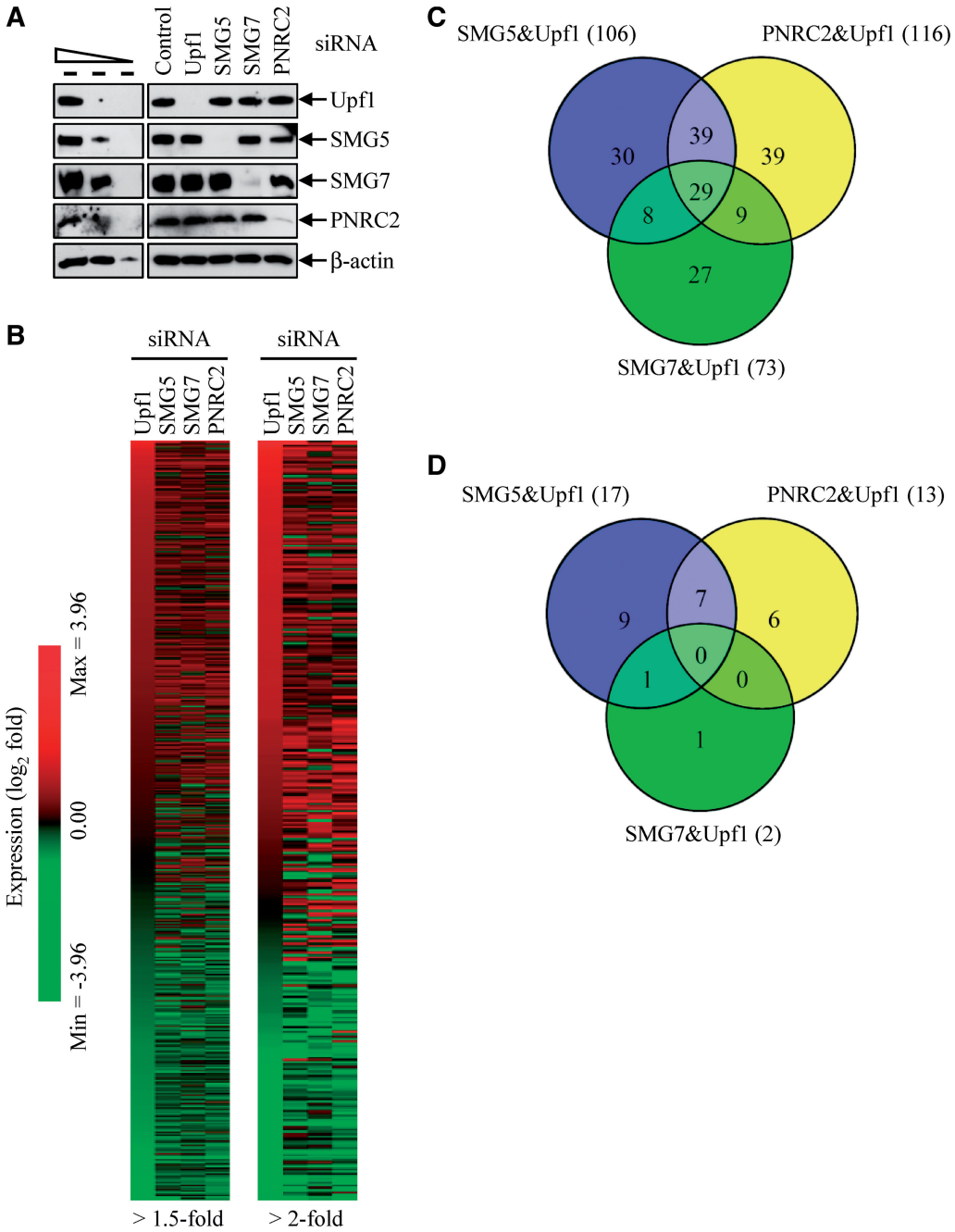

Figure 5. PNRC2-dependent NMD substrates significantly overlap with SMG5-dependent NMD substrates. (**A**) Western blotting showing the specific siRNA-mediated downregulations. (**B**) Comparative analysis of normalized microarray data using total-cell RNAs purified from HeLa cells depleted of Upf1, SMG5, SMG7 or PNRC2. Transcripts upregulated or downregulated by at least 1.5-fold (left) or 2-fold (right) were chosen for analysis. (**C**) Venn diagrams showing the number of transcripts that were commonly upregulated by at least 1.5-fold upon downregulation of Upf1-interacting factors (SMG5, SMG7 or PNRC2) and upon downregulation of Upf1. The total number of commonly upregulated transcripts after siRNA downregulation is depicted in parentheses. (**D**) Venn diagrams showing the number of transcripts that were commonly upregulated by at least 2-fold upon downregulation of Upf1-interacting factors (SMG5, SMG7 or PNRC2) and upon downregulation of Upf1.

transcripts were commonly upregulated by at least 2-fold upon SMG5, SMG7 and PNRC2 downregulation, respectively (Figure 5D). Among the 17 transcripts commonly upregulated in SMG5&Upf1, 7 (41%) and 1 (6%) transcripts were commonly upregulated in PNRC2&Upf1 and SMG7&Upf1 (Figure 5D), respectively. The specific regulation of cellular NMD substrates by each Upf1-interacting factor was further confirmed by qRT–PCRs (Supplementary Figure S1).

For the statistical analysis, transcripts commonly upregulated by at least 1.5-fold and 2-fold, respectively, were further analyzed by Kendall's rank correlation test (Supplementary Table S3). The test results showed that Kendall's tau coefficient between SMG5&Upf1 and

PNRC2&Upf1 was higher than that between SMG5& Upf1 and SMG7&Upf1 or that between SMG7&Upf1 and PNRC2&Upf1, indicating that SMG5&Upf1 was more closely related to PNRC2&Upf1 than SMG7& Upf1. All these results strongly suggest that SMG5 preferentially functions together with PNRC2 rather than with SMG7 under normal conditions.

Next, we compared the commonly downregulated transcripts (Supplementary Figure S2). Interestingly, many cellular transcripts were commonly downregulated upon Upf1, SMG5, SMG7 or PNRC2 downregulation, suggesting that Upf1 and Upf1-interacting factors are commonly involved in some cellular events other than NMD. Alternatively, it is also possible that upregulation of transcripts directly targeted for NMD causes a downregulation of these transcripts as a consequence of indirect or secondary effect.

Next, we analyzed the correlations between Upf1-interacting factors and NMD-inducing features (35): the upstream open reading frames (uORFs), introns downstream of translation termination codon, alternative splicing variants that harbors nonsense codons or frameshifts and selenocysteine incorporation codons (Supplementary Figure S3 and Supplementary Table S2). Transcripts commonly upregulated by at least 1.5-fold (Figure 5C) were analyzed. The results revealed that (i) there was no significant correlation between different Upf1-interacting factors and the NMD-inducing features and (ii) half of the transcripts in each category (SMG5&Upf1, SMG7&Upf1 and PNRC2&Upf1) contained uORF, which is an NMD-inducing feature.

DISCUSSION

Based on our observations and previous reports, two combinatory interactions among Upf1-interacting factors would be possible: SMG5–PNRC2 (in this study) and SMG5–SMG7 (31,36). It is likely that PNRC2 and SMG7 bind to SMG5 in a mutually exclusive manner, since PNRC2 and SMG7 did not detectably co-exist in the same complex (Figure 2). Furthermore, the data presented in this study indicate that SMG5–PNRC2 is a functionally more predominant complex than SMG5–SMG7 under normal conditions, since (i) downregulation of SMG5 or PNRC2, but not SMG7, abrogated NMDs of Gl and GPx1 mRNAs (Figure 1); (ii) decapping complex and PNRC2 were detectably complexed with SMG5 but not with SMG7 (Figure 2); (iii) rapid degradation of β-6bs mRNA elicited by tethered Upf1 was dependent on SMG5 and PNRC2, but not SMG7 (Figure 3); (iv) β-6bs mRNA degradation elicited by tethered SMG5 was completely abrogated by downregulation of PNRC2, but not SMG7 (Figure 3) and (v) a large fraction of SMG5-dependent cellular NMD substrates overlapped with PNRC2-dependent cellular NMD substrates more significantly than SMG7-dependent cellular NMD substrates (Figure 5).

Based on our microarray analysis (Figure 5), experiments using NMD reporters (Figure 1) and a recent study (37), and considering the critical roles of NMD in mRNA surveillance and post-transcriptional regulation, it is not surprising that each NMD substrate used multiple Upf1-interacting adaptors or effectors as a kind of backup system for eliminating PTC-containing mRNAs. However, it should be noted that the NMDs of many cellular mRNAs were dependent on only a single known Upf1-interacting adaptor or effector in our study (Figure 5C, D, Supplementary Figure S1 and Supplementary Table S2). Considering that SMG5-, SMG7- and PNRC2-dependent NMDs merge into Dcp2-dependent decapping pathway (16,19) and that, so far, there is no report showing direct interaction between Dcp2 and Upf1-interacting factors, it is most likely that unidentified additional adaptor proteins, other decapping enhancers (38,39) or unknown interactions between Dcp2 and Upf1-interacting factors link hp-Upf1 to the decapping enzyme, Dcp2 (Figure 6). In support of this idea, we observed that PNRC2 selectively associated with Dcp1a and Hedls, but not with Rck/p54 and Edc3 (Supplementary Figure S4), suggesting that PNRC2 has its own preference for decapping enhancers in order to link hp-Upf1 and Dcp2.

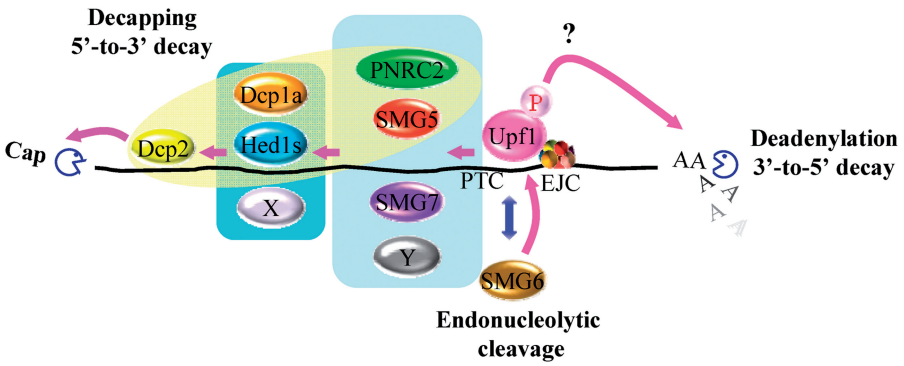

Figure 6. Model illustrating the diverse combinations of Upf1-interacting factors involved in triggering NMD. Hp-Upf1 may interact with one of binding partners including SMG5-7 and PNRC2. SMG6 triggers endonucleolytic cleavage, whereas SMG5, SMG7 and PNRC2 trigger decapping activity of Dcp2 in either Dcp1a-dependent or Dcp1a-independent (unknown protein X-dependent) manner. Decapped mRNA would be vulnerable to 5′-to-3′-degradation. For exosome-mediated 3′-to-5′-decay, it is not clearly known how the hp-Upf1 activates 3′-to-5′-decay and what kinds of proteins are involved in this connection. The light-yellow oval indicates a functionally dominant connection shown in this study. P, phosphate group; X and Y, unidentified proteins.

It should be noted that our microarray results showed that subsets of transcripts are upregulated in abundance by downregulation of SMG5, SMG7 or PNRC2, but not Upf1. By definition of NMD, these transcripts seem not to belong to NMD substrates. Instead, considering a variety of biological roles of each Upf1-interacting factor (40–46), the change of these transcripts in abundance could be due to other cellular pathways rather than NMD. Alternatively, it is also possible that each Upf1-interacting factor (at least SMG7) could be directly or indirectly recruited to mRNAs with the help of its interacting RNA-binding protein, eliciting rapid mRNA degradation in an Upf1-independent manner. The similar case can be exemplified by Upf1 recruitment: Upf1 can be recruited to mRNAs either by associating with a terminating ribosome in NMD or by directly interacting with Staufen1 in Staufen1-mediated mRNA decay (21,29).

The preferential use of different Upf1-interacting factors by different NMD substrates would be dependent on various factors including (i) the relative expression levels of Upf1-interacting factors within the cells; (ii) the preferential interaction among Upf1-interacting factors; (iii) the level or residues of Upf1 that are phosphorylated, since, for instance, different Upf1-interacting factors exhibit different binding activities to Upf1 depending on the level (Supplementary Figure S5) or residues (28) of Upf1 that are phosphorylated and (iv) *cis*-acting mRNA sequences. The selective use of different Upf1-interacting factors might be beneficial to cells, in that the specific regulation of a subset of NMD substrates is possible in response to environmental changes. The underlying mechanism by which NMD substrates select their own Upf1-interacting factor(s) and the biological relevance for preferential use of Upf1-interacting factors should be addressed in the future.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–5, Supplementary Tables 1–4, Supplementary Materials and Methods and Supplementary references [47–50].

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