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Inhibition of nonsense-mediated mRNA decay (NMD) by a new chemical molecule reveals the dynamic of NMD factors in P-bodies

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Abstract

In mammals, nonsense-mediated mRNA decay (NMD) is a quality control mechanism that degrades mRNAs harboring a premature termination codon, in order to prevent the synthesis of truncated proteins. To gain insight into NMD mechanism, we identified NMDI 1 (for nonsense-mediated mRNA decay inhibitor 1) as a small molecule inhibitor of the NMD pathway. We characterized the mode of action of this compound and demonstrated that it acts upstream of hUPF1. NMDI 1 induced the loss of interactions between hSMG5 and hUPF1 and the stabilization of hyperphosphorylated isoforms of hUPF1. Incubation of cells with NMDI 1 allowed us to demonstrate that NMD factors and mRNAs subject to NMD transit through processing bodies (P-bodies) as it is the case in yeast. The results suggest a model in which mRNA and NMD factors are sequentially recruited to P-bodies.

Introduction

Nonsense-mediated mRNA decay (NMD) is a quality control process found in all eukaryotic organisms studied to date (Conti and Izaurralde, 2005; Maquat, 2004a). One role of this process is to degrade mRNA harboring a premature termination codon (PTC) in order to prevent the synthesis of truncated proteins which could be non-functional, or whose function may be deleterious to cells. The NMD pathway has been shown to be involved in the regulation of gene expression in yeast, drosophila and mammals (He et al., 2003; Mendell et al., 2004; Rehwinkel et al., 2005; Sureau et al., 2001; Wollerton et al., 2004).

In mammalian cells, NMD takes place after pre-mRNA splicing, and, in most cases, is mediated by a protein complex deposited 20-24 nucleotides upstream of exon-exon junctions (Conti and Izaurralde, 2005; Lejeune and Maquat, 2005; Maquat, 2004b). This protein complex, called the exon-junction complex (EJC), is thought to recruit the evolutionary conserved UPF proteins that play an essential, but still not fully characterized, role in NMD. During what is referred to as the “pioneer round of translation” (Ishigaki et al., 2001), PTCs are recognized and the targeted mRNAs are degraded both by 5'-to-3' decay involving decapping, and 5'-to-3' exoribonucleases such as hXRN1 and hXRN2/hRAT1, and by 3'-to-5' decay involving deadenylation and the exosome (Chen and Shyu, 2003; Couttet and Grange, 2004; Lejeune et al., 2003).

NMD implicates the participation of hUPF proteins such as hUPF1, hUPF2, hUPF3 (also named hUPF3a) and hUPF3X (also called hUPF3b). The function of these hUPF proteins is still unclear. However, it has been proposed that they are sequentially recruited by the EJC: hUPF3/3X first, then hUPF2 and finally hUPF1 in mammalian cells (Kim et al., 2005; Lykke-Andersen et al., 2001). Interestingly, the function of hUPF2 has been

demonstrated to not be indispensable in some NMD cases, suggesting the existence of different pathways to elicit NMD (Gehring et al., 2005).

UPF1 is a phosphoprotein that undergoes phosphorylation/dephosphorylation cycles during NMD (Ohnishi et al., 2003; Page et al., 1999; Pal et al., 2001). UPF1 has been shown to interact with release factors in yeast (Czaplinski et al., 1998) and in mammals (Kashima et al., 2006), and could link the EJC and the translation termination complex. A direct interaction between hUPF1 and the cap binding protein CBP80 has also recently been demonstrated in mammalian cells (Hosoda et al., 2005), indicating that hUPF1 establishes a complex interaction network either before or during the pioneer round of translation. Phosphorylation of hUPF1 has been shown to be carried out by hSMG1, a PI3 kinase-related kinase (Page et al., 1999; Pal et al., 2001; Yamashita et al., 2001), and requires the presence of hUPF2 and hUPF3 (Kashima et al., 2006). In contrast, dephosphorylation of hUPF1 requires the presence of a multi-protein complex composed of hSMG5, hSMG6, hSMG7 and the protein phosphatase (PP)-2A (Chiu et al., 2003; Ohnishi et al., 2003). For the most part, hSMG5 and hSMG7 proteins are distributed evenly throughout the cytoplasm, but a fraction is also present in processing bodies (P-bodies) (Unterholzner and Izaurralde, 2004). hSMG6 is a cytoplasmic protein that concentrates in cytoplasmic foci distinct from P-bodies and whose nature is still unclear (Unterholzner and Izaurralde, 2004).

P-bodies have been described in lower and higher eukaryotic cells (Cougot et al., 2004; Ingelfinger et al., 2002; Sheth and Parker, 2003; van Dijk et al., 2002). In mammals, these cytoplasmic structures contain many factors involved in mRNA decay including components of the decapping machinery such as DCP1a (Ingelfinger et al., 2002), DCP2 (Ingelfinger et al., 2002; van Dijk et al., 2002), GE1 (also called HEDLS) (Fenger-Gron et al., 2005; Yu et al., 2005), p54/RCK (Cougot et al., 2004), the deadenylase CCR4 (Cougot et al., 2004), XRN1 (Bashkurov et al., 1997), the LSM1-7 complex involved in different aspects

of RNA processing (Cougot et al., 2004; Ingelfinger et al., 2002), and the hUPF1, hSMG5 and hSMG7 components of the NMD machinery (Fukuhara et al., 2005; Unterholzner and Izaurralde, 2004). The function of P-bodies is still unclear but they may serve as a storage compartment for both untranslated RNAs and proteins involved in RNA decay (Brenques et al., 2005; Franks and Lykke-Andersen, 2007; Pillai et al., 2005; Teixeira et al., 2005), and/or as a site for RNA decay (Cougot et al., 2004; Sheth and Parker, 2006).

In a recent work, we showed that hydrophobic tetracyclic indole derivatives block the function of specific splicing factors (Soret et al., 2005). In light of these findings, we decided to look further at this collection to determine if certain of these compounds also inhibit NMD. The underlying idea was that such small molecule inhibitors could represent powerful tools to decipher the NMD process. In this study, we report the identification of one such molecule, NMDI 1 (for nonsense-mediated mRNA decay inhibitor 1), that inhibits nucleus-associated as well as cytoplasmic NMD. The inhibitory mechanism appears to be due to the loss of the interaction between hSMG5 and hUPF1, thereby leading to the stabilization of the hyperphosphorylated forms of hUPF1 and to its concentration in P-bodies. Interestingly, NMDI 1-mediated inhibition revealed that other NMD factors and PTC-containing mRNAs can traffic through P-bodies as it is the case in yeast (Sheth and Parker, 2006).

Results

Identification of a novel NMD inhibitor

In a previous report we identified a series of polycyclic indole derivatives which block the function of specific splicing factors (Soret et al., 2005). Since certain molecules from this family inhibited the function of proteins involved in mRNA maturation, we decided to assess their capacity to inhibit NMD. HeLa cells were transfected by two test plasmids coding for β -Globin (G1) and for glutathione peroxidase 1 (GPx1) mRNA, harboring either a premature termination codon (Ter) or not (Norm). G1 mRNA is subject to nucleus-associated NMD in non-erythroid cells (Thermann et al., 1998; Zhang et al., 1998) whereas GPx1 mRNA is subject to cytoplasmic NMD (Moriarty et al., 1998). Additionally, a reference plasmid coding for the mouse major urinary protein (MUP) mRNA was also introduced into the cells (Ishigaki et al., 2001). 24 hours after transfection, cells were incubated for 20 hours with 5 μ M of indole compound (supplemental data table 1), or with DMSO (-) as a control. Then, total RNAs were purified and analyzed by RT-PCR (Fig. 1) to measure NMD efficiency. Among the 25 indole derivatives tested, only the compound 70 (referred to as NMDI 1), stabilized G1 Ter mRNA level about 3-fold, indicating that this molecule is an inhibitor of nucleus-associated NMD (Fig. 1A and data not shown). Interestingly, NMDI 1 also stabilized the level of GPx1 Ter mRNA by approximately 2-fold (Fig. 1B and data not shown). In order to confirm these results, we measured the NMD inhibition by RNase protection assay (RPA) as it represents a more reliable approach for RNA quantification. The results are presented in the supplemental data S1 and confirm the 2-3 fold of NMD inhibition by NMDI 1. Altogether, these data allowed us to conclude that NMDI 1 is an inhibitor of nucleus-associated as well as

cytoplasmic NMD. Notably, the inhibition level obtained with NMDI 1, is similar to that observed with other NMD inhibitors such as cycloheximide or to the down-regulation of hDCP2 or hPARN (Ishigaki et al., 2001; Lejeune et al., 2003). In order to show a more direct correlation between NMDI 1 and NMD inhibition, we measured NMD efficiency on PTC-containing Gl or GPx1 mRNAs in cells that were treated with increasing amount of NMDI 1 (Fig. 1C). Interestingly, we observed a progressive NMD inhibition from 0 to 5 μ M of NMDI 1 for both Gl and GPx1 constructs. Above 5 μ M, we were unable to get a substantially stronger inhibition, suggesting that NMD can not be abolished at 100% in our experimental conditions or that the 20-30% of mRNA which escaped from NMD inhibition represents the fraction of mRNAs already engaged in NMD process at the time of NMDI 1 treatment. In all subsequent experiments, we used 5 μ M of NMDI 1 as our working concentration. Notably, NMDI 1 does not exhibit any cellular toxicity, as measured by trypan blue staining, even at concentrations as high as 125 μ M (data not shown).

At this stage, some controls were performed in order to investigate the specificity of inhibition mediated by NMDI 1. First, NMDI 1 failed to have any effect on splicing of several pre-mRNA reporter transcripts (Soret et al., 2005) and did not affect the level of pre-mRNA (Supplemental data S1A and S1B). Second, unlike cycloheximide (CHX) which inhibits translation, NMDI 1 does not alter the expression of transfected firefly luciferase (Fig. 1D), suggesting that NMDI 1 is not a general translation inhibitor. To further demonstrate the absence of any effects of NMDI 1 on translation, we performed metabolic labeling of proteins with ³⁵S-methionine in HeLa cells and showed that treatment with NMDI 1 had no effects on ³⁵S incorporation (supplemental data S1C). Third, to assay the integrity of the miRNA decay pathway in the presence of NMDI 1, we used a renilla luciferase construct that is subject to degradation by *let-7* miRNA (pRL-Perf) or immune to miRNA decay pathway (pRL-3XBugleMut) (Pillai et al., 2005). Our results indicate that NMDI 1 does not increase Renilla

activity which is under the control of let7 miRNA, confirming that targeted mRNA degradation by miRNA is not altered by NMDI 1 (Fig. 1E). Finally, we also tested whether NMDI 1 could induce the formation of stress granules which provides a sensitive assay for proper mRNA metabolism. Indeed, these structures are aggregates of mRNPs that form when cells are subjected to several stresses, including mild translational inhibition. Unlike sodium arsenite treatment which is commonly used to induce stress granules formation (Kedersha et al., 2005), NMDI 1 treatment did not change the localization of G3BP protein, a well characterized marker of stress granules (Tourriere et al., 2003) (supplemental data S1D). Taken together, these results indicate that NMDI 1 is a new and a specific NMD inhibitor.

NMDI 1 abrogates NMD upstream of hUPF1 functions

In order to gain insight into the mode of inhibition of NMDI 1, we analyzed its effects on a tethering system that mimics the sequential recruitment of NMD factors on mRNA (Kim et al., 2005; Lykke-Andersen et al., 2000). Cells were transfected with two types of constructs. The first one codes for a **Firefly Luciferase (Fluc)** mRNA containing 8 binding sites for the MS2 protein in its 3'UTR, and the second one codes for either the MS2 protein, or one of the following fusions: MS2-hUPF1, MS2-hUPF2 or MS2-hUPF3X. Additionally, we transfected HeLa cells with a construct coding for the **Renilla Luciferase (Rluc)** mRNA in order to normalize the amount of analyzed RNA. Cells were then incubated for 20 hours with NMDI 1 or with DMSO (-) as negative control and **Rluc** as well as **Fluc** mRNAs levels were measured by RT-PCR as previously described (Hosoda et al., 2005). The expression of each MS2 fusion was controlled by western-blot in order to verify that the observed effects were not due to a variation in protein expression (Fig. 2A). In each case, the compound did not affect expression of the MS2 fusion, which was itself never higher than the level of the endogenous protein. As expected, control experiment performed in the presence of DMSO

revealed that the level of **FLuc** mRNA was lower in cells expressing one of the MS2-hUPF fusion proteins as compared to cells expressing only MS2 (Fig. 2B). Remarkably, NMDI 1 counter-acted the degradation induced by MS2-hUPF2 or MS2-hUPF3X, but had no effect against MS2-hUPF1 (Fig. 2B). Notably, the inhibition level obtained with NMDI 1 are very similar to that observed when NMD was inhibited through down-regulation of hCBP80 (Hosoda et al., 2005). To have a more accurate measure of the NMD inhibition, **Rluc** and **Fluc** mRNAs levels were also measured by RPA. The results are presented in supplemental Figure S2A and reproduce the quantification of mRNAs levels by RT-PCR (Fig. 2B). Altogether, these results indicate that NMDI 1 inhibits NMD downstream of hUPF3X or hUPF2 recruitment and upstream of hUPF1 functions.

NMDI 1 does not prevent the interactions between hUPF1 and hUPF3X

In the light of the results described **in the previous paragraph**, we hypothesized that NMDI 1 could prevent the recruitment of hUPF1 to the EJC *via* its interactions with the other hUPF proteins. To test this, we immunoprecipitated hUPF1 from HeLa cell extracts under conditions that preserve the integrity of mRNPs (Lejeune and Maquat, 2004). NMDI 1 or DMSO (-) was added to the cell culture 20 hours before immunoprecipitation (IP). Since hUPF2 was shown to be dispensable in some NMD cases (Gehring et al., 2005), we focused our analysis on the presence of hUPF3X protein in each IP (Fig. 3A). As a control for IP specificity, we did not detect TUBULIN protein in any of the hUPF1 IPs and no proteins were present in the IP performed with Normal Rabbit Serum (NRS). The results show that hUPF3X was present in hUPF1 IP even when cells were incubated with our NMD inhibitor. Thus, these data demonstrate that the interaction between hUPF1 and hUPF3X is not abolished by NMDI 1 and suggest that NMDI 1 would not prevent the recruitment of hUPF1 to the EJC.

NMDI 1 stabilizes hyperphosphorylated forms of hUPF1

Since hUPF1 requires a cycle of phosphorylation and dephosphorylation during NMD (Ohnishi et al., 2003), we next investigated the possibility that NMDI 1 may affect hUPF1 function by interfering with its phosphorylation level. We thus measured the level of hUPF1 phosphorylation in cells incubated with NMDI 1 or, as a control, with DMSO (-) by 2D gel analysis. Since hUPF1 phosphorylation is influenced by serum (Pal et al., 2001), we used 293T cells that, unlike HeLa cells, can be synchronized by serum deprivation. Cells were transfected with the expression vector pCI-neo-FLAG-hUpf1 (Sun et al., 1998), synchronized for 24 hours by serum deprivation 12 hours after transfection. Finally DMSO or 5 μ M of NMDI 1 was added for 3 hours before adding back serum for one hour. Our results show that when serum was not added back to the cell culture, the FLAG-hUPF1 protein electrofocalized in one spot corresponding to the unphosphorylated protein (Pal et al., 2001) (Fig. 3B). After serum addition, we observed a mild phosphorylation of FLAG-hUPF1 protein in the presence of DMSO and the stabilization of hyperphosphorylated isoforms of FLAG-hUPF1 when cells were incubated with NMDI 1 (Fig. 3B). We concluded that NMDI 1 stabilized hyperphosphorylated isoforms of hUPF1.

As it has been proposed that hUPF1 would localize to P-bodies when hyperphosphorylated (Fukuhara et al., 2005; Unterholzner and Izaurralde, 2004), we analyzed the cellular localization of FLAG-hUPF1 in HeLa cells in the absence or the presence of NMDI 1 (Fig. 3C). With the exception of co-expression experiments with hSMG7 which induces the recruitment of hUPF1 to P-bodies (Unterholzner and Izaurralde, 2004) (Fig. 3C), exogenous hUPF1 was equally distributed through the cytoplasm when cells were incubated with DMSO (-) as previously reported for untreated cells (Lykke-Andersen et al., 2000; Mendell et al., 2002) (Fig. 3C). When cells were treated with NMDI 1, we observed cytoplasmic concentrations of FLAG-hUPF1 in some structures that colocalize with the three

commonly used markers of P-bodies GFP-GE1, YFP-hSMG7 or CFP-hDCP1a (Fig. 3C). We also verified that FLAG-hUPF1 accumulated in P-bodies in the presence of NMDI 1 in 293T cells under the same experimental conditions as we used to study the phosphorylation level of FLAG-hUPF1 (supplemental data S2B). We used hXRN1 protein as a P-body marker in order to avoid an additional transfected DNA. After addition of serum, we observed some FLAG-hUPF1 cytoplasmic concentrations that colocalize with hXRN1 only when cells were treated with NMDI 1 but not in its absence. To definitively demonstrate that hyperphosphorylated isoform of hUPF1 accumulates in P-bodies, HeLa cells were treated for 20 hours with either DMSO (-) or NMDI 1 and the cellular localization of endogenous phosphorylated hUPF1 was determined using a specific antibody raised against a phosphoepitope of this protein (Ohnishi et al., 2003). The results presented in the supplemental data S6 indicate that in the presence of NMDI 1 phosphorylated hUPF1 isoforms colocalize with CFP-hDCP1a foci. We conclude that NMDI 1 induces the accumulation of hyperphosphorylated hUPF1 isoforms in P-bodies. This may occur via either stimulation of phosphorylation or alternatively, by blocking dephosphorylation.

To distinguish between these two possibilities, we subsequently investigated the association of hUPF1 with other NMD partners in HeLa cells treated or not with NMDI 1 (Fig. 3A). We first analyzed the interaction of hUPF1 with its dephosphorylation complex. Immunoprecipitation of hUPF1 allowed recovery of hSMG5, hSMG6 and hSMG7 from DMSO-treated cells. However, following treatment of HeLa cells with NMDI 1 only hSMG6 and hSMG7 but not hSMG5 were still associated with hUPF1 (Fig. 3A). Thus, we conclude that NMDI 1 destabilizes the interaction between hUPF1 and hSMG5. The fact that NMDI 1 does not alter the association of hUPF1 with hSMG1 and hUPF3X strongly suggests that NMDI 1 does not influence the interactions between hUPF1 and its phosphorylation complex (Fig. 3A). Altogether, our results indicate that the hyperphosphorylation of hUPF1 is most

likely due to a failure in dephosphorylation because of the loss of interaction between hUPF1 and hSMG5, rather than to an activation of phosphorylation. This conclusion is consistent with the fact that hSMG5 has been shown to be essential for hUPF1 dephosphorylation (Ohnishi et al., 2003).

hSMG5 is excluded from P-bodies in the presence of NMDI 1

Since NMDI 1 induces the localization of hUPF1 in P-bodies, hUPF1 hyperphosphorylation and the destabilization of interactions between hUPF1 and hSMG5, we assessed the cellular localization of the hUPF1 dephosphorylation complex during NMDI 1 treatment. hSMG5 and hSMG7 have been shown to localize mainly in the cytoplasm and, in particular, in P-bodies as shown by colocalization experiments with the endogenous LSM4 for hSMG7 and with hSMG7 for hSMG5 (Unterholzner and Izaurralde, 2004). hSMG6 similarly localizes mainly in the cytoplasm and also in some cytoplasmic foci that do not contain endogenous LSM4 (Unterholzner and Izaurralde, 2004). We transfected HeLa cells with expression vectors encoding YFP-hSMG5, YFP-hSMG6 or YFP-hSMG7 (Unterholzner and Izaurralde, 2004) and CFP-hDCP1a as a P-body marker. After 24 hours, we added DMSO (-) or 5 μ M of NMDI 1 to the cells. As previously shown, in the absence of the inhibitor, YFP-hSMG5, YFP-hSMG6 and YFP-hSMG7 were concentrated in cytoplasmic foci (Fukuhara et al., 2005; Unterholzner and Izaurralde, 2004) which for a substantial fraction of them (33%, 72% and 100% respectively) colocalized with CFP-hDCP1a (Fig. 3D). The fact that we observed hSMG6 in P-bodies unlike what was previously observed (Unterholzner and Izaurralde, 2004) is likely due to the different markers used for detection of P-bodies, and may reflect heterogeneity of P-bodies in their protein composition (see below and discussion). In the presence of NMDI 1, the cytoplasmic foci containing YFP-hSMG6 or YFP-hSMG7 perfectly colocalized with CFP-hDCP1a P-bodies. Interestingly, hSMG5 was no longer

observed in cytoplasmic foci and became evenly distributed in the cytoplasm in cells treated with NMDI 1 (Fig. 3D).

Endogenous hSMG5, hSMG6 or hSMG7 cannot be detected in cytoplasmic foci due to their weak expression (Unterholzner and Izaurralde, 2004). Since NMDI 1 inhibits NMD and induces the accumulation of hUPF1 in P-bodies as shown with exogenous as well as endogenous hUPF1 (Fig. 3C and supplemental data S2B and S3), we tested whether the three hSMG proteins would also accumulate in cytoplasmic foci of treated cells. The results shown in the supplemental data S3 indicated that these three proteins were not detected in cytoplasmic foci in DMSO-treated cells. However when cells were incubated with NMDI 1, both hSMG6 and hSMG7 colocalized with CFP-hDCP1a in P-bodies. In agreement with transfection experiment (Fig. 3D), under the same conditions, hSMG5 was not detected in cytoplasmic foci. Altogether, our results indicate that NMDI 1 modifies the cellular localization of hSMG5 by excluding it from P-bodies. This is consistent with the failure of hUPF1 antibodies to IP hSMG5 from NMDI 1 treated cells (Fig. 3A).

hUPF3 and hUPF3X localize in P-bodies when NMD is blocked by NMDI 1

Since some NMD factors such as hUPF1, hSMG5, hSMG6 or hSMG7 localize to P-bodies (Unterholzner and Izaurralde, 2004) (this study) we envisaged that other NMD factors may pass through P-bodies in a transient manner. As NMDI 1 is able to block NMD at a step where hUPF1 is confined to P-bodies, we investigated the cellular localization of hUPF3 and hUPF3X in both treated and untreated cells. These two proteins have been previously shown to be primarily nuclear proteins in untreated cells (Serin et al., 2001). We transfected HeLa cells with expression vectors that code for hUPF3-FLAG or hUPF3X-FLAG together with one of the P-body markers YFP-hSMG6, YFP-hSMG7, GFP-GE1 or CFP-hDCP1a. The cells were treated with DMSO or with NMDI 1 before performing indirect immunofluorescence

experiments (Fig. 4 and data not shown for CFP-hDCP1a). As for untreated cells (Lykke-Andersen et al., 2000; Serin et al., 2001), hUPF3 or hUPF3X localized primarily in the nucleus when cells were incubated with DMSO (-) (Fig. 4). However, when cells were grown in the presence of NMDI 1, we observed a cytoplasmic localization of hUPF3 as well as hUPF3X, with some accumulation in foci. To further characterize these foci, we analyzed their colocalization with cotransfected P-body markers (Fig. 4 and data not shown). hUPF3-FLAG as well as hUPF3X-FLAG proteins colocalized with YFP-hSMG6 (68% and 57% respectively) or YFP-hSMG7 (50% and 51%, respectively) in cells treated with NMDI 1. Surprisingly, unlike with FLAG-hUPF1, GFP-GE1 did not colocalize with hUPF3-FLAG or hUPF3X-FLAG. We conclude that hUPF3/3X proteins can translocate to the cytoplasm, which is consistent with their shuttling properties (Lykke-Andersen et al., 2000; Serin et al., 2001), and can reach a subset of P-bodies.

PTC-containing mRNAs accumulate in P-bodies in the presence of NMDI 1.

Since NMD factors accumulate in P-bodies when NMD is inhibited, we were interested in determining whether NMD substrates also accumulate in P-bodies. In yeast, It has recently been shown that PTC-containing mRNAs accumulate in P-bodies when NMD is blocked (Sheth and Parker, 2006). We speculated that our NMD inhibitor would allow us to reach the same conclusion in mammalian cells. HeLa cells were transfected with pmCMV-Gl Ter or pmCMV-GPx1 Ter, and the localization of resulting mRNAs was analyzed with a number of P-body markers: GFP-GE1, YFP-hSMG6, YFP-hSMG7, CFP-hDCP1a, GFP-hCCR4, FLAG-hUPF1, hUPF3-FLAG or hUPF3X-FLAG (Fig. 5 and supplemental data S4). As expected, in the absence of the inhibitor we were unable to detect PTC-containing mRNAs, most likely because of their rapid decay by NMD. However, in the presence of NMDI 1, PTC-containing mRNAs were stabilized and detected mainly in cytoplasmic

aggregates. Interestingly, our results indicated a **substantial** colocalization between PTC-containing Gl or GPx1 mRNAs and each tagged version of the tested hUPF proteins, CFP-hDCP1a, YFP-hSMG6 or YFP-hSMG7 (Fig. 5A and supplemental data S4). Although the colocalization between Gl Ter or GPx1 Ter mRNAs and hUPF3/3X was total (Fig. 5 and data not shown for hUPF3X), it was only partial with other P-body components such as FLAG-hUPF1 (63% and 74%, respectively), YFP-hSMG6 (76% and 81%, respectively) or YFP-hSMG7 (71% and 94%, respectively) and infrequently with GFP-hCCR4 (11% and 33%, respectively) (Fig. 5A and supplemental data S4). Notably, we often observed that RNAs foci and P-bodies did not overlap perfectly. Additionally, we were unable to observe a colocalization between PTC-containing mRNAs and GFP-GE1 (Fig. 5A and supplemental data S4). Altogether, our results show that PTC-containing mRNAs were present in and adjacent to P-bodies when NMD was inhibited by NMDI 1. In addition, these data indicate heterogeneity in the composition of P-bodies since some markers colocalize with PTC-containing mRNAs while others do not.

The accumulation of PTC-containing mRNAs in P-bodies when NMD is blocked in mammalian cells was confirmed by a more resolute approach in U2OS cells. In this setting, we tagged the mRNA with a 24 MS2 repeat, since this approach can efficiently detect single mRNA molecules by in situ hybridization (Fusco et al., 2003) (Fig. 5B). In control cells, PTC-containing mRNAs were mostly detected in the nucleus, and the cytoplasmic molecules that were detected, did not accumulate in P-bodies labelled with CFP-hDCP1a. When NMD was inhibited with NMDI 1, higher levels of Gl Ter-MS2 mRNAs were detected in the cytoplasm, and these molecules accumulated in structures that colocalized with P-bodies. As previously observed, mRNAs did not perfectly colocalize with CFP-hDCP1a, but were rather adjacent and formed a ring at the periphery of P-bodies, similar to what was found with miRNA targets (Pillai et al., 2005). These data confirmed that mRNAs subjected to NMD

accumulate in P-bodies when their degradation is inhibited and this conclusion seems not be cell-type specific.

We also analyzed the localization of Gl or GPx1 Norm mRNAs in HeLa and U2OS cells treated with NMDI 1 (Fig. 6). Our results showed no specific accumulation of these mRNAs in cytoplasmic bodies typified by YFP-hSMG6, GFP-GE1 or CFP-hDCP1a in presence of NMDI 1. In contrast, we did not see any wild-type mRNAs in P-bodies either because Norm mRNAs do not go to P-bodies or because their degradation pathway is not affected by either DMSO or NMDI 1. These results support the idea that NMDI 1 is an NMD inhibitor rather than a general RNA decay inhibitor.

Only some steps of the NMD process occur in P-bodies.

In order to determine whether the accumulation of NMD factors in P-bodies can be triggered when any step of the NMD process is inhibited, we decided to interfere with the NMD process in 3 different ways. The first one relies on the down-regulation of hUPF2 using siRNA (Kim et al., 2005) (Fig. 7A). According to the current model of NMD in mammalian cells (Maquat, 2004b), depletion of hUPF2 will block NMD at an earlier step than the one induced by NMDI 1. Interestingly, hUPF2 down-regulation does not induce the accumulation of FLAG-hUPF1, hUPF3-FLAG or hUPF3X-FLAG. Additionally, while hUPF2 depletion induces a stabilization of Gl-Ter mRNA due to the inhibition of NMD, Gl-Ter mRNA was homogenously distributed in the cytoplasm with no accumulation in P-bodies (Fig. 7B). Thus, inhibition of NMD by hUPF2 depletion does not trigger accumulation of NMD factors and substrates in P-bodies.

The second one is based on the down-regulation by siRNA of a protein involved in a late step of the NMD process such as hXRN1 (Cougot et al., 2004) (Fig. 7C). As for the down-regulation of hUPF2, the cellular localization of NMD factors including FLAG-hUPF1

or hUPF3-FLAG was not modified by the cellular lack of the hXRN1 protein (Fig. 7D), whereas, Gl-Ter RNA was detected in P-bodies. This result may indicate that the recycling of NMD factors had already occurred before the function of hXRN1. We conclude that the presence of NMD factors in P-bodies depends on the NMD step, i.e. only some steps in the NMD process occur in P-bodies.

According to our results, hUPF1 dephosphorylation is one of the NMD steps that occur in P-bodies. In the presence of NMDI 1, NMD is inhibited because hUPF1 is stalled in a hyperphosphorylated form due to the release of hSMG5 from P-bodies. To further confirm this model, we aimed to mimic the effect of NMDI 1 by analyzing the cellular localization of NMD factors in the presence of the hUPF1 mutant protein (HA-hUPF1dNT) that has been shown to prevent its interaction with hSMG5 due to the lack of its N-terminal part, or in the presence of hSMG5 mutant proteins (HA-hSMG5dCT and HA-hSMG5DA) that can not dephosphorylate hUPF1 due to either the lack of the C-terminal part or the substitution of the aspartic acid 860 by an alanine (Ohnishi et al., 2003). As shown in Figure 8A, when HeLa cells express HA-hUPF1dNT protein, hUPF3-FLAG, hUPF3X-FLAG proteins, Gl-Ter and GPx1-Ter mRNAs localize to P-bodies as shown by the colocalization with CFP-hDCP1a. This result is similar to what we observed when cells were treated with NMDI 1. Interestingly, YFP-hSMG5 was not detected in P-bodies suggesting that by destabilizing the interaction between hSMG5 and hUPF1, hSMG5 is excluded from P-bodies. Additionally, expression of either HA-SMG5dCT or HA-SMG5DA induced accumulation of FLAG-hUPF1, hUPF3-FLAG, hUPF3X-FLAG proteins, Gl-Ter and GPx1-Ter mRNAs into P-bodies (Figure 8B and C, respectively). Thus, by specifically blocking the dephosphorylation of hUPF1, NMD factors and substrates can be observed into P-bodies.

Discussion

In this study, we have characterized an indole derivative, NMDI 1, as an NMD inhibitor. This molecule has allowed us to study specific steps of NMD. The power of this approach lies in the ability to freeze NMD at a step when hUPF1 and hUPF3/3X are detected in P-bodies (Fig. 3 and 4). Using biochemical and cellular biology approaches, we have determined the precise event blocked by NMDI 1 and established that this inhibitor prevents the interactions between hUPF1 and hSMG5, resulting in the subsequent exclusion of hSMG5 from P-bodies and the stabilization of hyperphosphorylated isoforms of hUPF1. Unlike other approaches, such as transfection-mediated down-regulation of NMD factors, where only a fraction of cells are subject to the inhibition of NMD, small chemical molecules have the ability to diffuse across the cell membrane and affect most cells in culture. Therefore, such inhibitors should enable NMD to be inhibited in more physiological and complex environments such as tissue or multi-cellular organisms in order to study NMD mechanism *in vivo* and to evaluate their potential therapeutic capacities (Kuzmiak and Maquat, 2006).

As in yeast (Sheth and Parker, 2006), PTC-containing mRNAs, hUPF1 and hUPF3/3X proteins are found in P-bodies of mammalian cells when NMD is prevented (Fig. 4, 5 and 8). Undoubtedly, yeast and human P-bodies share some similarities in their protein compositions and functions but clear differences can also be seen. Unlike in yeast, in mammalian cells, PTC-containing mRNAs accumulate in P-bodies, or more precisely at the periphery of P-bodies suggesting that P-bodies can be formed by several compartments. This observation is consistent with recent proposal (Pillai et al., 2005) showing that RNA can be localized at the periphery of P-bodies where it might be stored before to be degraded or to be released from P-bodies. Another difference between yeast and mammalian P-bodies is that a down-regulation of hUPF2 does not lead to the accumulation of hUPF1 into P-bodies (Fig. 7) unlike in yeast (Sheth and Parker, 2006). Our results suggest that hUPF2 is involved in NMD process before

the transit of NMD factors and NMD substrates through P-bodies. These differences likely reflect a divergence in the process of NMD in yeast and in mammalian cells. Surprisingly when we blocked NMD at a late step when RNAs are going to be degraded (i.e. by down-regulating hXRN1) we did not detect NMD factors in P-bodies, but we observed PTC-containing mRNAs in P-bodies (Fig. 7). This confirms that NMD involves trafficking to P-bodies, and suggest that by inhibiting the RNA degradation step, we did not prevent the recycling of NMD factors from P-bodies.

Interestingly, we did not observe any differences in the cellular distribution and in the protein composition between P-bodies containing Gl Ter mRNAs and those containing GPx1 Ter mRNAs, even though these two mRNAs are subject to nucleus-associated or cytoplasmic NMD, respectively. However, we cannot exclude the possibility that NMDI 1 would freeze a series of dynamic events that occurs during NMD, and that the block of these events would induce a drift of nucleus-associated P-bodies to the cytoplasm. It is also possible that P-bodies with nucleus-associated NMD substrates and P-bodies with cytoplasmic NMD substrates have different biochemical or physical properties that would lead to the cosedimentation of one with the nuclear fraction and of the other with the cytoplasmic fraction. Further investigations will be necessary to clarify that point.

NMDI 1 allowed us to show that P-bodies display a large degree of variability in their NMD factors composition. For example, while hSMG6 colocalizes with CFP-hDCP1a or FLAG-hUPF1 when NMD is inhibited (Fig. 3 and data not shown), it only shows a partial overlap with CFP-hDCP1a in DMSO-treated cells (Fig. 3), and no colocalization with LSM4 (Unterholzner and Izaurralde, 2004). As another example, we detected hUPF3/3X-FLAG proteins in P-bodies positive for YFP-hSMG6 or YFP-hSMG7 but not in P-bodies containing GFP-GE1 (Fig. 4). Similarly, PTC-containing mRNAs are found in all P-bodies holding hUPF3/3X-FLAG proteins, in most P-bodies containing FLAG-hUPF1, YFP-hSMG6 or

YFP-hSMG7, but rarely or never in P-bodies stained with GFP-hCCR4 or GFP-GE1 (Fig. 5 and supplemental data S4). Since the factors that do not colocalize upon NMDI 1 treatment, such as hUPF3/3X (or PTC-containing mRNAs), and GE1, still form foci that colocalize with other P-body markers (such as hSMG7), these data suggest that P-bodies can exist in several “flavors” or forms that would differ in protein composition (at least in mammalian cells). These variations in P-body composition could reflect different functional states. In this view, hUPF3/3X, hSMG6, hSMG7, hUPF1 and hDCP1a, would be involved at early steps of RNA processing in P-bodies or as it has been recently proposed would nucleate the formation of P-bodies on the PTC-containing mRNA (Franks and Lykke-Andersen, 2007). hCCR4 would then join the structure, followed by GE1, which would induce degradation of PTC-containing mRNAs and recycling of hUPF3/3X. Then other NMD factors are recycled for a new turn of NMD (Fig. 9). This evolution in P-body composition could arise by fusion of different sub-categories of P-bodies, by the shuttling of individual components, or by a combination of these processes. An attractive approach to answer these questions would be to characterize NMD inhibitors that target other steps than the dephosphorylation of hUPF1.

Materials and methods

All results presented in this article are representative from 3 independent experiments at least.

Chemical molecules library

All the polycyclic indole compounds studied in this article issue from the Institut Curie-CNRS compound library (UMR 176, Institut Curie). Each compound was suspended at 20mg/ml in DMSO and then prepared at a working dilution of 5 μ M in 10% DMSO (v/v). The synthesis and the purification of these compounds have been described previously (Rivallée et al., 1981).

Constructs

β -globin Norm and Ter fused to MS2 binding sites constructs (Fig. 5B and 6B) were obtained by PCR amplification from β -globin WT and NS39 constructs (Thermann et al., 1998) using as sense primer 5'GCAACCTCAAGCTTACACCATGGTGCACCTGAC3' and antisense primer 5'AGAAAGCAGATCTGCTTAGTGATACTTGTG3'. Amplified fragment was cloned in HindIII/BglII of a modified pRSVbgal plasmid containing 24xMS2 sites (Fusco et al., 2003).

NMD measurements by RT-PCR

HeLa cells were cultured in 60mm dishes in Dulbecco's Modified Eagle Medium (DMEM, GIBCO-BRL) supplemented with 10% (v/v) fetal bovine serum at 37°C and 5% CO₂. Cells (1 x 10⁶) were cotransfected with 3 μ g of test plasmids pmCMV-GI (Norm or 39Ter)(Sun et al., 1998) or 3 μ g of test plasmids pmCMV-GPx1 (Norm or 46Ter)(Moriarty et al., 1998) and 1 μ g of reference plasmid phCMV-MUP (Belgrader and Maquat, 1994) using Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer's protocol. 24 hours after transfection, cells were treated for 20 hours with 5 μ M of chemical molecules or DMSO 0.01% (v/v) as a control. Total RNA was purified using TRI Reagent (SIGMA-ALDRICH) and GI, GPx1 and MUP mRNA were reverse transcribed before amplification by PCR in

presence of ^{32}P radiolabeled dCTP. The PCR conditions and analysis method have been previously described (Ishigaki et al., 2001). PCR products were quantified on Typhoon 9200 (Amersham Biosciences).

Luciferase activity for translation efficiency assay

HeLa cells were transfected with 2 μg of pFluc and 1 μg of pRluc. 24 hours after transfection, cells were incubated with DMSO 0.01% (v/v), NMDI 1 (5 μM) for 20 hours or 100 $\mu\text{g}/\text{ml}$ of cycloheximide for 4 hours before to harvest cells. Luciferase activity was quantified on 2×10^5 cell equivalent on microLumat LB 96P (EG&G Berthold) using Dual Glo Luciferase kit (Promega) according to manufacturer's protocol. The luciferase activity was normalized according to the level of Fluc and Rluc mRNAs level measured by RPA (data not shown).

Luciferase activity for miRNA decay pathway integrity

Cells were grown in 6 well plates and transfected with Lipofectamine Plus reagent (Invitrogen) using 50ng of RLperfect RNA reporter (Pillai et al., 2005), 200ng of pG13 plasmid coding for the **Firefly luciferase** and 4 μg of pTzU6 plasmid (Good et al., 1997). 24 hours after transfection, NMDI 1 was added to corresponding wells. 48 hours after transfection, luciferase activity was measured with Dual Glo Luciferase kit (Promega) according to manufacturer's protocol.

Measure of FLAG-hUPF1 phosphorylation level by 2D gel analysis

293T cells (10^6) were transfected with 1 μg of pCI-neo-FLAG-hUpf1 (Sun et al., 1998) using Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer's protocol. After 12 hours, serum was removed from the culture medium for 24 hours before

adding 5 μ M of NMDI 1 or as a control, DMSO 0.01% (v/v) for 3 hours at 37°C and 5% CO₂. Then 10% serum was added back for 1 hour at 37°C and 5% CO₂. Total proteins were purified in lysis buffer containing 8M urea, 2% CHAPS and 40mM Tris base. First dimension was then performed according to the protocol guide from AMERSHAM-BIOSCIENCES for 2D electrophoresis with Immobilized pH Gradient. ImmobilineTM DryStrip pH 3-10, 18cm (GE Healthcare Bio-Sciences AB) was used to separate proteins according to their isoelectric point. Then the second dimension was done by loading the first dimension on a 10% SDS-PAGE. Finally proteins were transferred to a nitrocellulose membrane before incubation with 1 μ g of mouse anti α -FLAG antibody (SIGMA-ALDRICH) in TBS containing 0.05% Tween overnight at 4°C, followed by incubation with a peroxidase-conjugated goat anti-mouse antibody (Pierce). Proteins were then detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Tethering assay

This experiment was carried out as described in (Hosoda et al., 2005).

Immunofluorescence, FISH assays and images analysis

HeLa cells were cultured on 12mm glass cover slips in DMEM 10% (v/v) FBS. Cells (10⁵) were transiently transfected with 500ng of plasmids pGFP-Ge1 (Yu et al., 2005), pYFP-hSmg5, pYFP-hSmg6, pYFP-hSmg7 (Unterholzner and Izaurralde, 2004), pGFP-hCcr4, pCFP-hDcp1a (Cougot et al., 2004), pCI-neo-FLAG-hUpf1 (Sun et al., 1998), pcDNA3-hUpf3-FLAG, pcDNA3-hUpf3X-FLAG (Lykke-Andersen et al., 2000), pmCMV-Gl (Norm or 39Ter) (Sun et al., 1998), pmCMV-GPx1 (Norm or 46Ter) (Moriarty et al., 1998), pHA-hUpf1dNT, pHA-hSmg5dCT or pHA-hSmg5DA (Ohnishi et al., 2003). 24 hours after transfection, cells were treated with 5 μ M of NMDI 1 or DMSO 0.01% (v/v) as control.

Finally 20 hours later cells were fixed using formalin solution (SIGMA-ALDRICH) for 10 min at room temperature and permeabilized in 70% ethanol overnight at 4°C. For immunofluorescence assays, fixed cells were incubated with a mouse anti-FLAG antibody (SIGMA-ALDRICH) for 2 hours at room temperature, washed three times with PBS and then incubated with Cy3-conjugated or FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) for 1 hour at room temperature. Finally, cells were washed three times with PBS and then incubated with Hoechst (2ng/μl) (SIGMA-ALDRICH) for 2 min at room temperature. For FISH experiments, fixed cells were incubated in pre-hybridization buffer (tRNA 125μg/ml, herring DNA 500μg/ml, BSA 1mg/ml, dextran sulfate 0.1g/ml, 50% formamide, 2X Sodium Saline Citrate (SSC) buffer) at 37°C for 1 hour in a tissue culture incubator. Then, fixed cells were incubated overnight in a tissue culture incubator with hybridization buffer (pre-hybridization buffer with Cy3-labeled probes), washed three times in 2X SSC at 37°C, three times in 1X SSC at room temperature, and finally incubated with Hoechst (2ng/μl) (SIGMA-ALDRICH) for 2 minutes at room temperature. Cy3 5'- and 3'-end labeled probe 5'CGATCTGCGTTCTACGGTGTT3' was used to detect Gl mRNA Ter or GPx1 mRNA Ter. For figures 5B and 6B, the MS2 probe sequence was 5'AT*GTCGACCTGCAGACAT*GGGTGATCCTCAT*GTTTTCTAGGCAATT*A3' (where * indicates the internal Cy3 modification).

Fixed cells were observed in VECTASHIELD mounting medium (Vector Laboratories, Inc.) with a DMRA microscope (Leica), a PL APO 63x oil objective (NA 1.32) (Leica) and A4 (for hoechst), GFP and Y3 (for Cy3) filter sets (Leica). Images were captured at 0.3μm intervals along the z-axis using a piezzo stepper (E662 LVPTZ amplifier; Servo) and a cooled CCD Micromax camera (1,300 x 1,030 pixels, RS; Princeton Instruments Inc.) driven by MetaMorph (v 6.2; Universal Imaging Corp.). Pixel sizes were 106 x 106nm, and voxel sizes were 106 x 106 x 100nm. For deconvolution and image reconstruction, xyz image

stacks of fixed cells were processed using Huygens 2.3 (Scientific Volume Imaging b.v.; Hilversum) using a MLE algorithm. Three-dimensional restored stacks were processed with Imaris 4 (Bitplane) for volume rendering and quantification.

Down-regulation of hUPF2 or hXRN1

HeLa cells (10^5 cells) were transfected with 100nM of siRNA hUpf2 (Eurogentec) (Kim et al., 2005) using JetPEI reagent (PolyPlus Transfection) for 48 hours before to harvest cells and analyze the down-regulation efficiency by western-blot or by immunofluorescence.

Down-regulation of hXRN1 has been previously described (Cougot et al., 2004).

Immunopurification and western-blot analysis

hUPF1 immunopurification and western-blot analysis were performed according to the protocol described in (Lejeune and Maquat, 2004) using rabbit anti α -hUPF1 antibody (Ohnishi et al., 2003). Western-blot analyses were performed using 1/250 rabbit anti α -hUPF1, rabbit α -hSMG5, rabbit α -hSMG6 or rabbit α -hSMG7 antibody (Ohnishi et al., 2003), 1/1000 rabbit α -hUPF3/3X (Ishigaki et al., 2001) or 1/1000 mouse anti α -TUBULIN (SIGMA-ALDRICH). For figure 2, Western-blot analysis was done with 1/1000 rabbit anti α -hUPF1, 1/1000 rabbit anti α -hUPF2 and 1/1000 rabbit anti α -hUPF3/3X (Ishigaki et al., 2001). Proteins were detected using SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Online supplemental material

Figure S1 shows that pre-mRNA splicing and mRNA translation are not affected by NMDI 1. Figure S2 confirms the results of Figure 2 by RPA approach and presents the evidence of the accumulation of FLAG-hUPF1 in P-bodies of 293T cells in the presence of NMDI 1 and

serum. Figure S3 shows that endogenous phosphorylated hUpf1, hSmg6 and hSmg7 are detected in P-bodies in the presence of NMDI 1. Figure S4 demonstrates that GPx1-Ter mRNA is present in P-bodies when NMD is blocked by NMDI 1. Finally, Table 1 shows the structures of compounds that have been used in this study.

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Legends

Figure 1: Identification of an NMD inhibitor. A. RT-PCR analysis of Globin and MUP mRNAs. HeLa cells (10^6) were transfected with test and reference plasmids that code for Globin mRNA wild-type (Norm) or harboring a premature termination codon (Ter) and for MUP mRNAs. After transfection, cells were incubated with DMSO (-) or with 5 μ M of a chemical compound for 20 hours. Purified RNA was reverse transcribed in order to serve as a substrate for specific amplification by PCR. The three leftmost lanes correspond to serial 2-fold dilutions of PCR template to ensure that the amplification conditions are quantitative. B. RT-PCR analysis of GPx1 mRNA wild-type (Norm) or harboring a premature termination codon (Ter). Experiment was performed as described in A. The measure of the level of Gl or GPx1 mRNA was normalized with the level of MUP mRNA. The level of each Gl Ter or GPx1 Ter was normalized with the level of the corresponding Gl Norm or GPx1 Norm and is reported as a percentage of Norm (number below each lane). C. Dose-response effect of NMDI 1 on Gl or GPx1-Ter. Hela cells were transfected with pmCMV-Gl-Ter or pmCMV-GPx1-Ter and with phCMV-MUP. 24 hours after, cells were incubated with increasing amount of NMDI 1. NMD was measured by quantitative radiolabeled RT-PCR and confirmed by RPA. D. Measure of Firefly luciferase activity. Cells were transfected with pRLuc and pFluc expression vectors and then treated with DMSO (-), NMDI 1 or cycloheximide (CHX). Firefly luciferase activity was measured by a luminometer and normalized according to the expression level of Firefly luciferase and Renilla luciferase mRNAs. E. NMDI 1 does not inhibit miRNA induced mRNA decay. HeLa cells were transfected with either pRL-3xBulgeMut (RLm) or pRL-Perf (RLp) (Pillai et al., 2005) and incubated for 24h with DMSO (-) or NMDI 1. Histogram represents the ratio Renilla luciferase/ Firefly luciferase. Results

were normalized to RLuc which was set at 10 arbitrary units. Error bar denotes standard deviation. All results are representative of at least three independent experiments.

Figure 2: NMDI 1 inhibits NMD before the functions of hUPF1. A. HeLa cells were transiently transfected with plasmids that encode the Renilla luciferase (Rluc) mRNA, the Firefly luciferase (Fluc) mRNA containing MS2 binding sites in its 3'UTR and mRNA coding for MS2 protein either alone or fused with one of the hUPF proteins. 24 hours after transfection, HeLa cells were incubated either with DMSO (-) or with NMDI 1 for 20 hours. The expression level of each MS2 fusion protein was determined by Western-blot. 10µg of protein extract were loaded on 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane before incubation with antibodies against each of the hUPF proteins. The position of endogenous and exogenous proteins is indicated on the right. B. The level of Fluc mRNA was normalized with the level of the corresponding Rluc mRNA and is reported as a percentage of normalized Fluc mRNA when only MS2 protein was expressed. The three leftmost lanes correspond to serial 2-fold dilutions of PCR template to show that the amplification conditions are quantitative.

Figure 3: NMDI 1 modifies the cellular distribution of hUPF proteins and stabilizes hyperphosphorylated isoforms of FLAG-hUPF1. A. Endogenous hUPF1 protein was immunopurified using rabbit anti-hUPF1 antibodies from HeLa cell extracts that were incubated with DMSO (-) or with 5µM of NMDI 1 (+). In parallel, an immunopurification control was performed using normal rabbit serum (NRS) in order to verify the specificity of the immunopurification protocol. The three leftmost lanes correspond to serial 2-fold dilutions of a whole HeLa cell extract. * Uncharacterized band that represents presumably a degradation product. B. 2 dimensional gel analysis of the FLAG-hUPF1 phosphorylation

level. 293T cells (10^6) were transfected with 1 μ g of pCI-neo-FLAG-hUPF1 plasmid. 12 hours later serum was removed from the culture medium for 24 hours and then either DMSO or 5 μ M of the NMDI 1 was added to the culture medium for 3 hours before adding back 10% serum to the culture medium (sample (-+)) except in the sample ((--)). Proteins were extracted and loaded on a 2D gel analysis system (see materials and methods). C. Immunofluorescence assay. HeLa cells were transfected with pCI-neo-FLAG-hUpf1 and either pYFP-hSmg7, pGFP-Ge1 or pCFP-hDcp1a. 24 hours after transfection cells were incubated with DMSO (-) or 5 μ M of NMDI 1 for 20 hours. The blue staining present in the Merge of the two leftmost set of images correspond to nuclei staining by Hoechst. The white line specifies a 10 μ m length. The white square is a magnification of a cell area. D. Immunofluorescence assay. HeLa cells were transfected with pYFP-hSmg5, pYFP-hSmg6 or pYFP-hSmg7 and with pCFP-hDcp1a expression vectors. 24 hours after transfection, cells were cultured in presence of DMSO (-) or 5 μ M of NMDI 1 for 20 hours. The white line on each microscopy picture specifies a 10 μ m length. The white square on microscopy pictures is a magnification of a cell area.

Figure 4: hUPF3 localizes to P-bodies when NMD is inhibited. HeLa cells were transfected with pcDNA3-hUpf3-FLAG or pcDNA3-hUpf3X-FLAG plasmid and either pGFP-Ge1, pYFP-hSmg6 or pYFP-hSmg7. 24 hours after transfection, cells were incubated either with DMSO (-) or with 5 μ M of NMDI 1 for 20 hours. In Merge, nuclei are visualized by a Hoechst staining in blue. The white line on microscopy picture specifies a 10 μ m length. The white square is a magnification of a cell area.

Figure 5: PTC-containing mRNAs accumulate in P-Bodies under conditions of NMD inhibition. A. HeLa cells were transfected with pmCMV-GI Ter and an expression vector encoding a P-body component: pCI-neo-FLAG-hUpf1, pYFP-hSmg6, pYFP-hSmg7, pcDNA3-hUpf3-FLAG, pGFP-Ge1 or pGFP-hCcr4. 24 hours after transfection, DMSO (-) or 5 μ M NMDI 1 was added to the culture medium for 20 hours. B. U2OS cells were transfected with pGI Ter-MS2 plasmid and with pCFP-hDcp1a expression vector. These cells were used for their properties to highly express transfected genes which is crucial for this approach. Cells were treated as described in A. Nuclei are shown in Merge by Hoechst staining in blue. The white line on microscopy picture specifies a 10 μ m length. The white square is a magnification of a cell area.

Figure 6: WT mRNAs do not accumulate in P-bodies when NMD is inhibited by NMDI 1. A. HeLa cells were transfected with pmCMV-GI Norm and an expression vector encoding a P-body component: pYFP-hSmg6 or pGFP-Ge1. Cells were submitted to the same treatment as in Figure 5A. B. Same as in Figure 5B except that cells were transfected with pGI Norm-MS2 plasmid rather than pGI Ter-MS2. The white line on microscopy picture specifies a 10 μ m length. The white square is a magnification of a cell area.

Figure 7: Down-regulation of hUPF2 or hXRN1 does not lead to the accumulation of NMD factors in P-bodies. The efficiency of the hUPF2 (A) or hXRN1 (C) down-regulation was evaluated by western-blot from 10 μ g of total protein. The three leftmost lanes represent 2 times dilutions of total extract from HeLa cells that were not transfected. (B) HeLa cells were transfected with pCFP-hDcp1a and either pCI-neo-FLAG-hUPF1, pcDNA3-hUPF3-FLAG, pcDNA3-hUPF3X-FLAG or pmCMV-GI-Ter in the presence of siRNA luciferase (Luc) or siRNA hUPF2 (Upf2). (D) Same as (B) except siRNA hXRN1 replaced siRNA hUPF2 and

pcDNA3-hUPF3X-FLAG was not tested. The white line on microscopy picture specifies a 10 μ m length. The white square is a magnification of a cell area.

Figure 8: NMDI 1 effect on the localization of NMD factors and substrates can be reproduced by using hUPF1 or hSMG5 mutants that prevent the phosphorylation of hUPF1. HeLa cells were transfected with pCFP-hDcp1a and pHA-hUpf1dNT (A), pHA-hSmg5dCT (B) or pHA-hSmg5DA (C), and either pCI-neo-FLAG-hUpf1, pcDNA3-hUpf3-FLAG, pcDNA3-hUpf3X-FLAG, pmCMV-GI-Ter, pmCMV-GPx1-Ter, pYFP-hSmg5 (A) or pCI-neo-FLAG-hUpf1 (B and C). The white line on microscopy picture specifies a 10 μ m length. The white square is a magnification of a cell area.

Figure 9: Model of P-body composition dynamics. At the early stage, phosphorylated hUPF1, hUPF3/3X, hSMG5, hSMG6, hSMG7 and PTC-containing mRNAs transit to P-bodies (1). hCCR4 accumulates later (2) followed by GE1 which induces the degradation of PTC-containing mRNAs and the recycling of hUPF3/3X (3). Finally, others NMD factors will be recycled as in particular hUPF1.

Supplemental Data

Figure S1: NMDI 1 is a specific NMD inhibitor. (A) Measure of NMD inhibition by NMDI 1 using RPA. 3 μ g of total RNAs from cells treated with DMSO (-), 5 μ M of compound 17 (17) or 5 μ M of NMDI 1 were analyzed by RPA and loaded on 6% denaturing gel. The levels of GI pre-mRNA and mRNA either wild-type (Norm) or harboring a premature termination codon

(Ter), and MUP mRNA are measured. (B) Analysis of GPx1 pre-mRNA and mRNA either wild-type (Norm) or harboring a premature termination codon (Ter), and MUP mRNA. Each probes lanes represent 10% of the radioactivity loaded in the sample lanes. The quantification of the radioactivity was performed on Typhoon 9200 (Amersham Biosciences) and indicated below each lane. The level of Gl or GPx1 pre-mRNA was measured and compared to the level of the corresponding Norm pre-mRNA in cells treated with DMSO in order to show that NMDI 1 does not alter pre-mRNA splicing. The difference of migration between the free probes and the pre-mRNA species for Gl and GPx1 or mRNA species for MUP is due to the digestion of the non target region that was transcribed. This experiment is representative of 3 independent experiments. (C) NMDI 1 does not interfere with translation. HeLa cells (5×10^6) were incubated for 20 hours with 10% DMSO as a control, with $5 \mu\text{M}$ of NMDI 1 or with $100 \mu\text{g/ml}$ of cycloheximide (for 4 hours) in DMEM (GIBCO-BRL) containing 10% (v/v) fetal bovine serum (FBS). 30 minutes before ^{35}S -methionine pulse, cells are cultured in DMEM supplemented with 10% (v/v) FBS without methionine and cysteine (GIBCO-BRL). Then, cells were incubated 10 minutes with ^{35}S -methionine ($50 \mu\text{Ci/ml}$ of medium; Amersham). After 4 washes with PBS, cells were harvested in RIPA buffer (50mM TRIS-HCl pH 8, 150mM NaCl, SDS 0,1% (w/v), sodium deoxycholate 0,5% (w/v), NP40 1%, 1mM PMSF). An acetone precipitation was performed, total proteins were quantified with BCA Protein Assay Reagent (PIERCE) and incorporated radioactivity was measured in Liquid Scintillation analyser Tri-Carb 2800TR (Perkin Elmer). (D) NMDI 1 does not induce stress granules formation. HeLa cells were treated either with DMSO, $5 \mu\text{M}$ of NMDI 1 for 20 hours or 0.5mM sodium arsenite for 1 hour. Nuclei are detected by Hoechst staining in blue.

Material and method:

Total RNA (3μg) were subject to RPA using RPA IIITM Ribonuclease Protection Assay (Ambion) according to the manufacturer protocol. Probes were synthesized *in vitro* using T7 RNA polymerase (New England BIOLABS) in presence of ³²P radiolabeled GTP or UTP. PCR fragments that contain T7 promoter and antisense region of Gl, or GPx1 pre-mRNA or MUP mRNA, were used as templates. 5'ACCACCGTAGAACGCAGATCG3' and 5'TAATACGACTCACTATAGGGTTGGTCTCCTTAAACCTGTC3' primers were used to synthesize Gl probe. GPx1 probe was amplified with 5'ACCACCGTAGAACGCAGATCG3' and 5'TAATACGACTCACTATAGGGGTACCAGAGGGAAAGTAAGCG3' primers. Finally MUP probe was obtained using 5'CTGATGGGGCTCTATG3' and 5'TAATACGACTCACTATAGGGTCCTGGTGAGAAGTCTCC3' primers.

Figure S2: NMDI 1 inhibits NMD after the recruitment of hUPF2 and before the functions of hUPF1. (A) The same RNA samples as used for the figure 2B have been analyzed by RNA protection assay using RPA IIITM Ribonuclease Protection Assay (Ambion) according to the manufacturer procedure. Each probes lanes represent 10% of the radioactivity loaded in the sample lanes. Probes were synthesized as described for Figure S1 using 5'ATGACTTCGAAAGTTTATG3' and 5'TAATACGACTCACTATAGGGTTCAGATTTGATCAACGCA3' primers for Rluc probe, and 5'AAGGCCCGGGCGCCATTCTATCC 3' and 5'TAATACGACTCACTATAGGGTTCACGTTTATTATAAATGTGCG3' for the Fluc probe. (B) NMDI 1 induces concentration of FLAG-hUPF1 in P-bodies in 293T cells. 293T cells were transfected with 1μg of pFLAG-hUpf1 and treated as described in Figure 3B. 48 hours after transfection, cells were fixed in formalin solution as described in Material and Methods. FLAG-hUPF1 was immunodetected using anti-FLAG antibody (SIGMA-ALDRICH) and

hXRN1 was detected using anti-hXRN1 antibody (gift from Dr. Jens Lykke-Andersen).

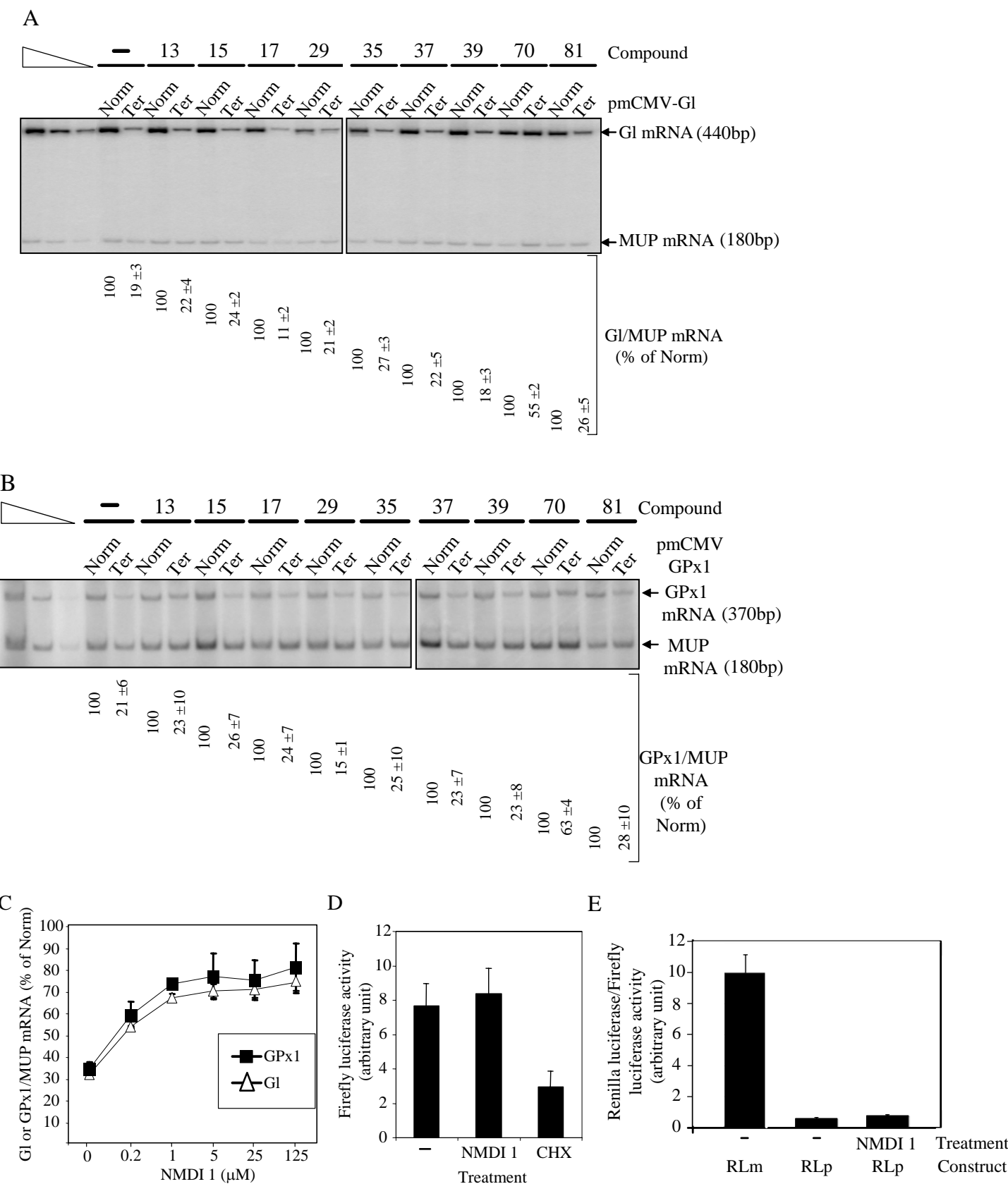
Nuclei are detected by Hoechst staining in blue. White arrow indicates the position of a P-body.

Figure S3: Endogenous phosphorylated hUpf1, hSmg6 and hSmg7 are detected in P-bodies under NMD inhibition. HeLa cells (10^5) were transfected with 100ng of pCFP-hDcp1a as a P-bodies marker. After 24 hours, cells were incubated either with DMSO (-) or with 5 μ M of NMDI 1 for 20 hours. Then cells were fixed in formalin solution as described in Material and Methods. The immunofluorescence assay was carried out as described in Material and Method excepted that cells were incubated with anti-phosphorylated hUpf1, anti-hSmg5, anti-hSmg6 or anti-hSmg7 antibodies (Ohnishi et al., 2003). The white square is a magnification of a cell area. The white line represents a 10 μ m length.

Ohnishi, T., Yamashita, A., Kashima, I., Schell, T., Anders, K. R., Grimson, A., Hachiya, T., Hentze, M. W., Anderson, P., and Ohno, S. (2003). Phosphorylation of hUPF1 induces formation of mRNA surveillance complexes containing hSMG-5 and hSMG-7. *Mol Cell* 12, 1187-1200.

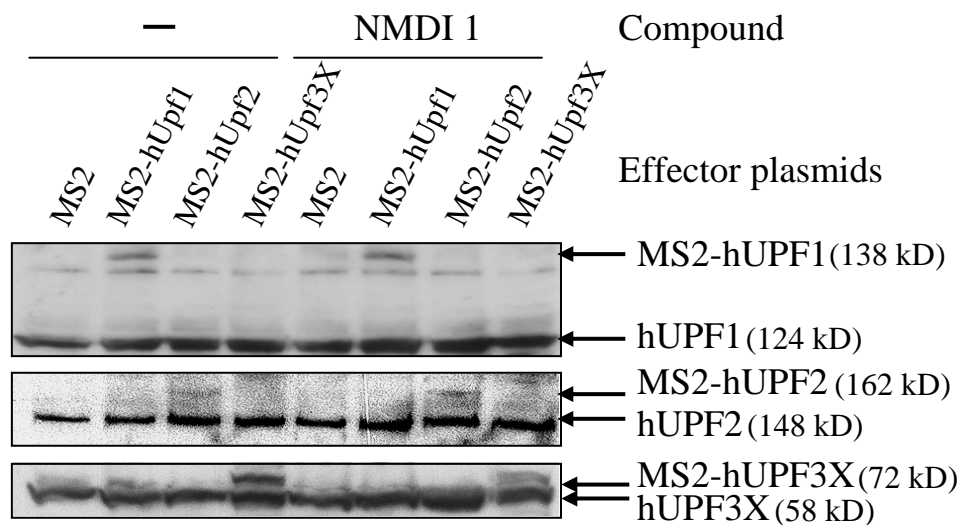
Figure S4: PTC-containing mRNAs accumulate in P-Bodies when NMD is blocked by NMDI 1. HeLa cells (10^5) were transfected with pmCMV-GPx1 Ter and one of the expression vectors for P-bodies components: pCI-neo-FLAG-hUpf1, pYFP-hSmg6, pYFP-hSmg7, pcDNA3-hUpf3-FLAG, pGFP-Ge1 or pGFP-hCcr4. 24 hours after transfection, DMSO (-) or 5 μ M NMDI 1 was added to the culture medium for 20 hours before fixing cells in formalin solution. Nuclei are shown in Merge by Hoechst staining in blue. The white square shows a magnification of cell details. The white line represents a 10 μ m length.

Table1: Structures of chemical compounds shown in figure 1.

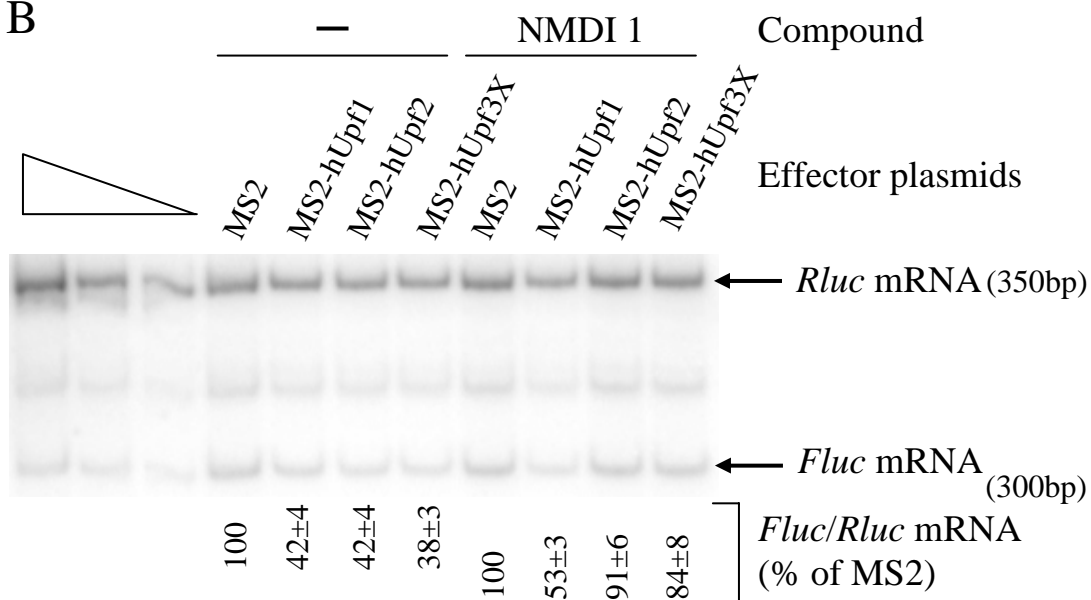


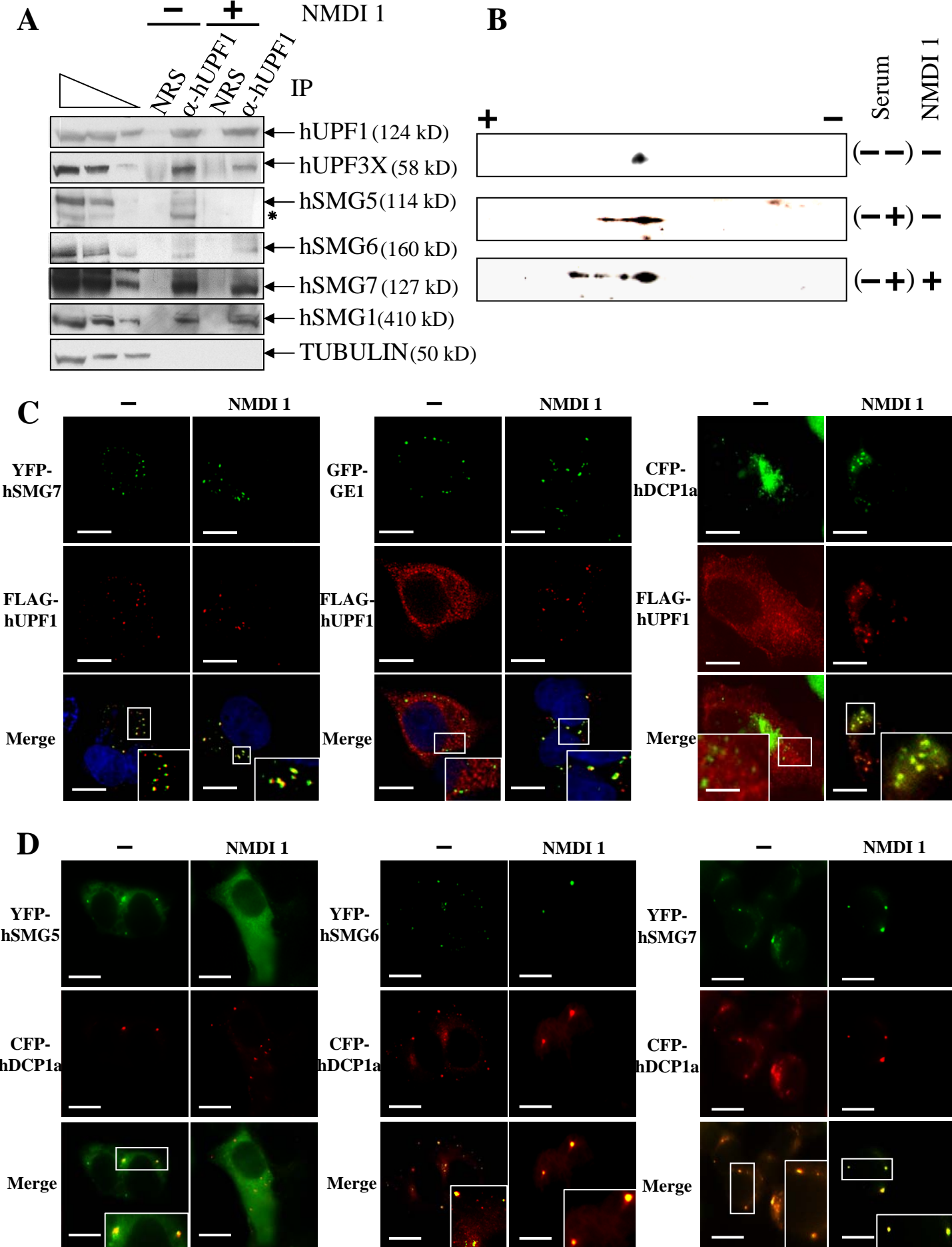
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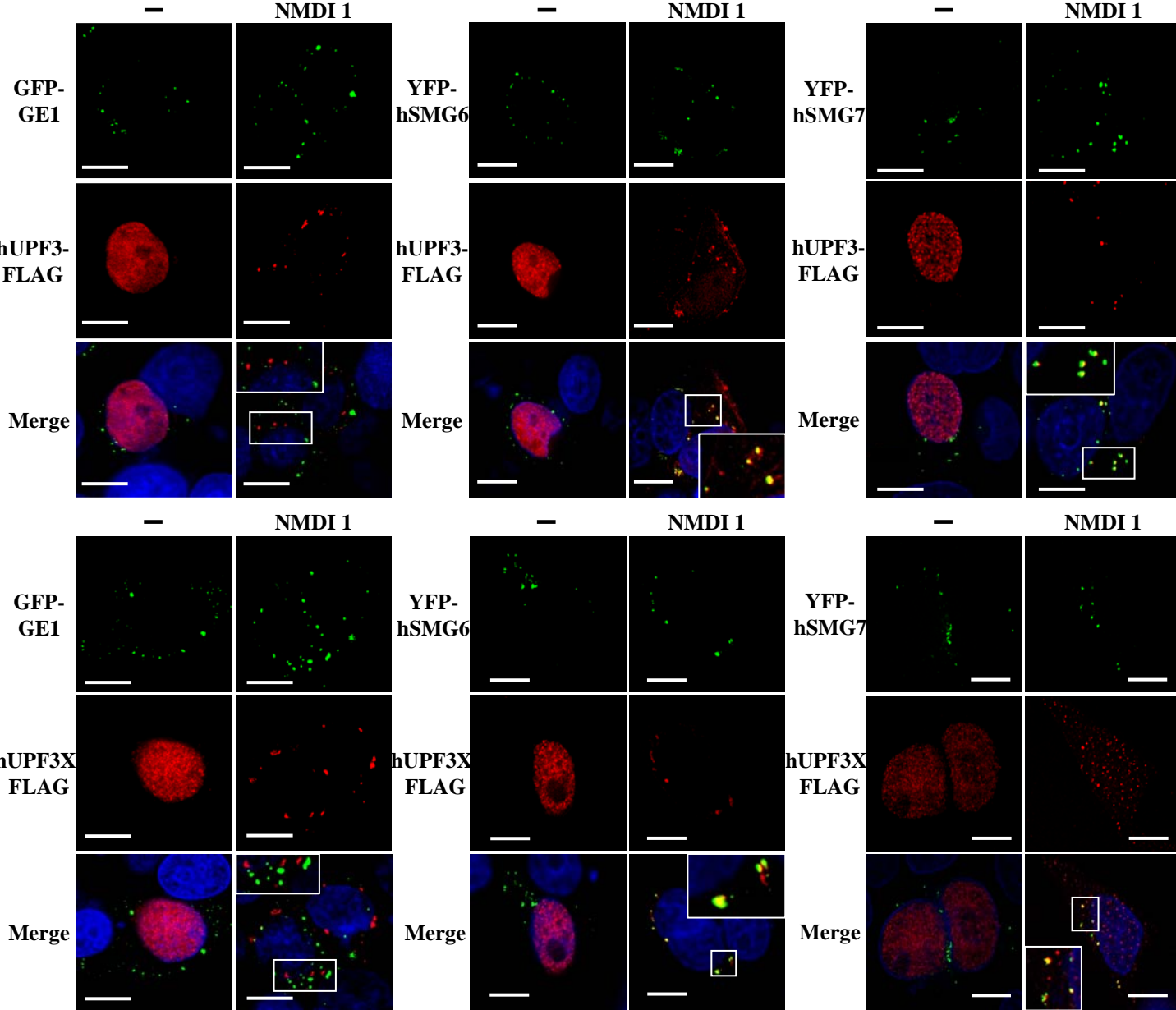
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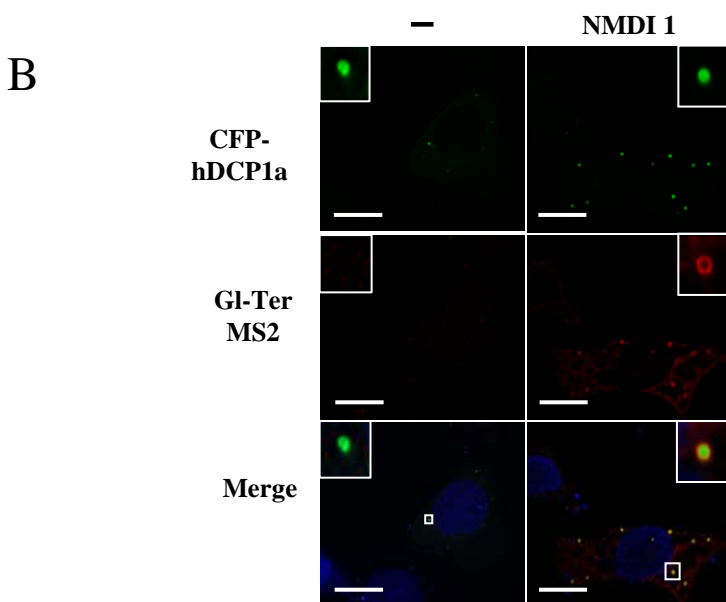
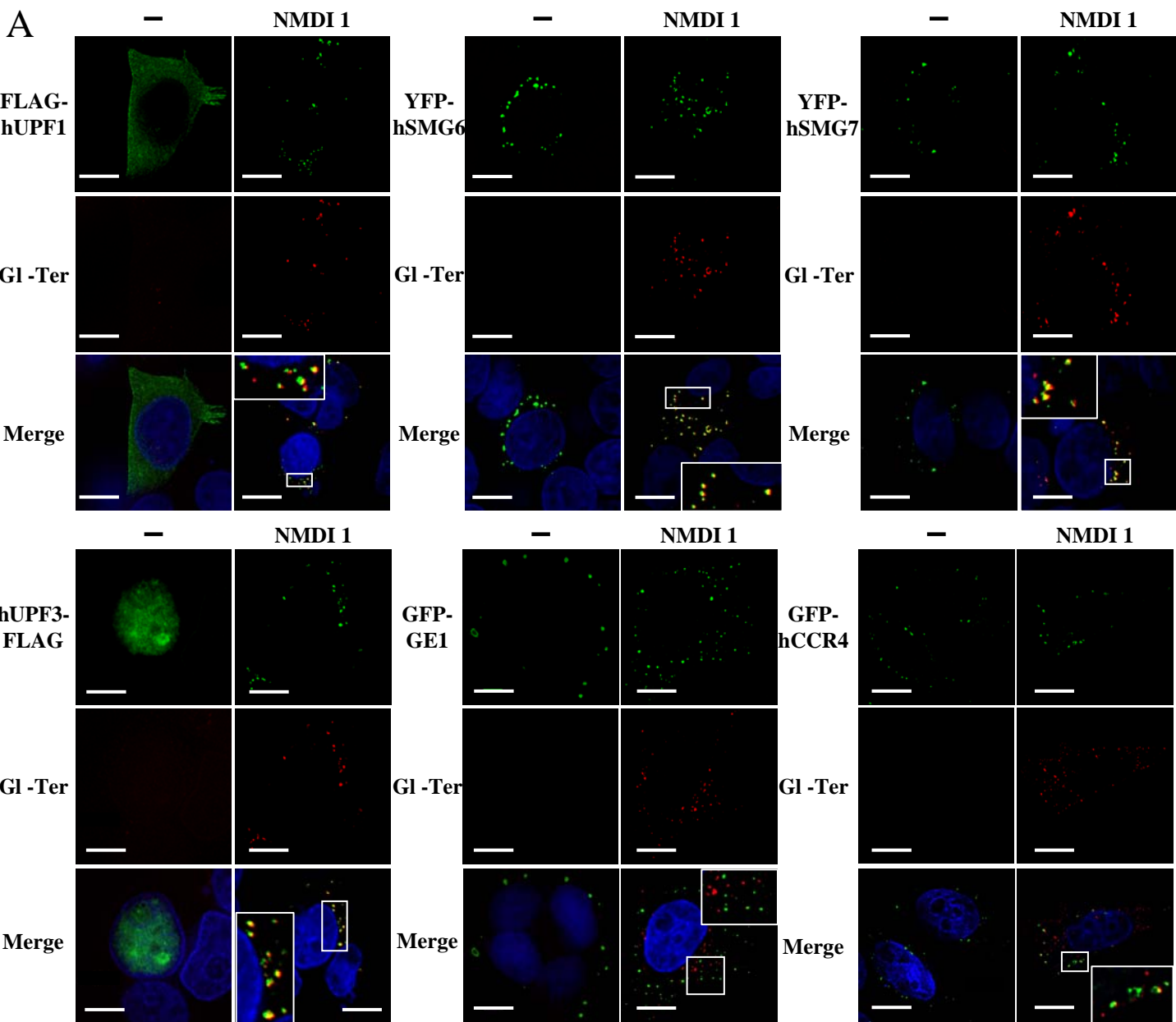


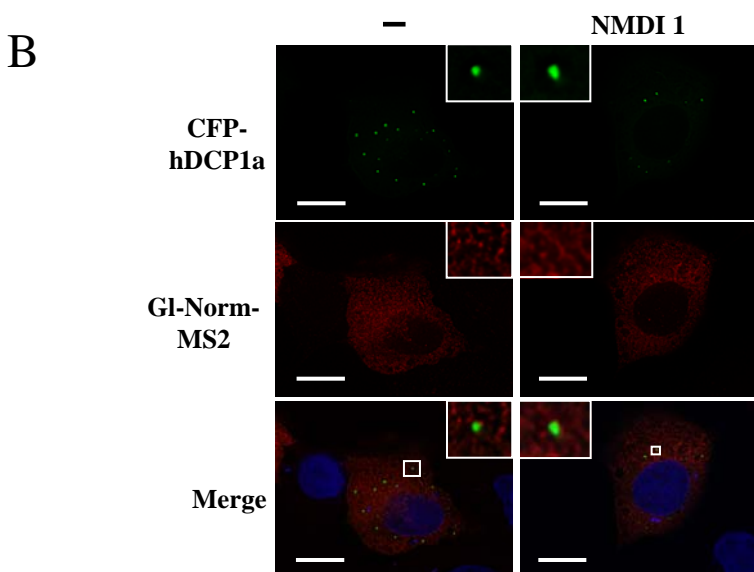
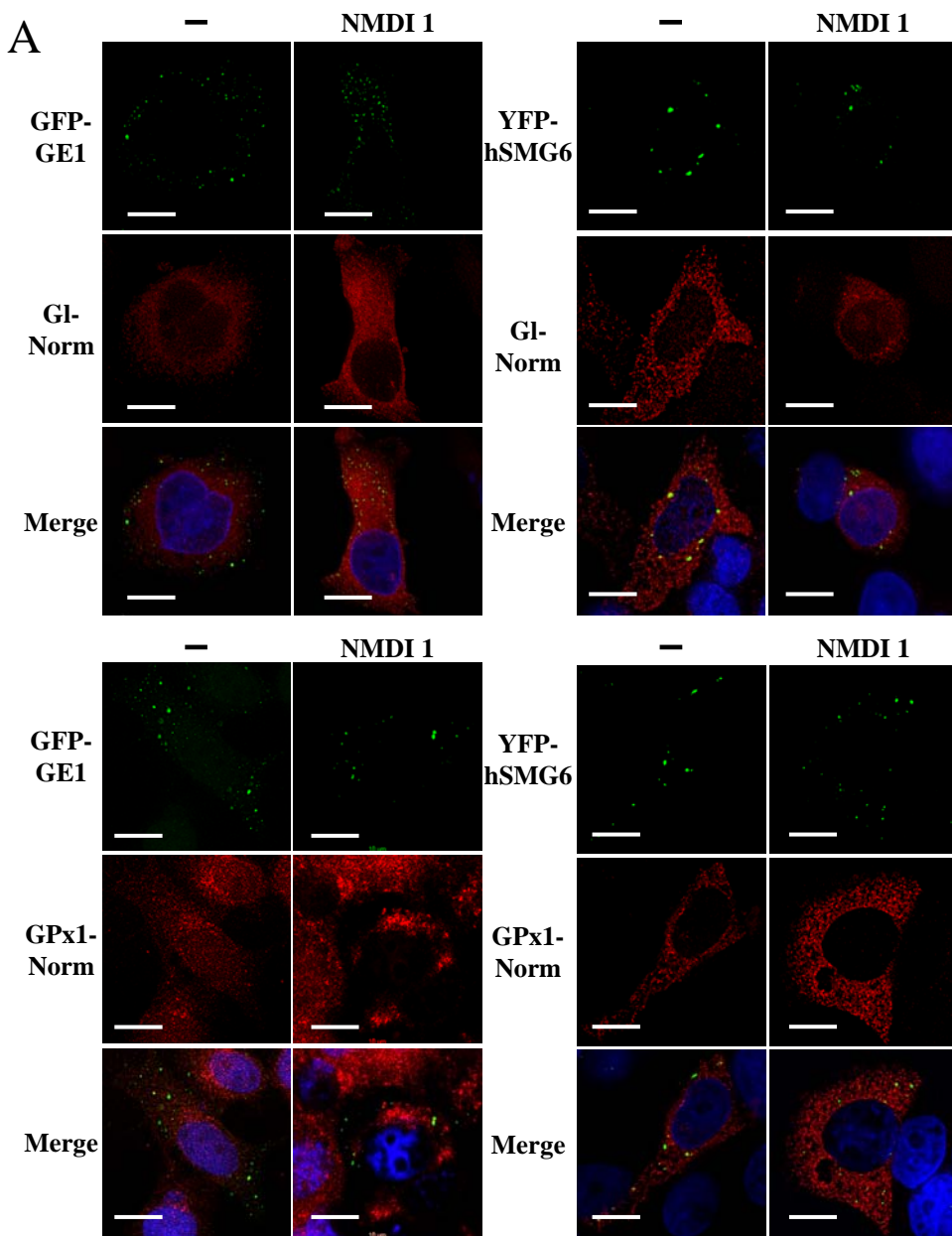
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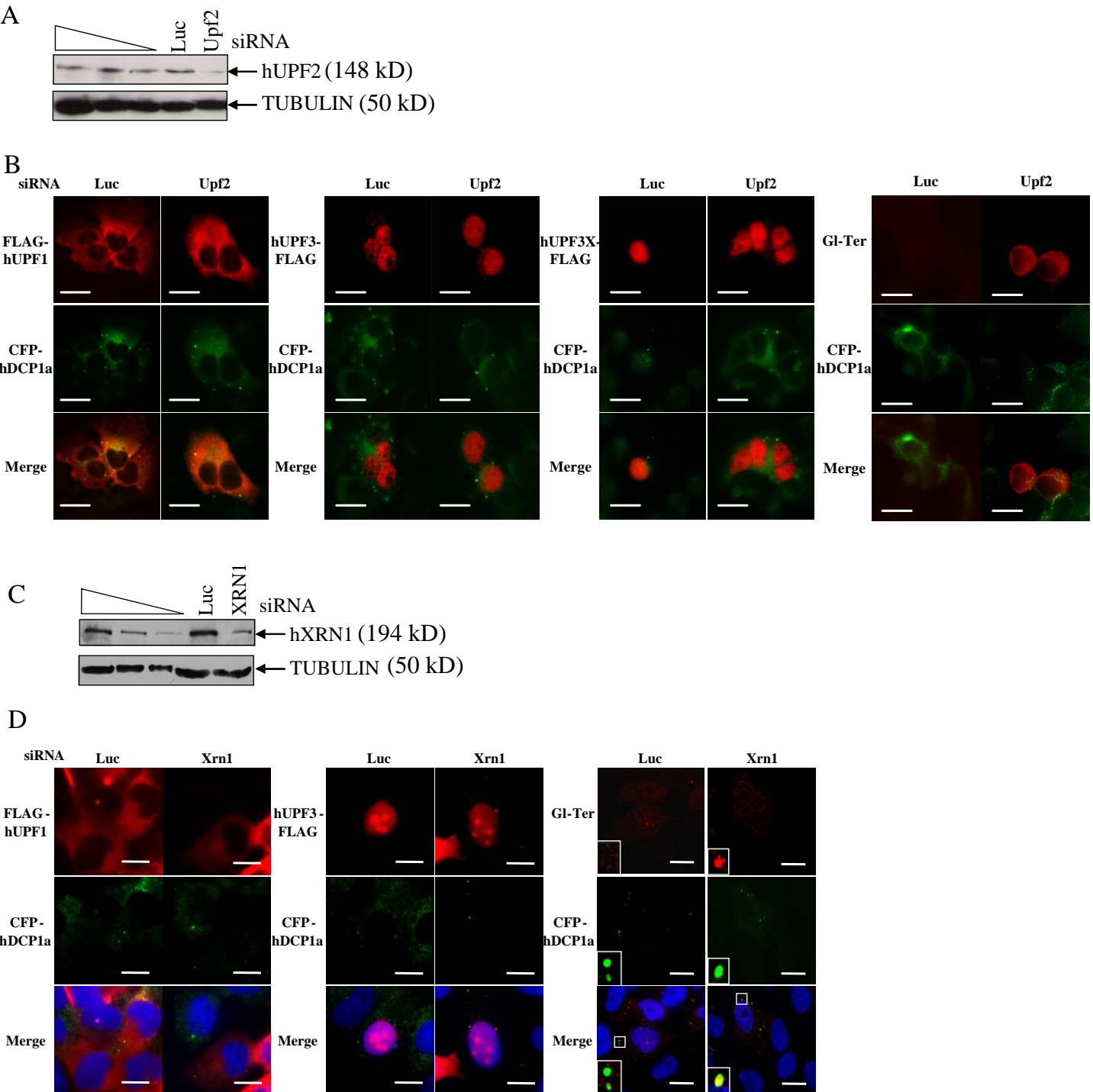




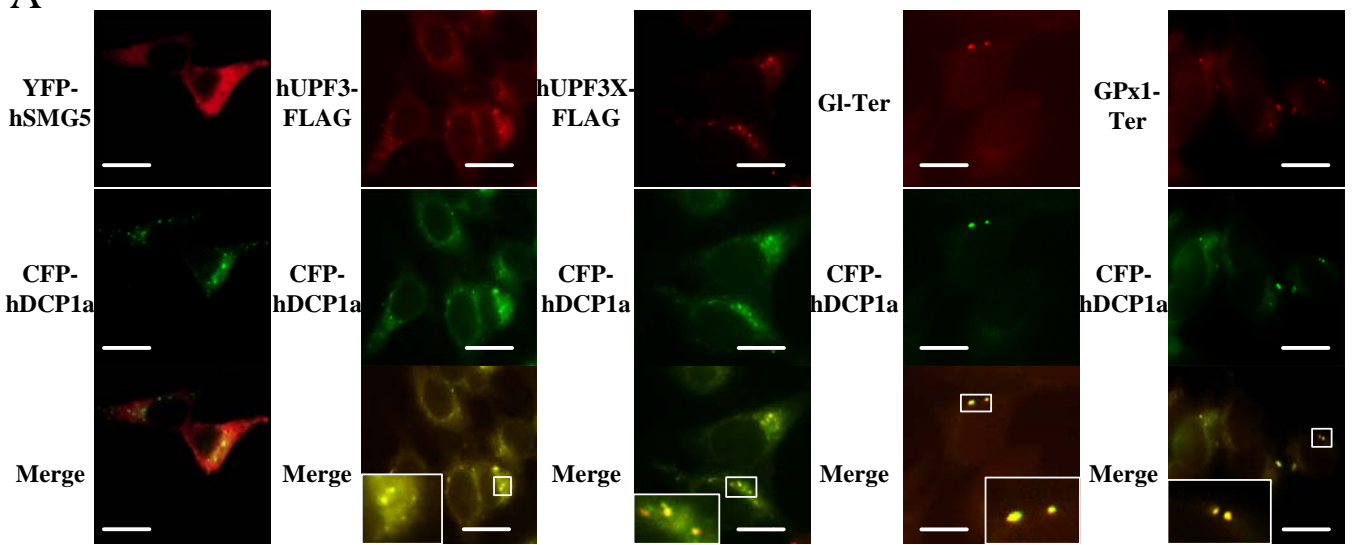




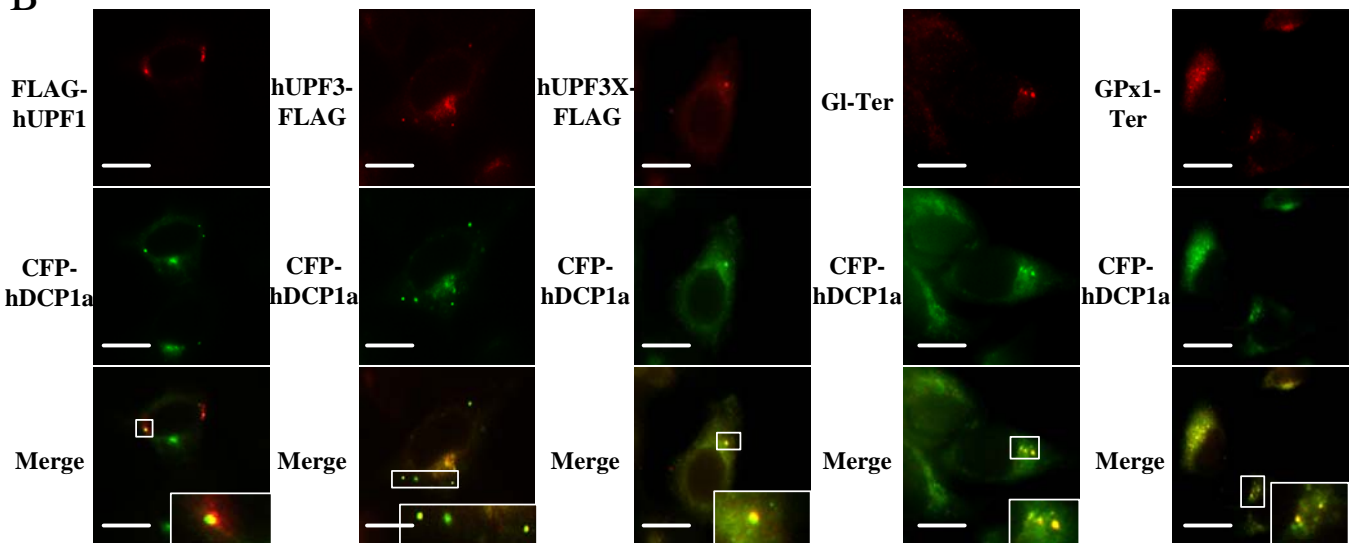




A



B



C

