Supplemental Materials

Molecular Biology of the Cell

Gasperini et al.

GASPERINI ET AL. SUPPLEMENTAL MATERIAL

SUPPLEMENTARY FIGURE S1. ***ALS-linked FUS mutant intracellular localization and stress granule (SG) recruitment in HeLa cells.*** **(A)** Immunofluorescence images of HeLa cells transfected with WT and mutant FUS-HA showing the cytoplasmic shift of P525L (arrowheads). Cells were stained with DAPI to mark the nucleus and CellMask^{TM} Deep Red plasma membrane stain to mark the cytoplasm. Scale bar=40 μm. **(B)** The graphs report the quantification of FUS-HA nucleus/cytoplasm signal, obtained by high-content image analysis, and confirm the cytoplasmic de-localization of FUS mutants. The analysis was performed on untreated (left panel) and arsenite-treated cells (right panel). (**C**) The graph reports FUS-HA spot number per cell induced by arsenite treatment, analyzed by high-content image assay. The results confirm that FUS mutants are more recruited to SGs compared to WT. In (**B**) and (**C**), bars indicate means ± SEM of 5 replicates, and p-values were calculated with unpaired two-tailed Student's t-test to compare RALY KO to control cells (*p<0.05; **p<0.01; ***p<0.001).

SUPPLEMENTARY FIGURE S2. ***Nuclear translocation of wild-type and mutant FUS is increased in RALY silenced cells.*** (**A**) The graphs report the quantification of FUS-HA nucleus/cytoplasm signal obtained by high-content image analysis, in untreated HeLa cells. (**B**) The graphs report the quantification of FUS-HA nucleus/cytoplasm signal obtained by high-content image analysis, in arsenite treated HeLa cells. (**C**) The graph reports the quantification of FUS-HA number of spots per cell, obtained by high-content image analysis. In (**A**), (**B**) and (**C**), RALY silenced cells were selected by staining with anti-RALY antibody and setting a fluorescence threshold as reference to distinguish silenced cells. Bars indicate means ± SEM of 5 replicates, and p-values were calculated with unpaired two-tailed Student's t-test to compare siRALY to si CTRL cells (**p<0.01; ***p<0.001). (**D**) The graphs show the number of spots per cell, in wild-type (WT) and RALY KO HeLa cells. The number of spots was measured in control and arsenite treated cells and stained with the following markers: Casc3, TIA1 and PABP1. Bars indicate means ± SEM of 5 replicates, and p-values were calculated with unpaired two-tailed Student's t-test to compare WT and RALY KO cells (*p<0.05; **p<0.01; ***p<0.001).

SUPPLEMENTARY FIGURE S3. ***Protein expression analysis in different cell lines and mouse central nervous system organs.*** (**A**) Western blot analysis showing the expression EWSR1, FUS, TAF-15, TDP-43 and RALY in HeLa, SH-SY5Y, NSC-34 cells, adult mouse spinal cord, hippocampus and cortex lysates. TUBULIN was developed as reference. (**B-C**) Specificity of the PLA assay. (**B**) PLA was performed on HeLa cells (panels i and ii) as well as on HeLa cells lacking RALY expression (RALY-KO, panel iii). In contrast to control HeLa cells, no PLA signal was detected when HeLa cells were incubated with only the primary antibody (ab-RALY, panel ii). Similarly, PLA signal was not detected in RALY-KO HeLa cells after the incubation with both anti-RALY and anti-FUS antibodies (panel iii). (**C**) Control PLA was also performed on NSC-34 and no signal was detected upon incubation only with each secondary antibodies.

SUPPLEMENTARY FIGURE S4. ***Validation of expression and localization of FUS-GFP constructs.*** (**A**) Schematic representation showing the domain composition of FUS-GFP constructs. QGSY, region enriched for glutamine, glycine, serine and tyrosine residues (Δ1-165); G1, glycine rich region; G2 and G3, RGG (arginine-glycines rich motifs); RRM, RNA-recognition motifs; Z, zinc-finger domain. ΔQGSY, deletion 1-165; ΔG1, deletion 165-276; ΔRRM, deletion 285-371; ΔG2ZG3, deletion 360-501. (**B**) Immunofluorescence images of HeLa cells transfected with FUS-GFP constructs to check their intracellular expression. Cells were stained with DAPI to mark the nucleus and CellMask^{TM} Deep Red plasma membrane stain to mark the cytoplasm. All constructs show a mainly nuclear localization, being not deleted the PY-NLS. Scale bar =40 μm.

SUPPLEMENTARY FIGURE S5. ***Validation of expression and localization of RALY-MYC constructs.*** (**A**) Schematic representation showing the domain composition of RALY-MYC constructs. The bipartite NLS is depicted by 2 black boxes, representing NLS1 and NLS2. ΔCterm, deletion 144-306; ΔG, deletion 226-306; ΔRRM, deletion 1-142; ΔNterm, deletion 1-224. (**B**) Immunofluorescence images of HeLa cells transfected with RALY-MYC constructs to check their intracellular expression. Cells were stained with DAPI to mark the nucleus. Scale bar=40 μm.

SUPPLEMENTARY FIGURE S6. Analysis of RALY activity in presence of FUS mutants. (**A**) Expression levels of the analyzed *Raly*, *H1fx*, *Dctn1*, *Sod1*, and *Sncb* mRNAs in NSC-34 cells expressing WT or mutated FUS. qRT-PCR data were normalized on *Gapdh*.

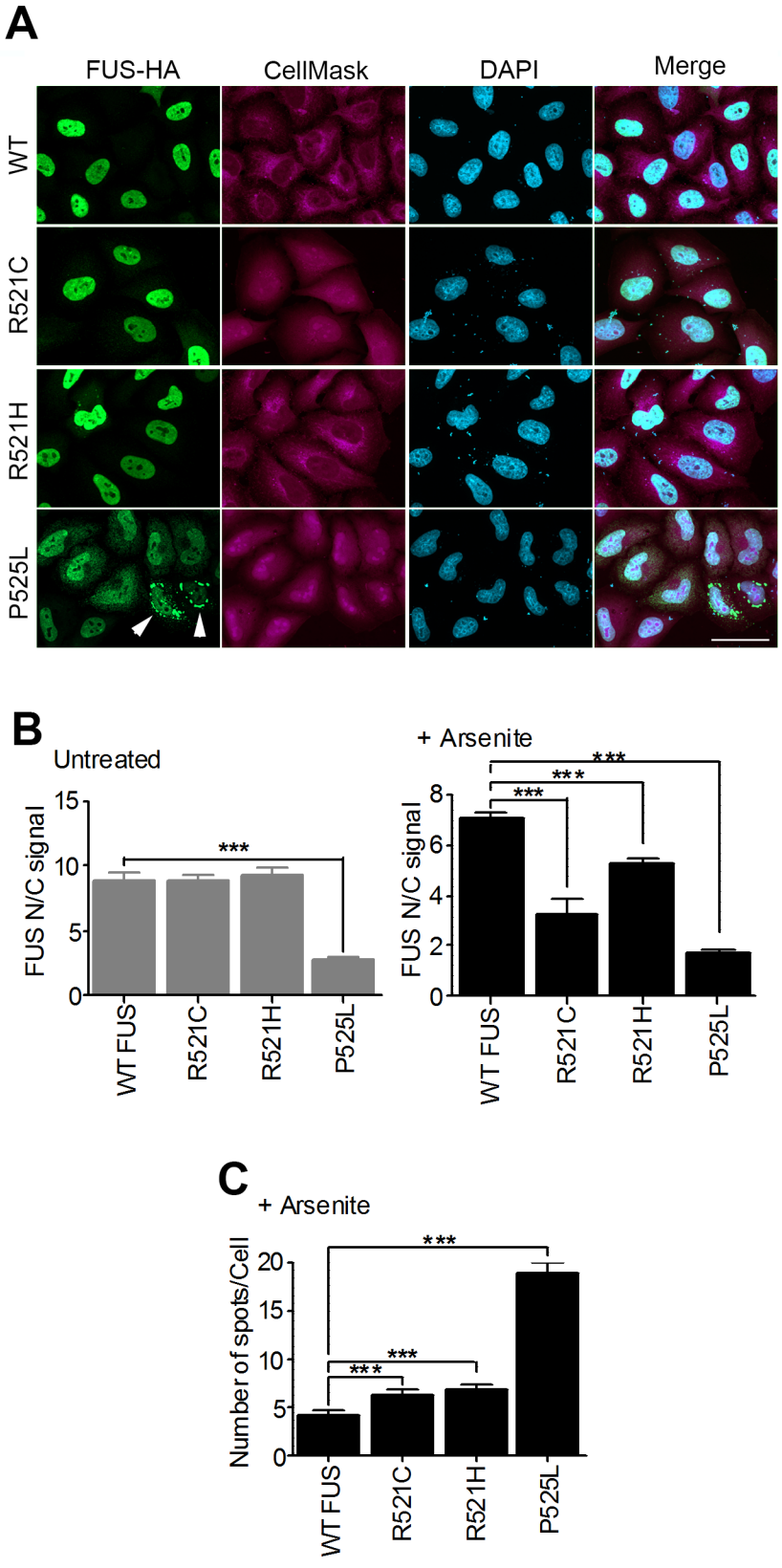
(**B**) *Dctn1*, *Sod1*, *Sncb*, *H1fx* and *Raly* mRNAs are enriched in RALY-containing RNPs. The RNA, purified after RALY or normal rabbit IgG IP in NSC-34 cells induced with doxycycline for 48 hours for WT or mutated FUS-HA expression, was analyzed by qRT-PCR. The mRNA enrichment was calculated relatively to the 10% of RNA input. Each graph represents one single experiment performed starting from WT (upper left panel), R521C (upper right panel), R521H (bottom left panel) and P525L (bottom right panel) FUS. Both *Gapdh* and *B2m* mRNAs were analyzed as negative controls for RALY binding. (**C**) DYNACTIN1, SOD1 and H1X proteins are less expressed in NSC-34 cells expressing FUS mutants. Equal amounts of protein extracts were loaded and checked by TUBULIN immunoblotting. (**D**) Quantification of the indicated proteins in NSC-34 cells expressing FUS mutants. The graph represents the mean of three independent experiments. Bars indicate means ± SEM and p-values were calculated with unpaired two-tailed Student's t-test to compare FUS mutant with WT FUS-overexpressing cells (*p <0.05; **p<0.01; ***p<0.001).

SUPPLEMENTARY FIGURE S7. ***RALY nuclear localization signal characterization.*** (**A**) Schematic representation of the GFP constructs with either full length (FL) RALY NLS, or NLS1, or NLS2 at the N-terminus. The NLS1 is highlighted in blue, the NLS2 in pink. (**B**) The graph reports the nuclear/cytoplasmic signal quantification by high-content image analysis of either GFP-tagged FL RALY NLS, or NLS1 or NLS2. The FL NLS induced the stronger nuclear translocation of GFP signal, while NLS1 and NLS2 induced a significant but lower translocation. Bars indicate means ± SEM of 5 replicates, and p-values were calculated with unpaired two-tailed Student's t-test to compare GFP to RALY NLS-GFP constructs (*p<0.05; ***p<0.001). (**C**) Images showing GFP nuclear translocation induced by RALY NLS, and confirming data reported in panel B. Nuclei were stained with DAPI. The scale bar corresponds to 40 μm. (**D**) The graph reports the nuclear/cytoplasmic signal quantification by high-content image analysis of either wild-type (WT RALY) or NLS mutated RALY-GFP (MUT RALY), and shows that MUT RALY is significantly more cytoplasmic compared to WT. Bars indicate means ± SEM of 5 replicates, and p-values were calculated with unpaired two-tailed Student's t-test to compare WT to MUT RALY-GFP (**p<0.01). (**E**) Images of HeLa cells transfected with either WT or MUT RALY-GFP, and treated or not with arsenite. Both WT and MUT RALY-NLS sequence are reported. Cells were also stained with PABP1 to delineate outline the cytoplasm and SGs. In contrast to WT RALY, MUT RALY is delocalized to the cytoplasm and forms SGs even without arsenite treatment. Scale bar=20 μm.

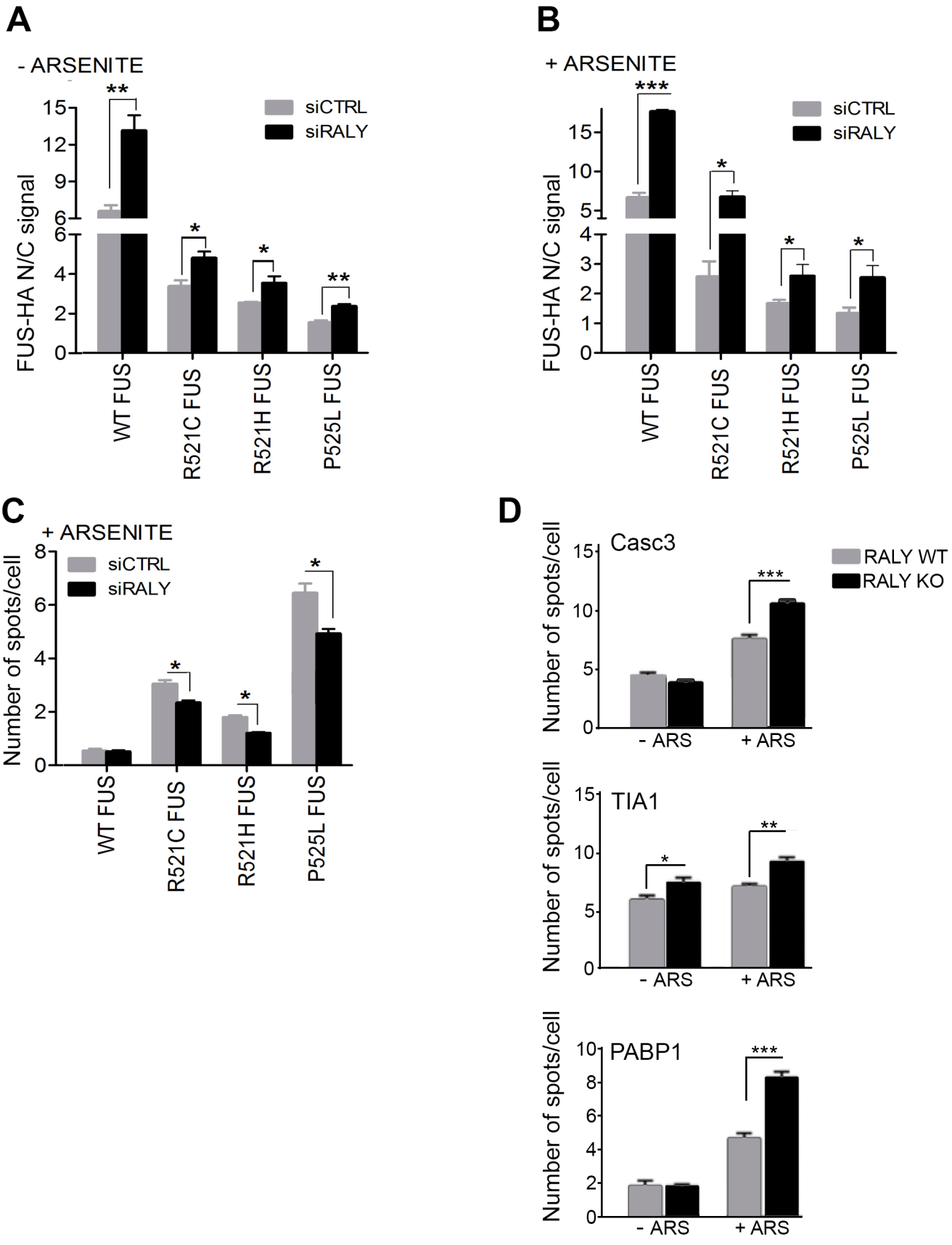
SUPPLEMENTARY FIGURE S8. ***RALY and FUS do not have an additive effect on their target expression.*** (**A**) DYNACTIN1, SOD1, BETA SYNUCLEIN and H1X proteins are less expressed in RALY KO HeLa cells. FUS transient silencing in both WT and RALY KO cells does not change their protein expression. Equal amounts of protein extracts were loaded and checked by TUBULIN immunoblotting. (**B**) Quantification of the indicated proteins in WT and RALY KO cells transfected with either si-CTRL or si-FUS. The graph represents the mean of four independent experiments. Bars indicate means ± SEM and p-values were calculated with unpaired two-tailed Student's t-test (*p <0.05; **p<0.01; ***p<0.001). (**C**) Expression levels of the analyzed mRNAs (*DCTN1*, *FUS*, *H1FX*, *SOD1*) was measured by qRT-PCR. Graphs reports the data normalized on *B2M*. The scatter plot represents results from three independent experiments. P-value was calculated with unpaired two-tailed Student's t-test (*p <0.05; **p<0.01; ***p<0.001).

SUPPLEMENTARY FIGURE S9. ***Prion domain prediction for RALY protein***. The top of the panel shows the probability of each residue belonging to the Hidden Markov Model state prion domain or 'background'. The middle part of the panel shows the primary sequence of RALY, with the predicted prion domain highlighted in red. The lower part of the panel shows the domain architecture of RALY. The predicted prion domain encompasses the RGG domain.

Gasperini et al., SUPPLEMENTARY FIGURE S1



Gasperini et al., SUPPLEMENTARY FIGURE S2



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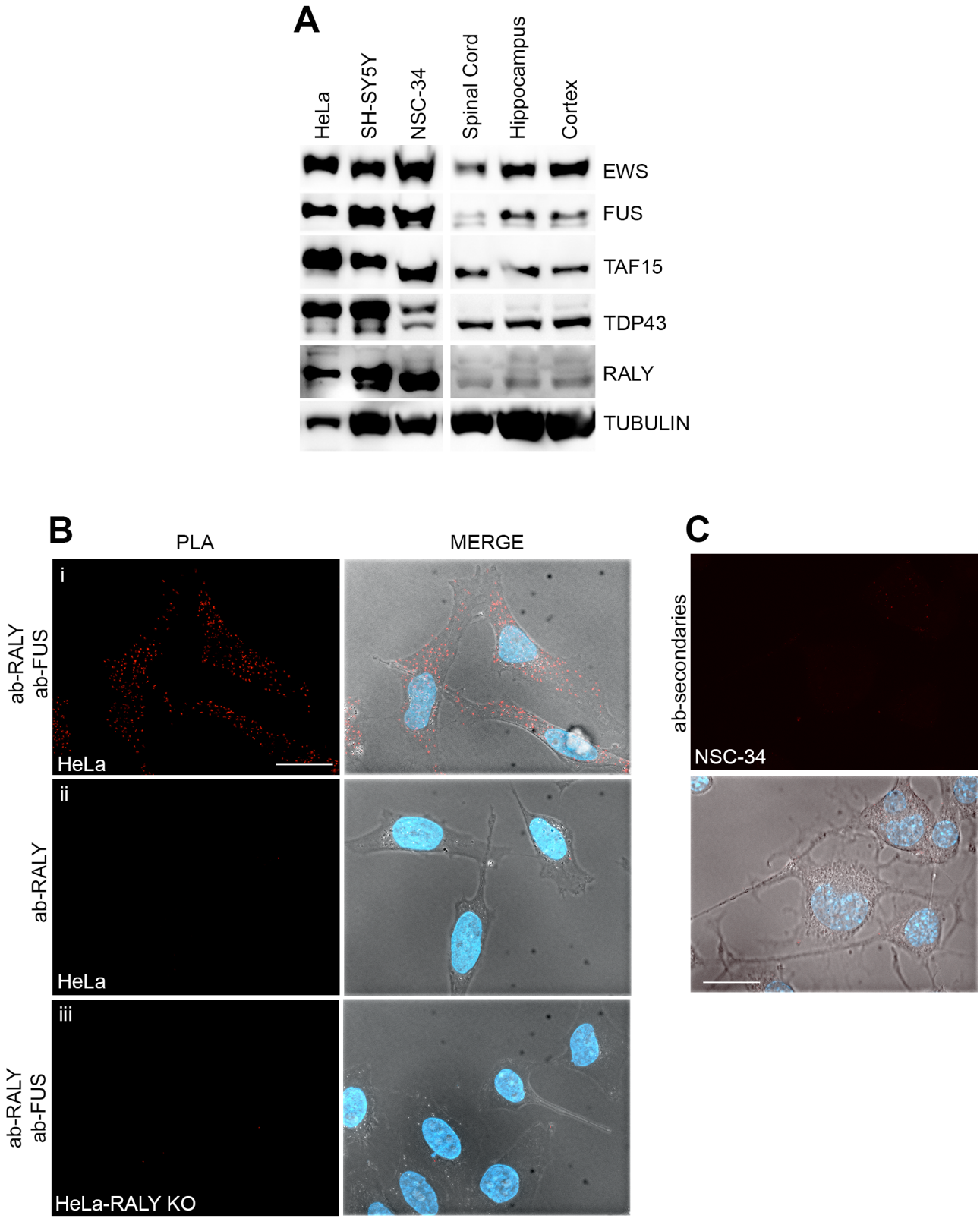

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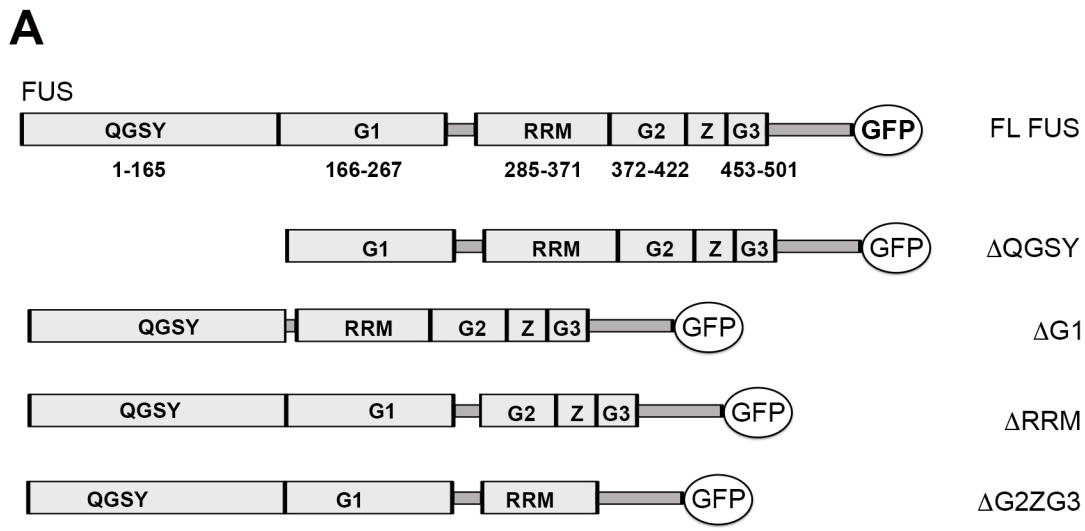

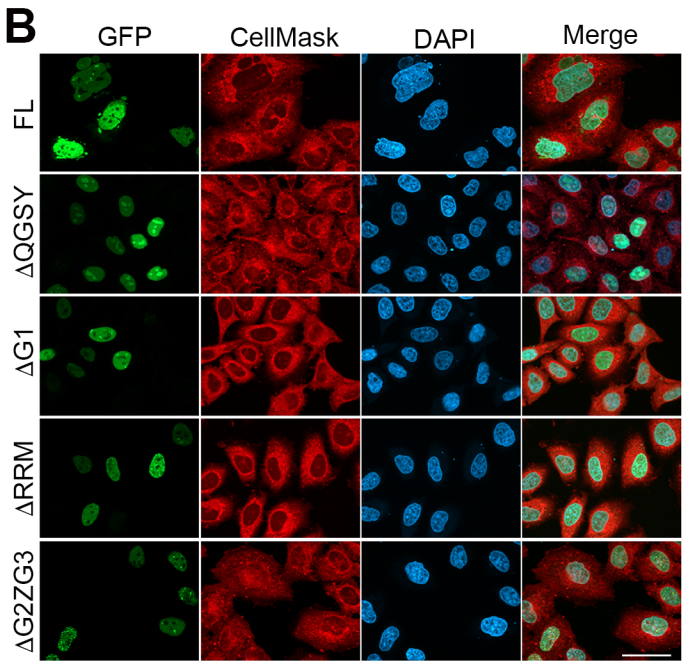

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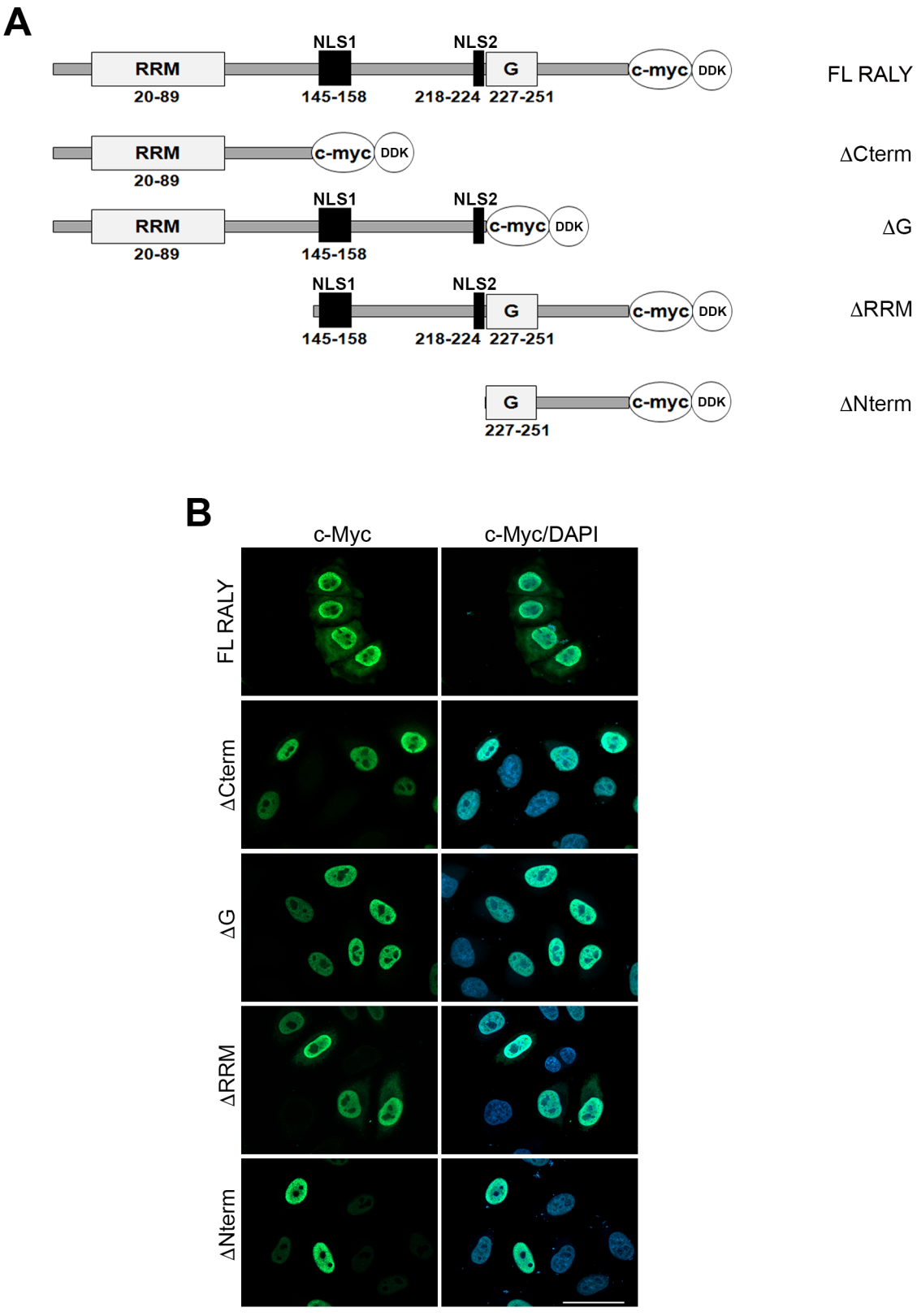

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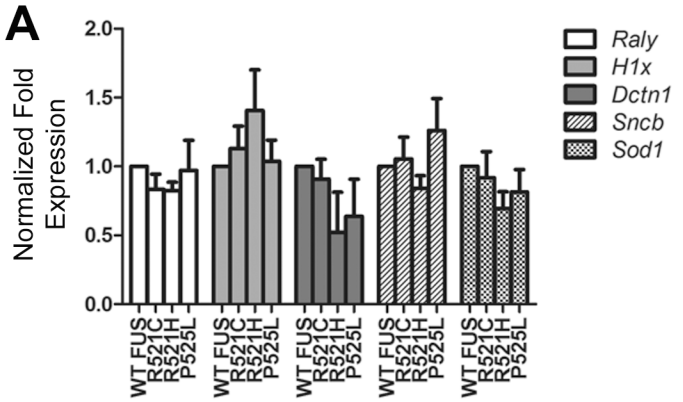

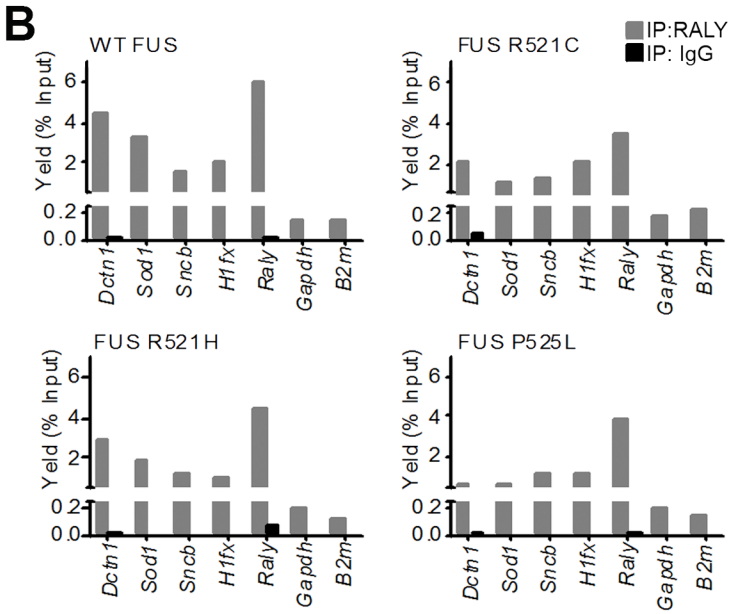

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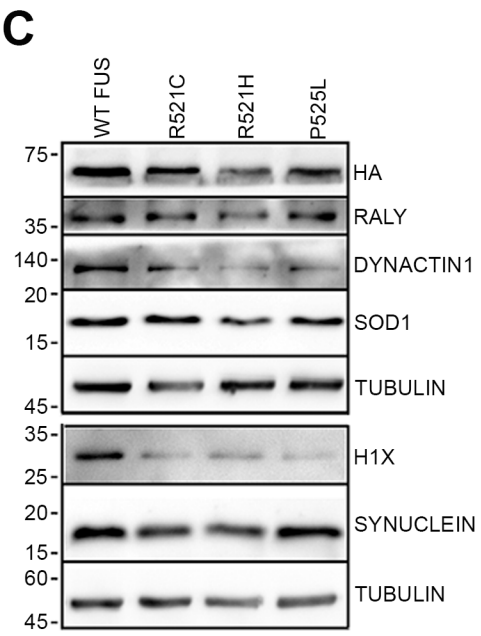

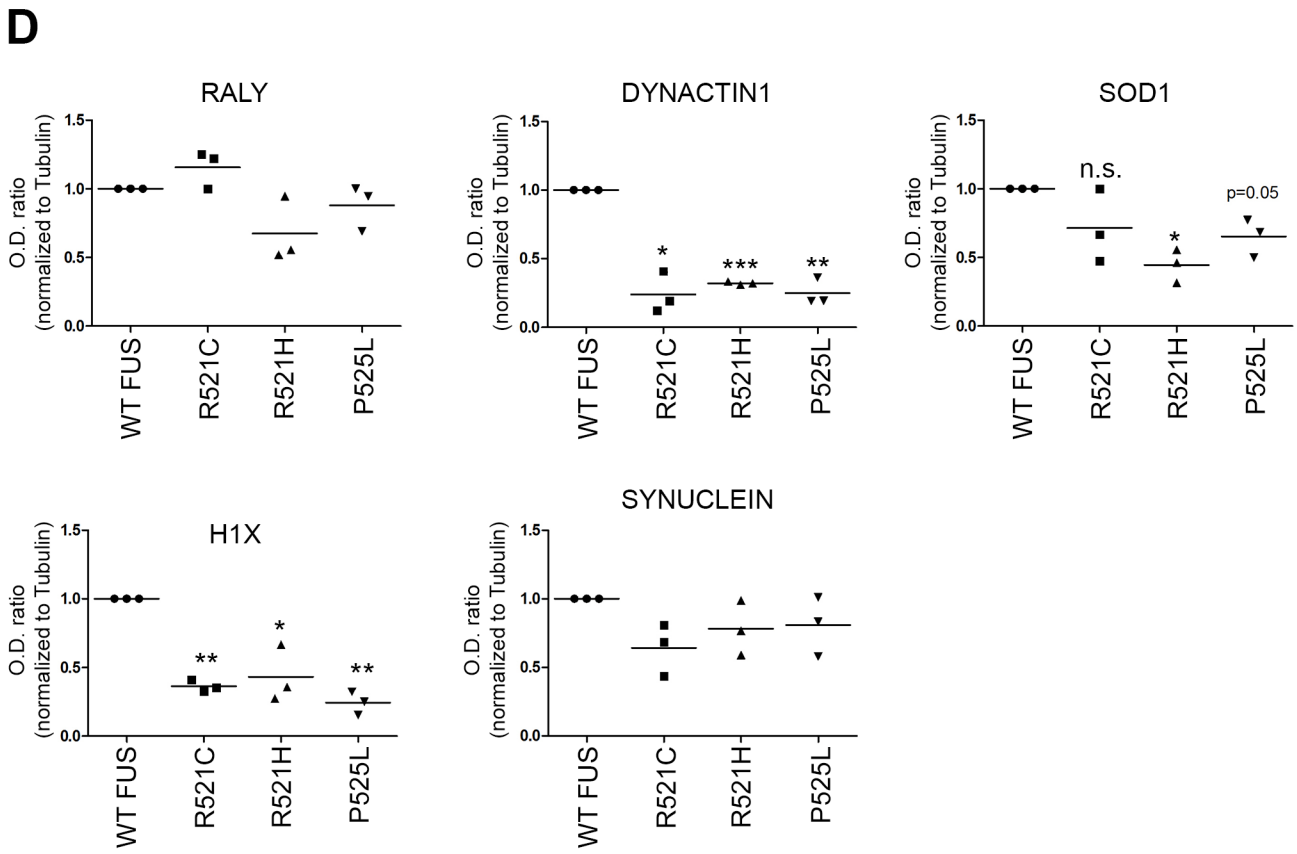

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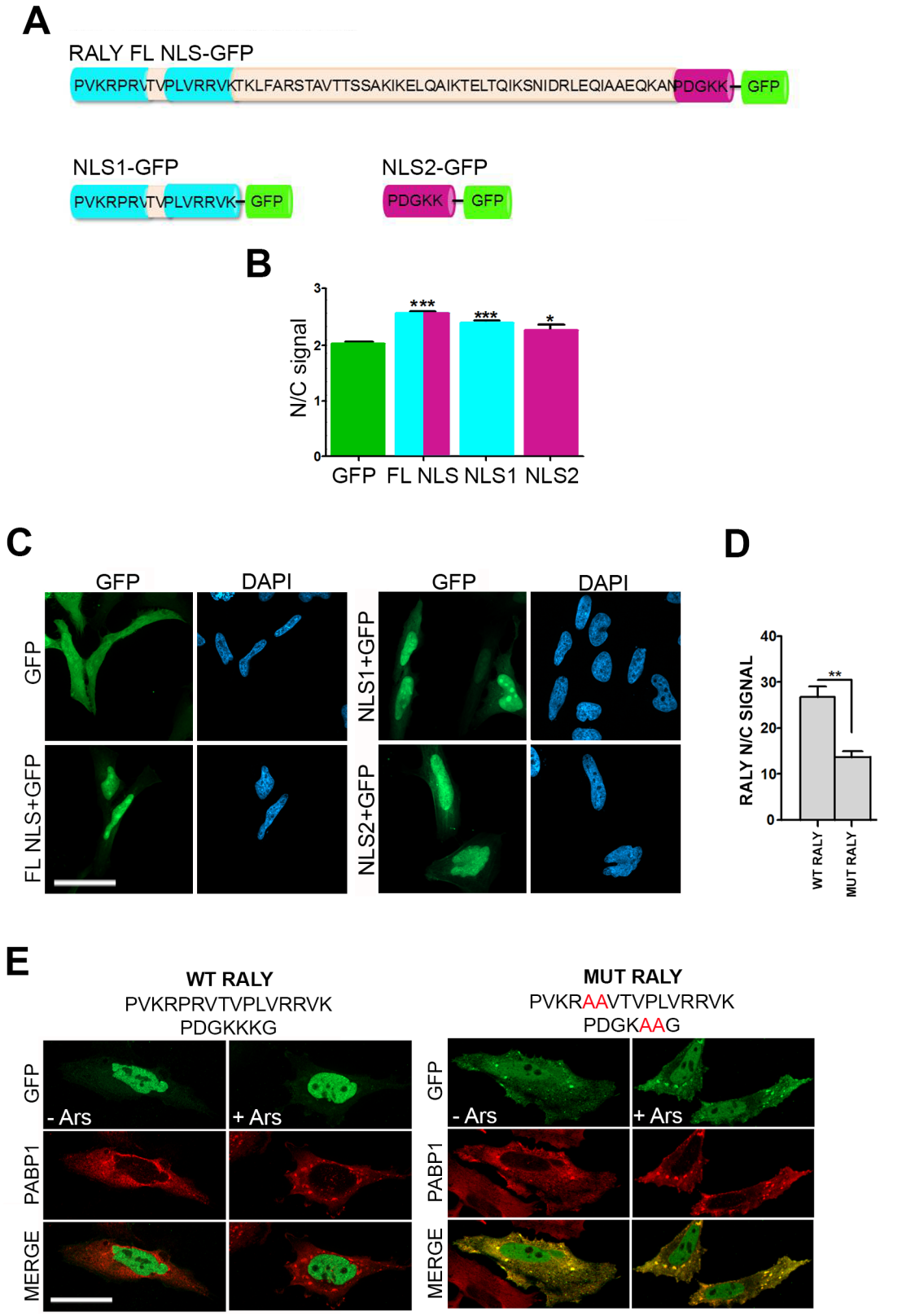

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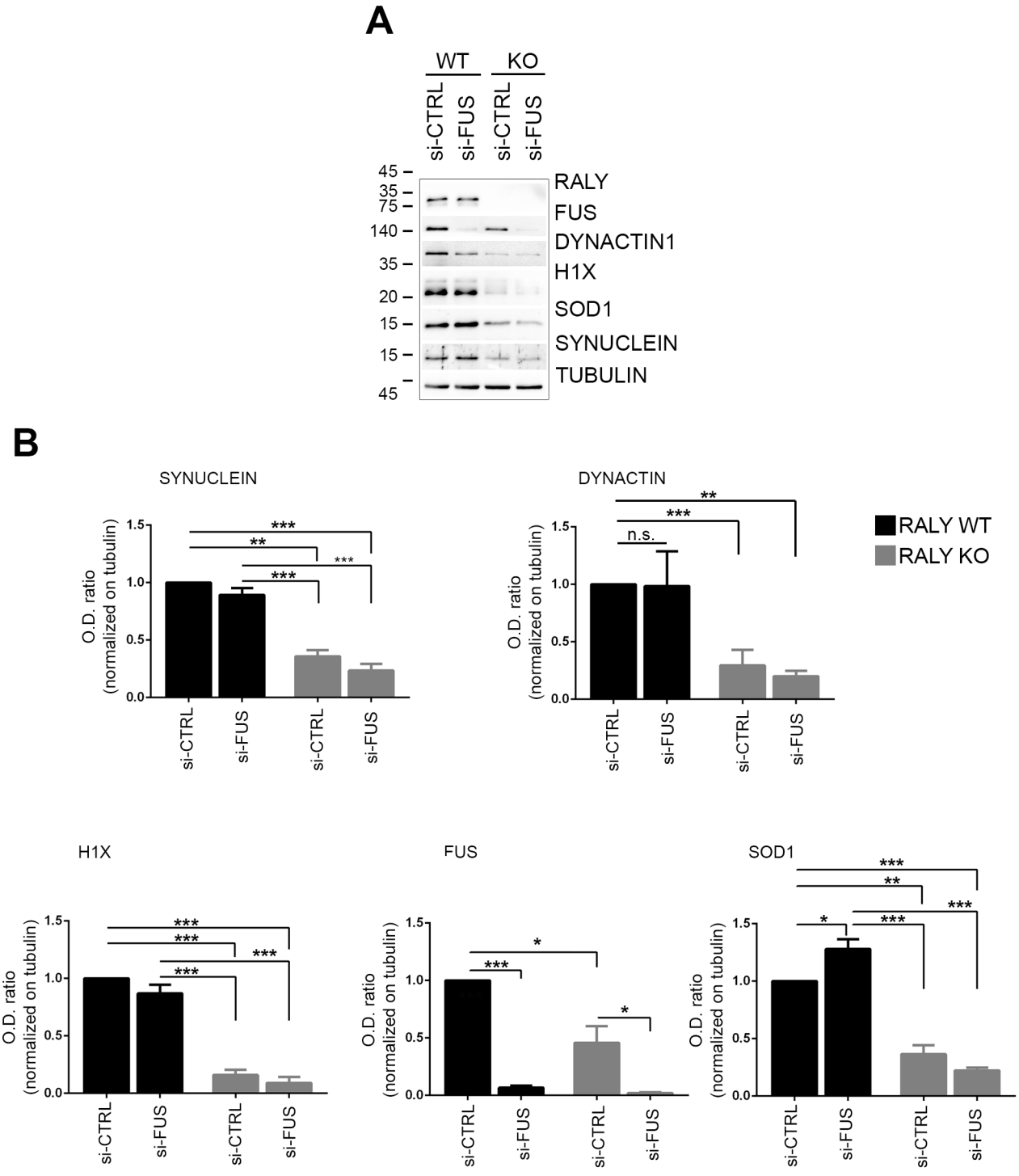

Gasperini et al., SUPPLEMENTARY FIGURE S8

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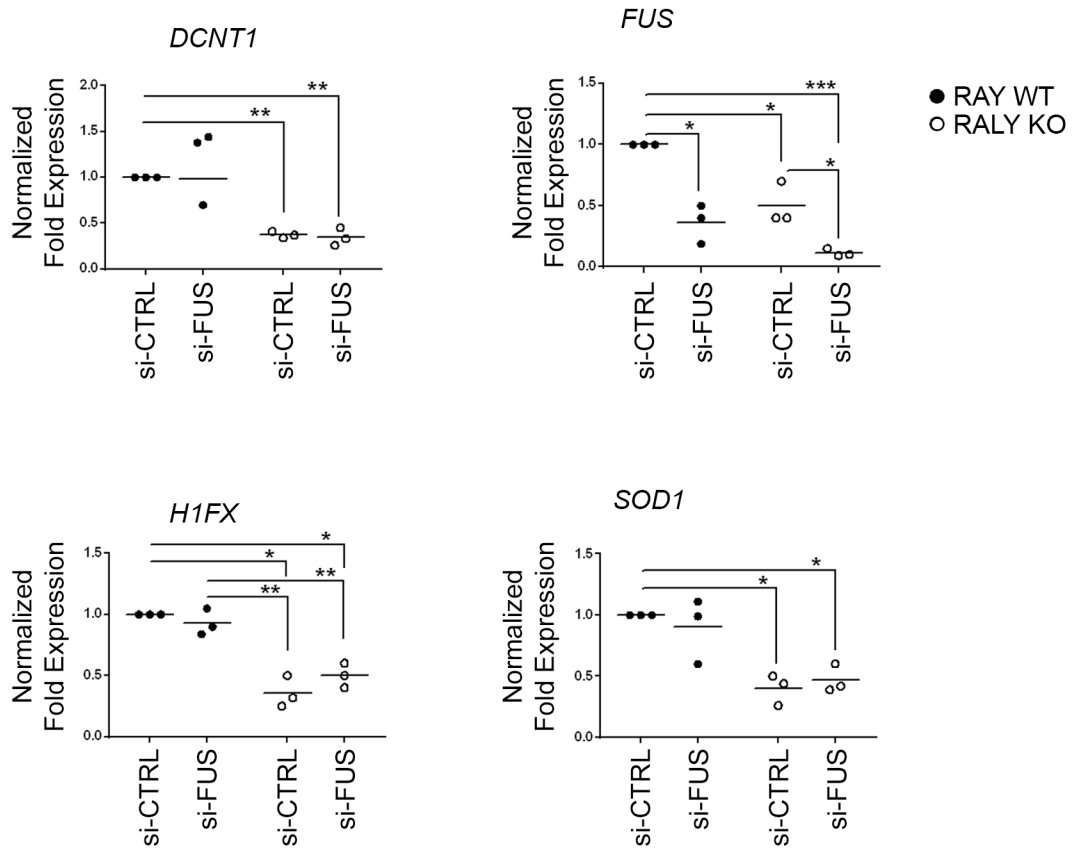

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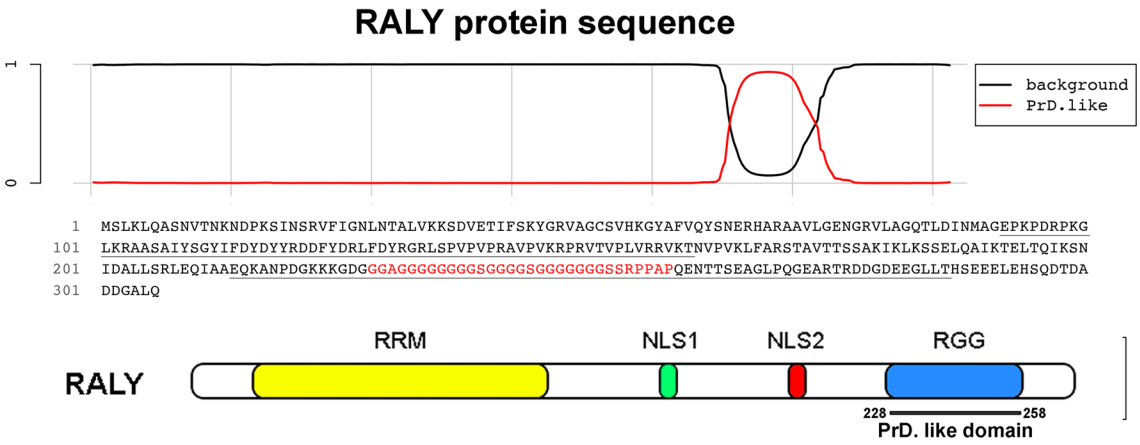

SUPPLEMENTARY TABLE S1. List of oligonucleotides used in this study.

PRIMER	VECTOR	SEQUENCE
RALY-cmyc 1-306 Forward	pCMV6-Entry vector	5'-GCTAGCATGTCCTTGAAGCTTCAGGC
RALY-cmyc 1-306 Reverse	pCMV6-Entry vector	5'-GCGGCCGCGTCTGCAAGG
RALY-cmyc 1-142 Forward	pCMV6-Entry vector	5'-GCTAGCATGTCCTTGAAGCTTCAGGCAAG
RALY-cmyc 1-142 Reverse	pCMV6-Entry vector	5'-GCGGCCGCGTGACCGCCC
RALY-cmyc 1-225 Forward	pCMV6-Entry vector	5'-GCTAGCATGTCCTTGAAGCTTCAGGC
RALY-cmyc 1-225 Reverse	pCMV6-Entry vector	5'-GCGGCCGCGTACCCTTCTTC
RALY-cmyc 143-306 Forward	pCMV6-Entry vector	5'-GCTAGCATGCCTGTGAAGCGACCCCG
RALY-cmyc 143-306 Reverse	pCMV6-Entry vector	5'-GCGGCCGCGTCTGCAAGGCC
RALY-cmyc 226-306 Forward	pCMV6-Entry vector	5'-GCTAGCATGGATGGAGGTGGCGCCG
RALY-cmyc 226-306 Reverse	pCMV6-Entry vector	5'-GCGGCCGCGTCTGCAAGGCC
RALY-EGFP 1-306 Forward	pEGFP-N1	5'- CTCAGATCTATGTCCTTGAAGCTTCAGGCA
RALY-EGFP 1-306 Forward	pEGFP-N1	5'- - TTTACCGGTTGCAAGGCCCCATCATCCGC
RALY-EGFP NLS-$1_{PR/AA}$	pEGFP-N1	AGGGCGGTCCCTGTGAAGCGAGCAGCGGTCACAGT CCCTTTGGTCCGG
RALY-EGFP NLS-$1_{PR/AA}$	pEGFP-N1	CCGGACCAAAGGGACTGTGACCGCTGCTCGCTTCA CAGGGACCGCCCT
RALY-EGFP NLS-$2_{KK/AA}$	pEGFP-N1	AAGGCCAATCCAGATGGCAAGGCTGCAGGTGATG GAGGTGGCGCCGGC
RALY-EGFP NLS-$2_{KK/AA}$	pEGFP-N1	GCCGGCGCCACCTCCATCACCTGCAGCCTTGCCAT CTGGATTGGCCTT
WT FUS-His-HA Forward	pCMV6-AN-His-HA vector	5'-GCG ATCGCGAGGCGATCGCCGCCTCAAACGATT ATACCCAAC
WT FUS-His-HA Reverse	pCMV6-AN-His-HA vector	5'-ACGCGTGCGACGCGTTTAATACGGCCTCTCCCTG CATC
$hFUS^{R521C}$ Forward	pCMV6-AN-His-HA vector	5'- GCACAGACAGGATTGCAGGGAGAGGCCGT
$hFUS^{R521C}$ Reverse	pCMV6-AN-His-HA vector	5'- ACGGCCTCTCCCTGCAATCCTGTCTGTGC
$hFUS^{R521H}$	pCMV6-AN-His-	5'- GCACAGACAGGATCACAGGGAGAGGCCGT

Forward	HA vector	
hFUS^{R521H} Reverse	pCMV6-AN-His-HA vector	5'- ACGGCCTCTCCCTGTGATCCTGTCTGTGC
hFUS^{P525L} Forward	pCMV6-AN-His-HA vector	5'- TCGCAGGGAGAGGCTGTATTAAACGCGTT
hFUS^{P525L} Reverse	pCMV6-AN-His-HA vector	5'- AACGCGTTTAATACAGCCTCTCCCTGCGA

Prime Time Mini qPCR Assay from IDT	
Gene Name	**Assay Name**
GAPDH	Hs.PT.39a.22214836
FUS	Hs.PT.58.580807
SOD1	Hs.PT.20593019
DCTN1	Hs.PT.5824832156
B2m	Mm.PT.58.10497649
Dctn1	Mm.PT.58.10787537.gs
H1fx	Mm.PT.58.6663383.g
Pink1	Mm.PT.58.23711353
Raly	Mm.PT.58.10295856
Sncb	Mm.PT.58.28818829
Sod1	Mm.PT.58.12368303

qPCR Assay primers designed with IDT tools and bought from Eurofin Genomics	
Gene Name	**Sequence**
PRMT1_Human_Fw	5'-TGACTCCTACGCACACTTTG
PRMT1_Human_Rev	5'-ATGGAGTTGCGGTAAGTGAG
Gapdh_mouse_Fw	5'-TTCACCACCATGGAGAAGGC
Gapdh_mouse_Rev	5'-GGCATGGACTGTGGTCATGA
H1FX_Human_Fw	5'-AAGAAGGTGAAGAAGGCGG-3'
H1FX_Human_Rev	5'-CGTGAGCCGTACAAAATCTAC-3'
B2M_Human_Fw	5'- GGCATTCCTGAAGCTGACAG-3'

B2M_Human_Rev	5’- TGGATGACGTGAGTAAACCTG-3’