Supporting Information

Title: The NBDY microprotein regulates cellular RNA decapping

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Figure S1| An anti-NBDY antibody is specific and reveals co-localization of endogenous NBDY with a decapping protein in P-bodies. (a) Detection of endogenous NBDY by immunofluorescence (additional field of view). Fixed HEK 293T cells were stained with antibodies detecting NBDY or DCP1A. Scale bars, 10 μm. (b) Analysis of *NBDY* knockout (KO) HEK 293T cell lines by Western blot using an anti-NBDY antibody, with comparison to wild type HEK 293T cells.

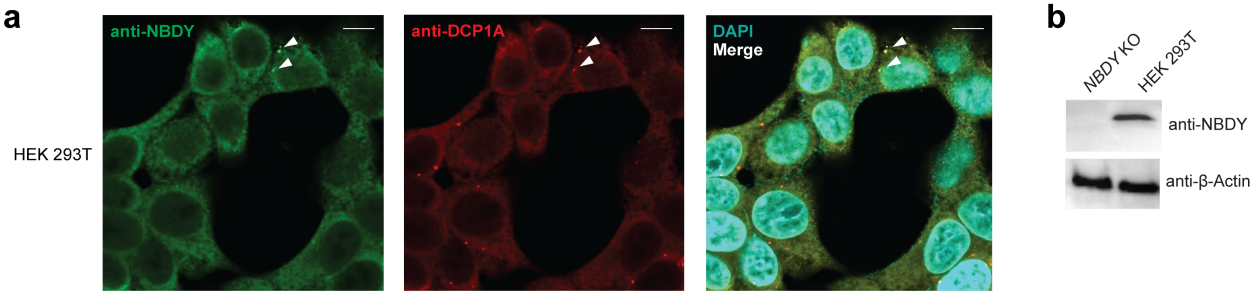


Figure S2| NBDY interacts with DCP1A via a C-terminal polyproline motif. A pulldown assay showing the direct interaction of GST-DCP1A-EVH1 with recombinant His_6-tagged NBDY constructs.

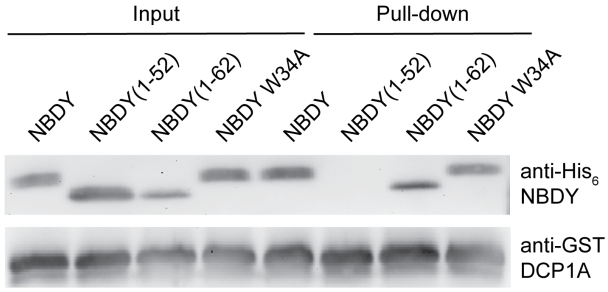


Figure S3| NBDY knockout causes secondary changes in RNA synthesis. (a,b) Top significant biological process GO terms of genes downregulated (a) or upregulated (b) by RNA synthesis in *NBDY* KO versus WT HEK 293T cells. Fisher's exact test was performed using PANTHER overrepresentation test with FDR<0.05. (c) The mean fold changes in RNA decay rate (red) vs synthesis rate (blue) for genes encoding RNA decay machineries that are exclusively downregulated in *NBDY* KO vs WT HEK 293T cells but not in *DCP2* KO.

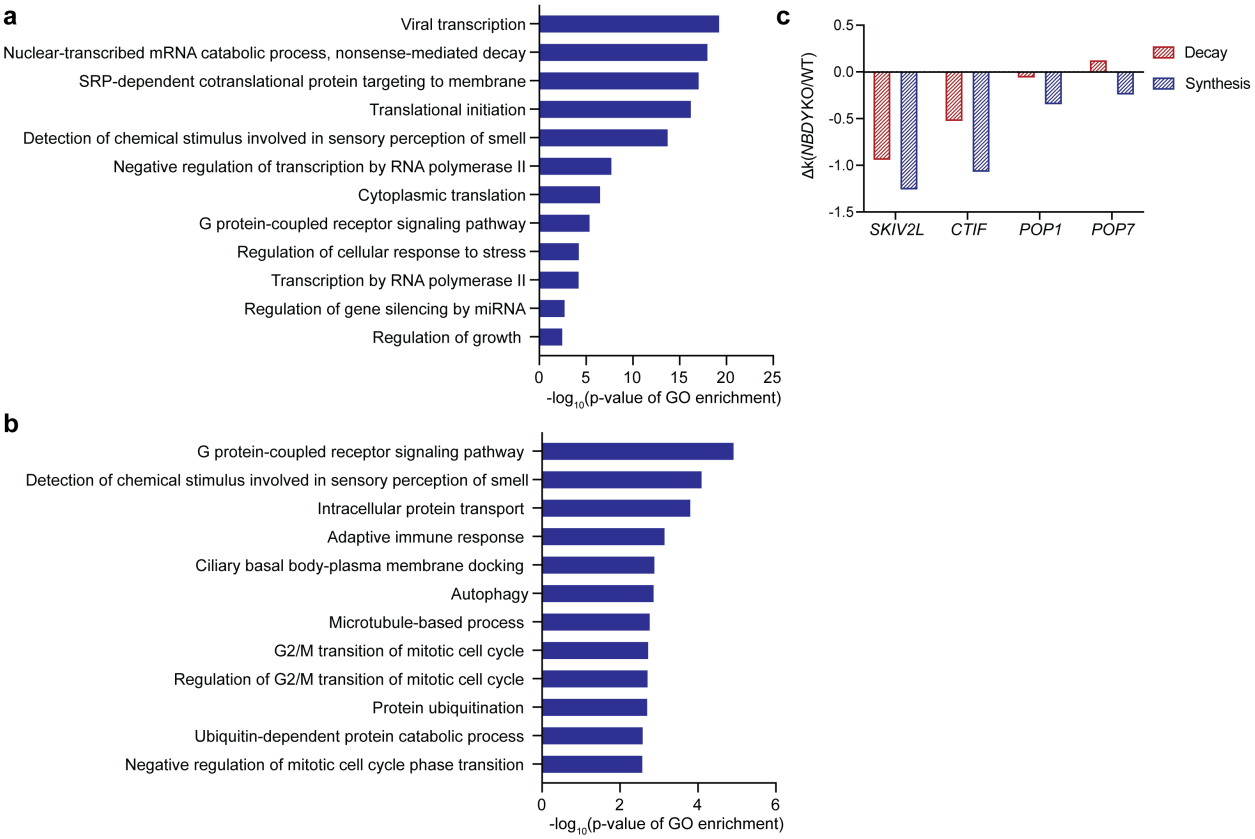


Figure S4| NBDY regulates RNA decay. (a) RNA stability measurement of selected genes belonging to the following classes: stabilized in both *DCP2* KO and *NBDY* KO (*LSM12*) and destabilized in both *DCP2* KO and *NBDY* KO (*MRE11*). Number of biological replicates: n=3. Error bars represent mean ± s.d. Significance was analyzed by ANOVA linear regression. **P < 0.01; ***P < 0.001, Dunnett's test. (b) Western blot confirmation of *NBDY/XRN1* double knockout (DKO) cells.

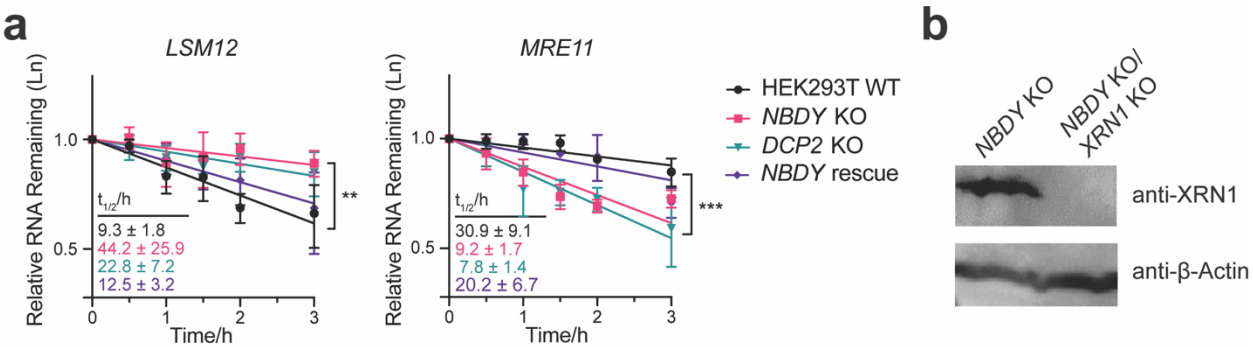


Figure S5| NBDY status does not affect the activity of the RNA decapping complex *in vitro*. (a) Silver stain of the decapping complex immunopurified from WT HEK 293T and *NBDY* KO cells. (b) Analysis of immunopurified decapping complex components by Western blot. (c) *In vitro* decapping assay with decapping complex immunopurified from *NBDY* KO or WT HEK 293T cells. EDTA inhibition served as a negative control.

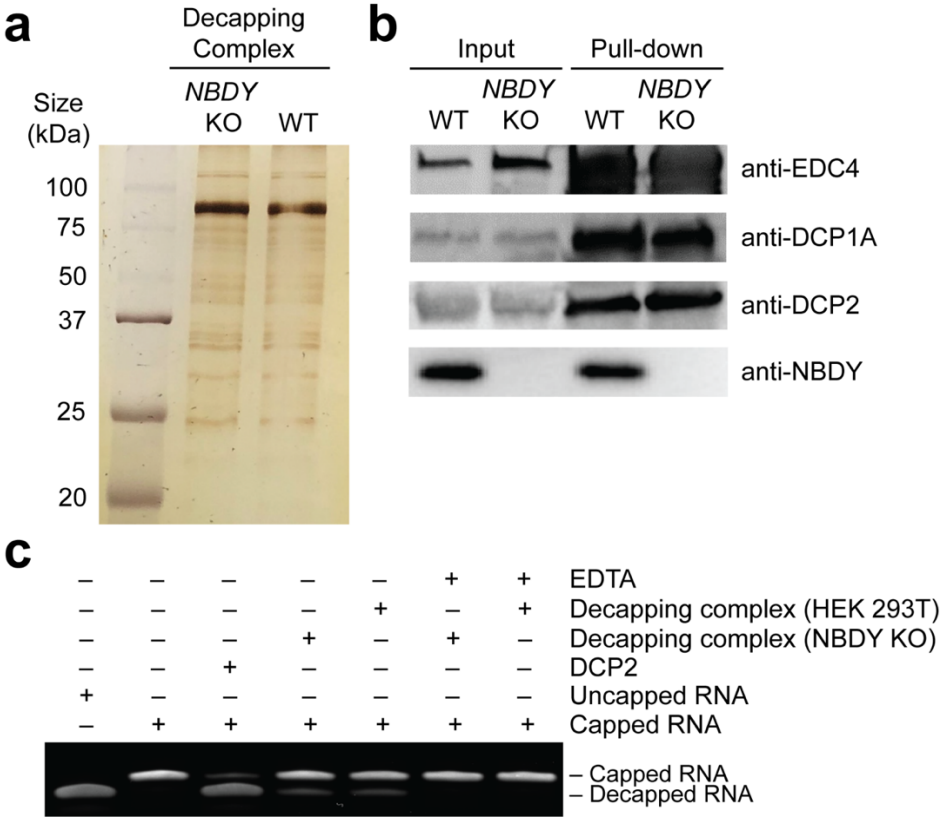


Figure S6| NBDY interaction with DCP1A does not affect stability of a specific endogenous reporter transcript. RNA life time of a DCP2 substrate, *RRP41*, was measured in NBDY rescue and NBDY (1-52) truncated construct (non-DCP1A-interacting) complementation cell line. Number of biological replicates: *n*=3. Error bars represent mean ± s.d. Significance was evaluated by linear regression *t*-test; ns, not significant.

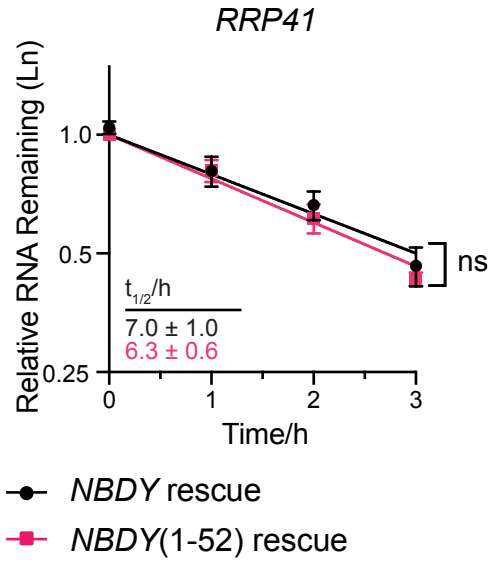
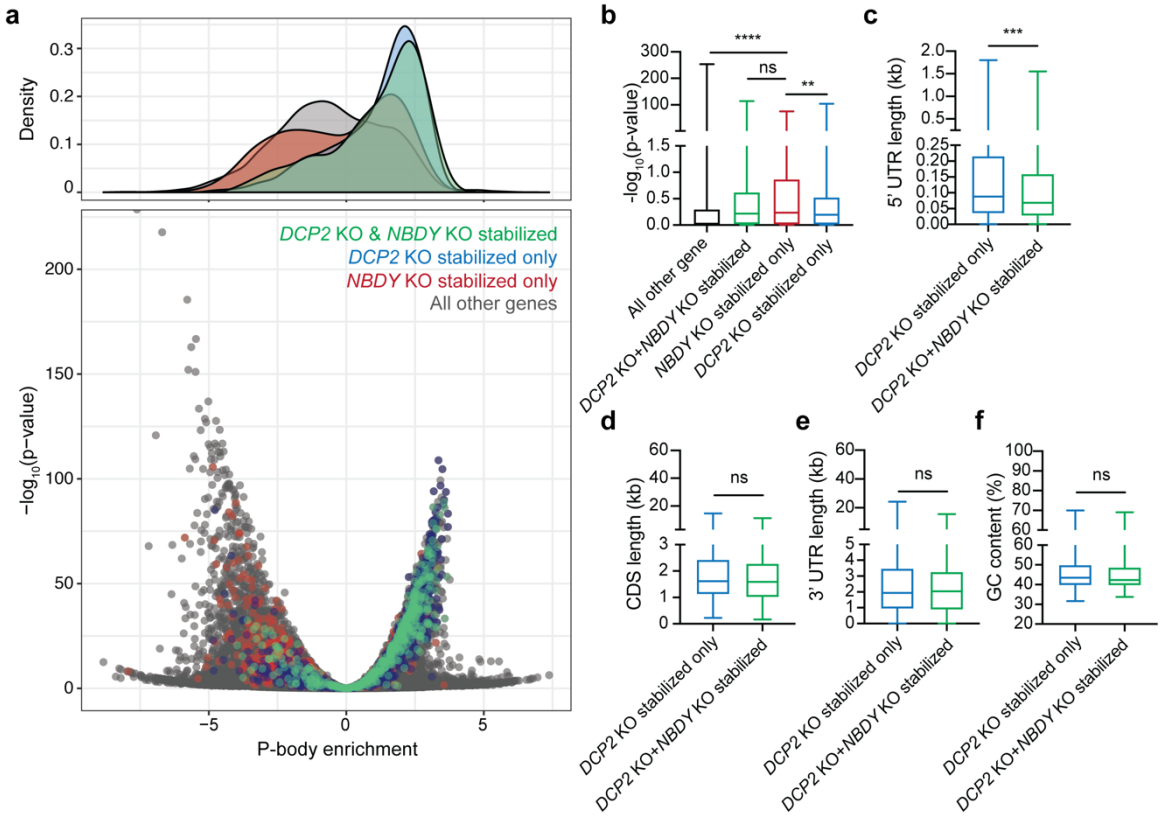


Figure S7| Physical basis of differential regulation of DCP2 substrates by NBDY. (a) P-body enrichment based on data from Hubstenberger *et al*. for each of the described classes of RNA stability changes in *DCP2* KO or *NBDY* KO vs WT HEK 293T cells. (b) Box plot depicting sum of p-values of NMD targeting based on data from Colombo et *al*. for indicated classes of RNA stability changes in *DCP2* KO or *NBDY* KO vs WT HEK 293T cells. Significance was evaluated by Mann Whitney *U* test; ns, not significant; **P<0.01; ****P<0.0001. (c-f) Box plots representing 5´ UTR length (c), coding sequence length (d), 3´ UTR length (e) and GC content (f) for RNAs stabilized in both *DCP2* KO or *NBDY* KO vs WT HEK 293T cells or that exclusively in *DCP2* KO vs WT HEK 293T cells. Data were obtained from ENSEMBL (version 101). Significance was evaluated by Mann Whitney *U* test; ns, not significant; ***P<0.001.



Gene/Primer Name	Primer Sequences
NBDY Fwd/Rev	5' GGAGAAAACTGACGACCCGTTTCTGT 3' 5' TCTCTACTTCTCCGGAGGAGGAGGG 3'
DCP2 Fwd/Rev	5' GCATGAGTCAGTTCCACATCATTGA 3' 5' CAGACAGAAGATGACTATCCCAATCA 3'
XRN1 Fwd/Rev	5' CACTTTTCCCTGCTGCTTAAGAT 3' 5' ATTTCTGGGGGAGTTTACGC 3'

Table S1.

Gene specific PCR primers used in this study.

Gene/Primer Name	Primer Sequences
MRE11 Fwd/Rev	5' ATGCAGTCAGAGGAAATGATACG 3' 5' CAGGCCGATCACCCATACAAT 3'
ATM Fwd/Rev	5' ATCTGCTGCCGTCAACTAGAA 3' 5' GATCTCGAATCAGGCGCTTAAA 3'
ZNF84 Fwd/Rev	5' AGCAGCCTAGTGTCACTGG 3' 5' TGCCACATCATGTTACCATCTAC 3'
GJC1 Fwd/Rev	5' AGCTGTAGGAGGAGAATCCATC 3' 5' TGCAAACGCATCATAACAGACA 3'
EPC2 Fwd-2/Rev-2	5' GGGAGACAATGAGTAAACTCTCC 3' 5' GACGCAGTCGTTGAGATCAG 3'
EPC2 Splint	5' CCAACATGGCGGACATTACCCATCAAAGCCAGCAAACGCAGTGTTCATTC 3'
Anchor Fwd	5' GCTGATGGCGATGAATGAACACTGC 3'

Table S2.

qRT-PCR and qSL-RT-PCR primers used in this study.