# Nemo-like kinase disrupts nuclear import and drives TDP43 mislocalization in ALS

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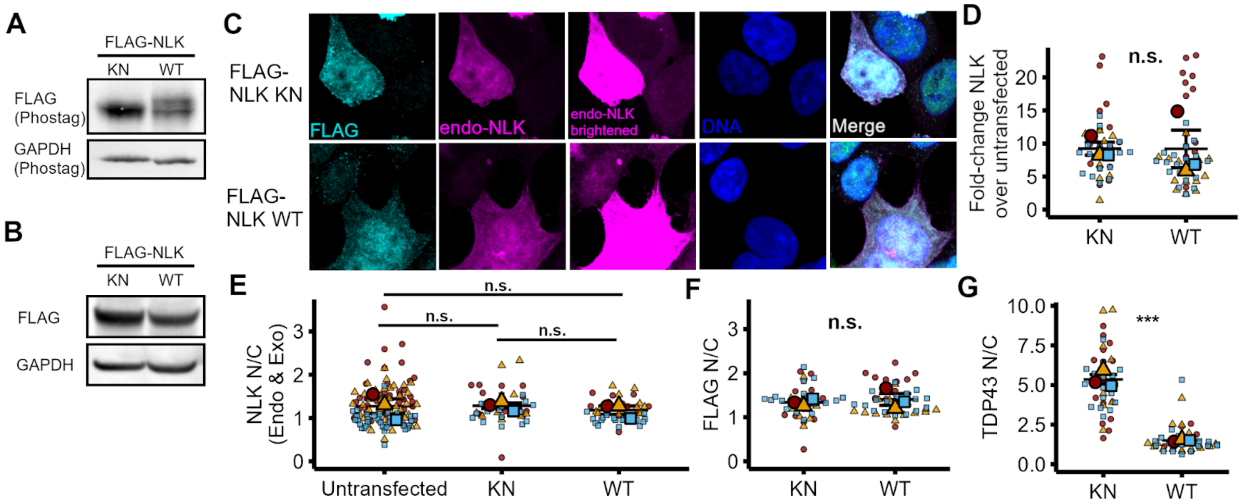
# Supplemental Material

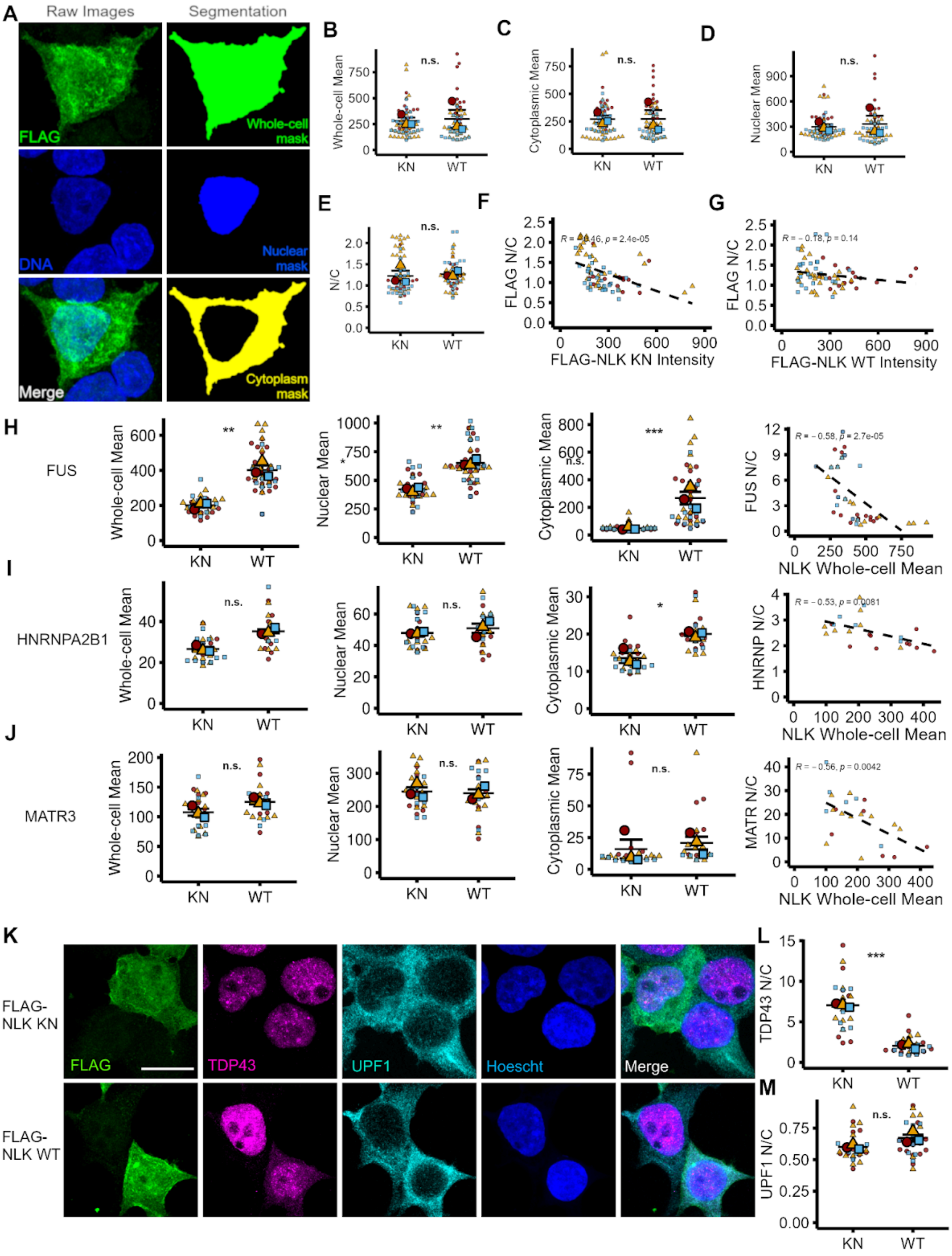
## Supplemental figures and legends



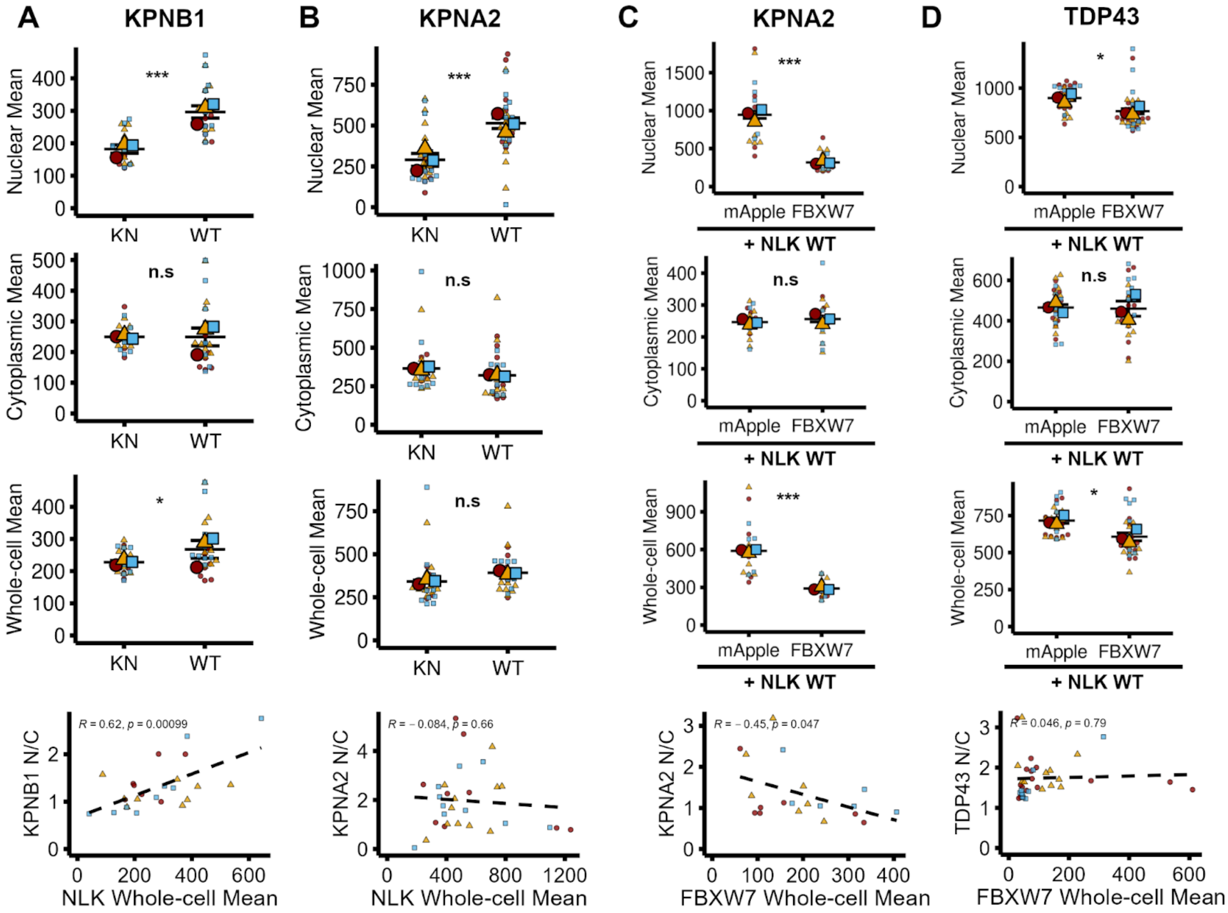
**Supplemental Figure 1. Validation of FLAG-NLK plasmids and analysis of endogenous versus exogenous NLK localization and levels related to Figure 1.**(**A**) Western blot of lysates from HEK293 cells transfected with FLAG-NLK constructs, run on PhosTag acrylamide SDS-PAGE, showing a phosphorylation-dependent mobility shift in FLAG-NLK WT but not FLAG-NLK KN, consistent with kinase activity and autophosphorylation. (**B**) Standard SDS-PAGE Western blot of the same lysates as in (**A**), demonstrating equivalent expression of FLAG-NLK WT and KN.

(**C**) Immunofluorescence using antibodies against endogenous NLK (magenta) and exogenous FLAG-tagged NLK (cyan) reveals similar subcellular localization to both the nucleus and cytoplasm. Nuclei were stained with Hoechst (blue). (**D**) Quantification of fold-change in total NLK levels (based on anti-NLK staining) in FLAG-NLK-expressing cells relative to untransfected controls. (**E**) Quantification of nuclear-cytoplasmic ratio (N/C) of total NLK (anti-NLK antibody) in untransfected and FLAG-NLK-expressing cells. (**F**) Quantification of N/C ratio of exogenous

FLAG-NLK, showing comparable nuclear and cytoplasmic localization for both WT and KN forms. (**G**) Quantification of TDP-43 N/C ratio in FLAG-positive cells from the same population as in (**F**), showing TDP-43 mislocalization in cells expressing FLAG-NLK WT but not KN.

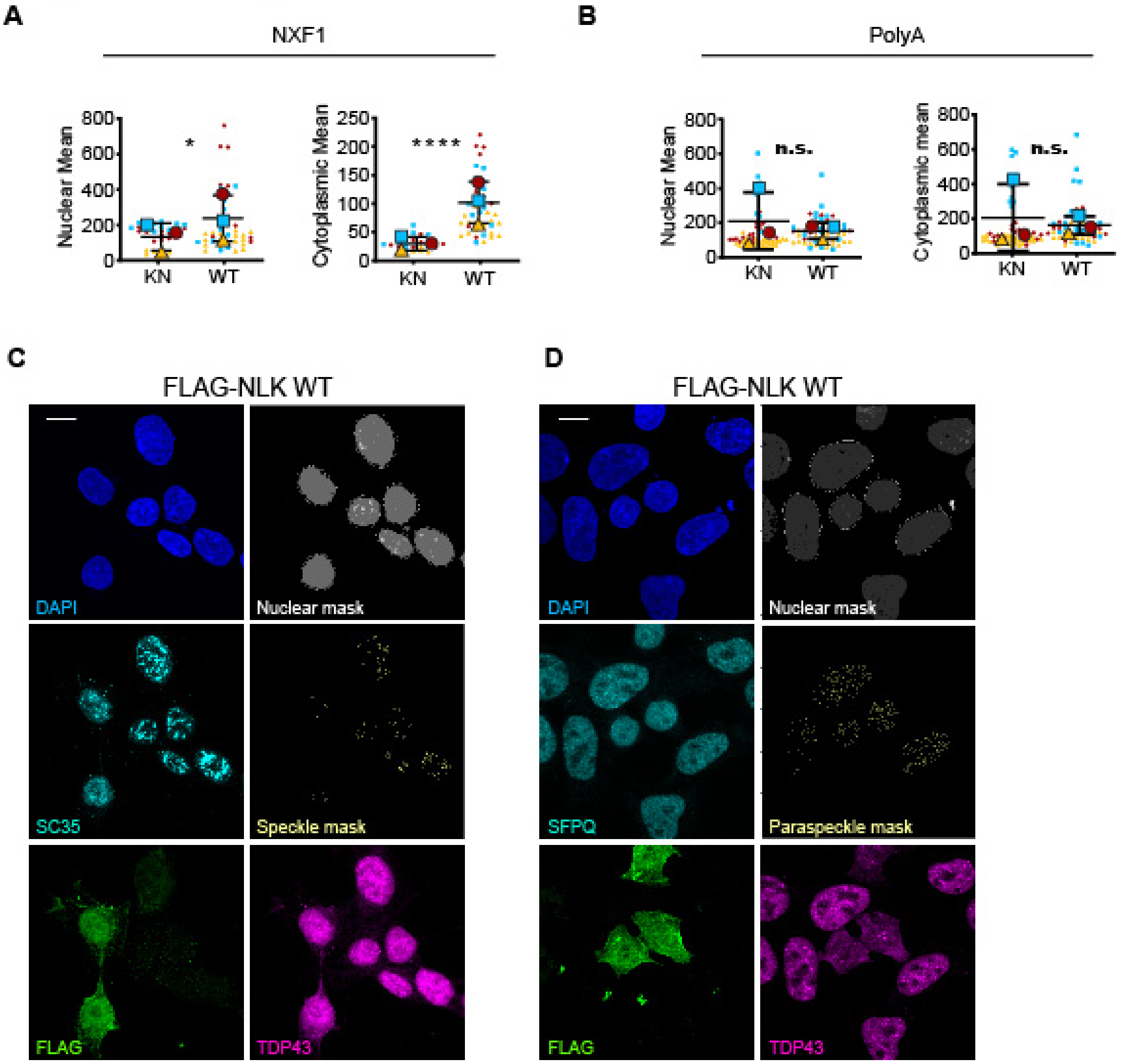

A
Raw Images
Segmentation
FLAG
Whole-cell mask
DNA
Nuclear mask
Merge
Cytoplasm mask
B
Whole-cell Mean
n.s.
KN
WT
C
Cytoplasmic Mean
n.s.
D
Nuclear Mean
n.s.
E
N/C
n.s.
F
FLAG N/C
R = −0.46, p = 2.4e-05
FLAG-NLK KN Intensity
G
FLAG N/C
R = −0.18, p = 0.14
FLAG-NLK WT Intensity
H
FUS
Whole-cell Mean
**
Nuclear Mean
**
Cytoplasmic Mean
***
FUS N/C
R = −0.58, p = 2.7e-05
NLK Whole-cell Mean
I
HNRNPA2B1
Whole-cell Mean
n.s.
Nuclear Mean
n.s.
Cytoplasmic Mean
*
HNRNP N/C
R = −0.53, p = 0.0081
NLK Whole-cell Mean
J
MATR3
Whole-cell Mean
n.s.
Nuclear Mean
n.s.
Cytoplasmic Mean
n.s.
MATR N/C
R = −0.56, p = 0.0042
NLK Whole-cell Mean
K
FLAG-NLK KN
FLAG-NLK WT
FLAG
TDP43
UPF1
Hoescht
Merge
L
TDP43 N/C
***
M
UPF1 N/C
n.s.

**Supplemental Figure 2. Supporting quantification from Figure 1.** (**A**) Representative images illustrating how nuclear and cytoplasmic compartments are defined in HEK293 cells. FLAG immunostaining is used to generate a whole-cell mask, while Hoechst DNA staining defines the nuclear mask. The cytoplasmic mask is derived by subtracting the nuclear area from the whole-cell area, enabling differentiation between nuclear and cytoplasmic compartments. (**B–G**) Supporting quantification for Figure 1A–E, showing FLAG-NLK whole-cell, cytoplasmic, and nuclear intensities, as well as nuclear-cytoplasmic ratio (N/C). Scatterplots show the relationship between FLAG-NLK whole-cell intensity and corresponding N/C ratios. (**H–J**) Supporting quantification for Figure 1G–L, showing whole-cell, cytoplasmic, and nuclear intensities of the indicated endogenous proteins, along with their N/C ratios. Scatterplots illustrate the relationship between whole-cell intensity and N/C ratio. (**K**) HEK293 cells were transfected with plasmids encoding either FLAG-NLK KN or FLAG-NLK WT, followed by immunofluorescence using antibodies against FLAG (green), TDP-43 (magenta), and UPF1 (cyan). Nuclei were stained with Hoechst (blue). Scale bar = 10 μm. (**L**) Superplot of UPF1 N/C ratios from images in (K). Line = mean; error bar = standard error. No significant difference observed (unpaired t-test with Welch’s correction). (**M**) Superplot of TDP-43 N/C ratios from the same cells as in (K), showing a significant reduction with NLK WT expression.



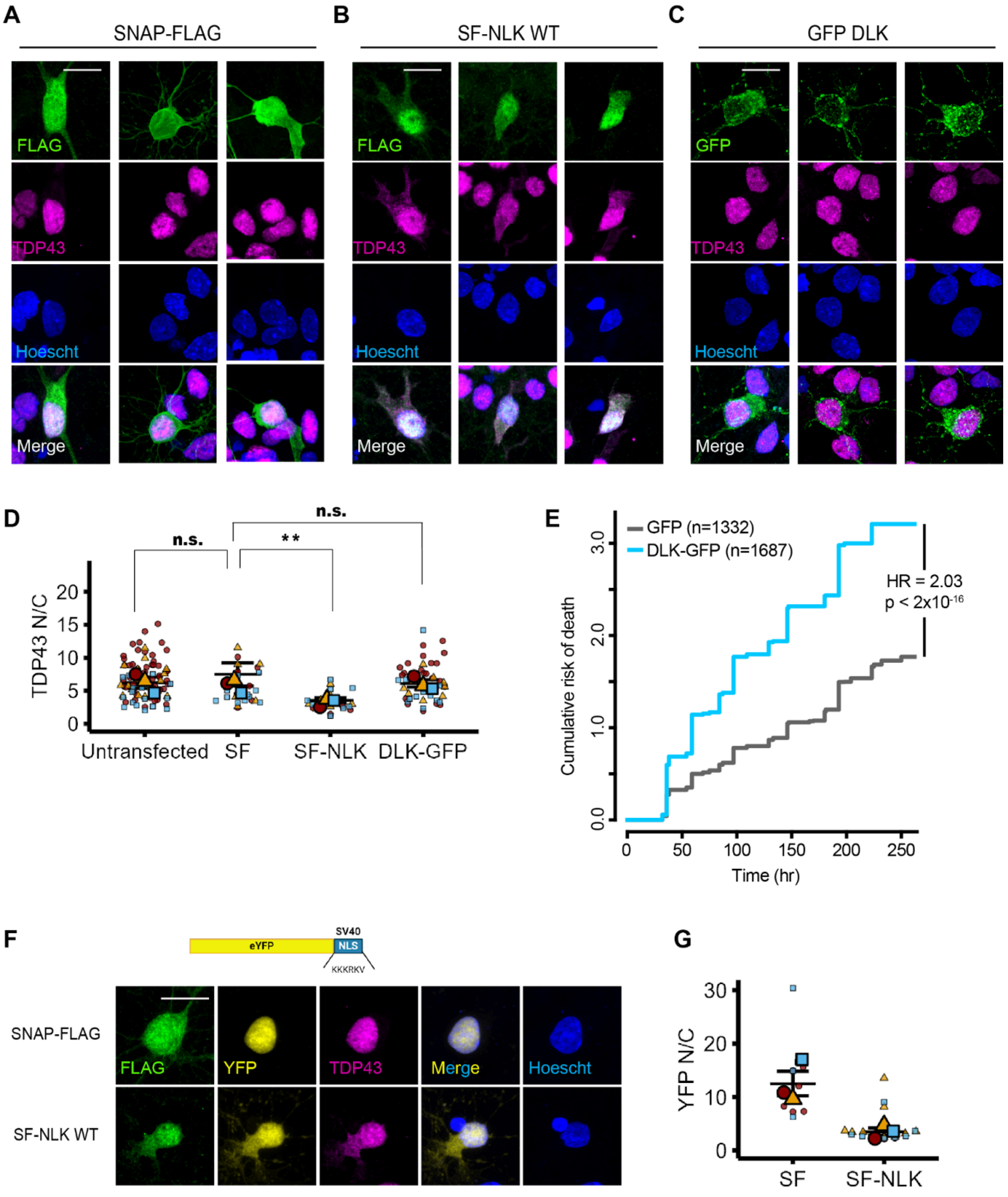
**Supplemental Figure 3. Supporting quantitation from Figure 3.** (**A**) Superplot of KPNB1 nuclear, cytoplasmic, and whole-cell intensities following overexpression of FLAG-NLK KN or FLAG-NLK WT (related to Figure 3A). Statistical significance is indicated as follows in all sub-panels: n.s. = not significant, *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired t-test with Welch's correction). (**B**) Superplot of KPNA2 nuclear, cytoplasmic, and whole-cell intensities following overexpression of FLAG-NLK KN or FLAG-NLK WT (related to Figure 3B). Line = mean; error bar = standard error. (**C**) Superplot of KPNA2 nuclear, cytoplasmic, and whole-cell intensities following co-expression of FLAG-NLK WT with either mApple or V5-FBXW7 (related to Figure 3C). Line = mean; error bar = standard error. (**D**) Superplot of TDP-43 nuclear, cytoplasmic, and whole-cell intensities following co-expression of FLAG-NLK WT with either mApple or V5-FBXW7 (related to Figure 3D). Line = mean; error bar = standard error.

**Supplemental Figure 4**



**Supplemental Figure 4. Additional quantifications from Figure 5.** (**A**) Superplot of NXF1 nuclear and cytoplasmic levels after NLK KN or NLK WT overexpression related to Figure 5B. *p<0.05, ****p<0.0001. (unpaired T-test with Welch's correction). (**B**) Superplot of PolyA nuclear and cytoplasmic levels after NLK KN or NLK WT overexpression related to Figure 5C. n.s., not significant as determined by unpaired T-test with Welch's correction. (**C**) Representative image of HEK cells transfected with plasmids encoding either FLAG-NLK KN or FLAG-NLK WT followed by immunofluorescence for FLAG (green), TDP43 (magenta) and markers of speckles

(SC-35; cyan); DNA was stained with Hoescht (blue). Nuclear masks (Hoescht signal) and speckle masks (SC35) were generated using CellProfiler. (**D**) Representative image of HEK293 cells transfected with plasmids encoding either FLAG-NLK KN or FLAG-NLK WT followed by immunofluorescence for FLAG (green), TDP43 (magenta), and markers of paraspeckles (SFPQ, cyan); DNA was stained with Hoescht. Nuclear masks (Hoescht signal) and paraspeckle masks (SFPQ) were generated using CellProfiler.

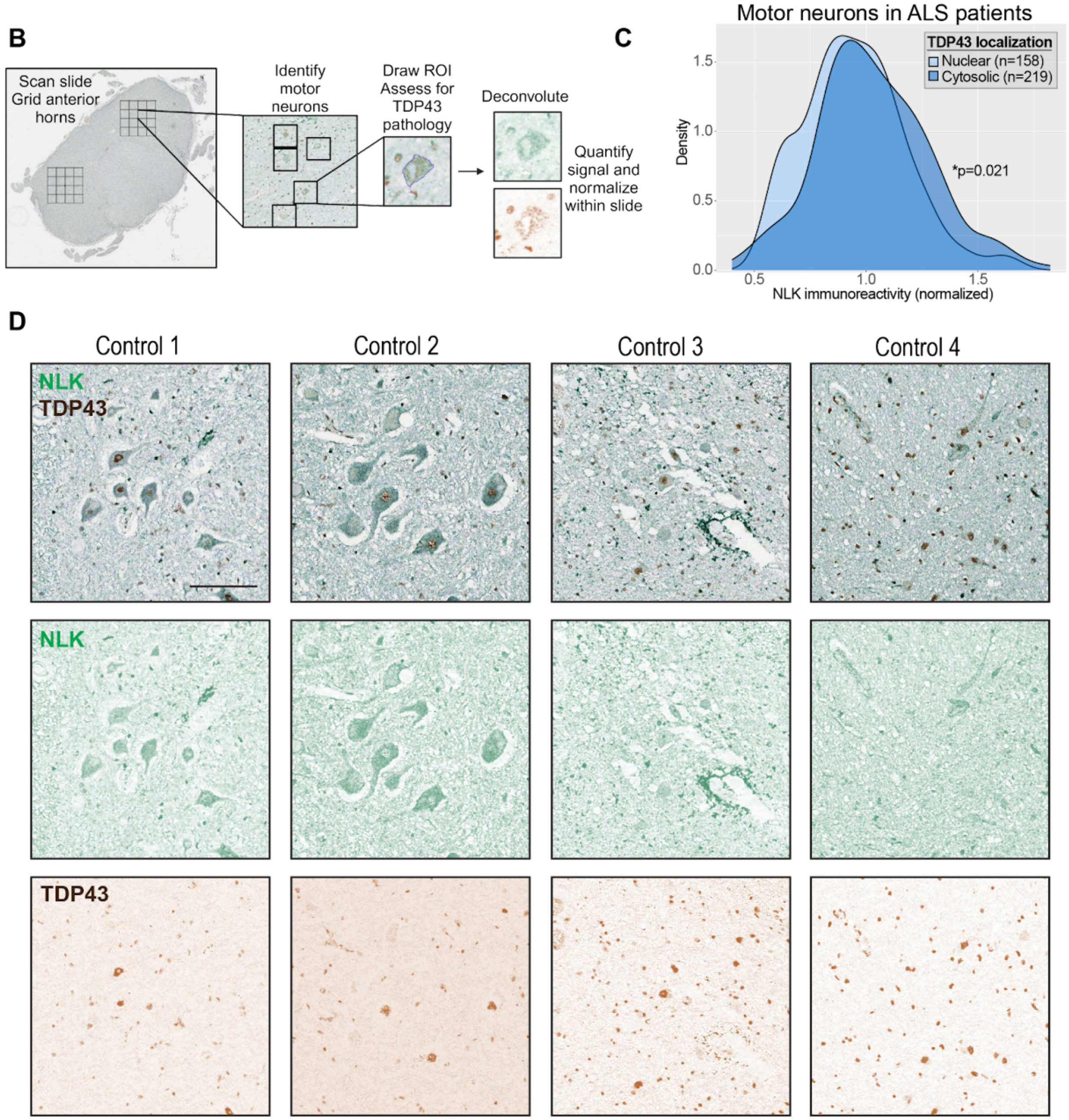


**Supplemental Figure 5. Supplemental images and quantification supporting Figure 5.**

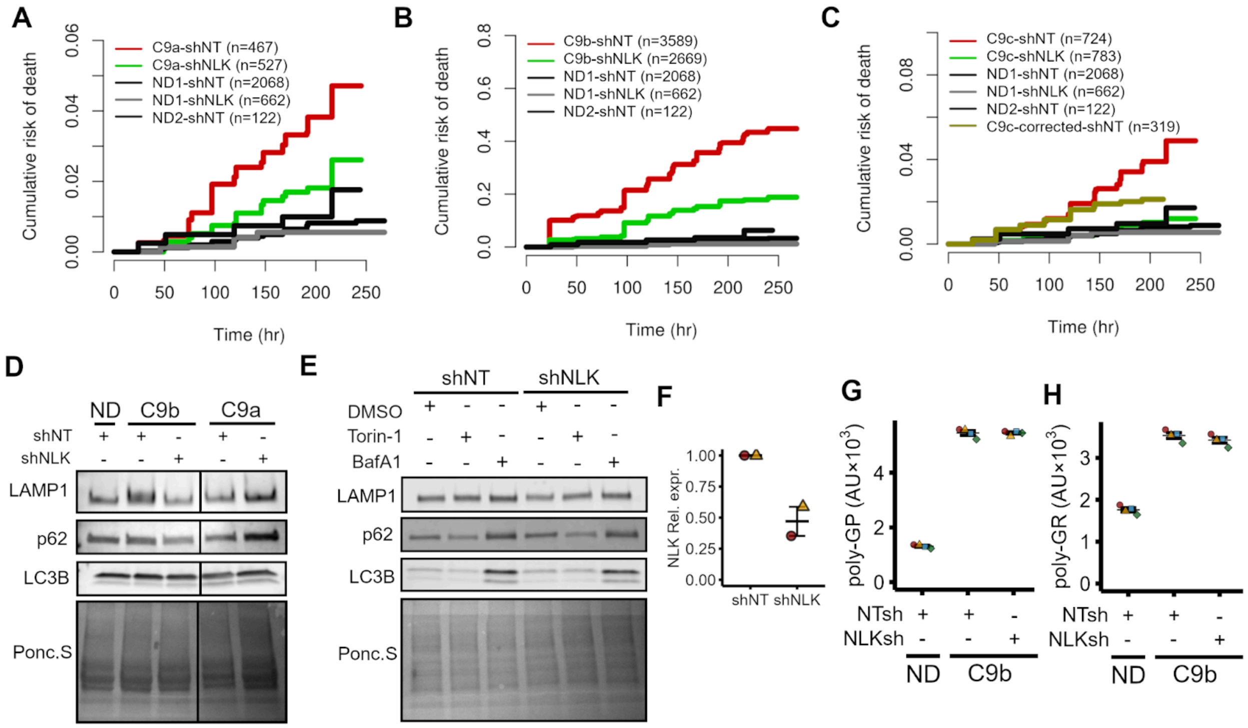
(**A-B**) Additional representative images of rodent primary cortical neurons transfected with either SNAP-FLAG (SF; negative-control) or SNAP-FLAG-NLK (SF-NLK) followed by immunofluorescence using antibodies against FLAG (green) and TDP43 (magenta). DNA was stained with Hoechst (blue)  (**C**) Rodent primary cortical neurons were transfected with DLK-GFP followed by immunofluorescence using antibodies TDP43 (magenta) and direct visualization of tagged protein (green). DNA was stained with Hoechst (blue). (**D**) Superplots of N/C ratio of TDP43 in untransfected rodent primary cortical neurons and those transfected with SNAP-FLAG (SF; negative-control), SNAP-FLAG-NLK (SF-NLK), and DLK-GFP, corresponding to representative images in A-C. n.s. not significant. ** $p<0.01$ (Dunnett's T3 multiple comparisons test). (**E**) Cumulative Hazard plot showing the relative risk of death in neurons expressing either GFP or DLK-GFP, calculated by Cox proportional hazards analysis, stratified among 3 biological replicates. HR, hazard ratio. (**F**) Rodent primary cortical neurons were co-transfected with YFP-NLS$^{SV40}$ and either SNAP-FLAG (SF; negative-control) or SNAP-FLAG-NLK (SF-NLK) followed by immunofluorescence using antibodies against FLAG (green) and TDP43 (magenta). DNA was stained with Hoechst (blue). Scale bar = 10 μm. (**G**) Superplots of N/C ratio of TDP43 in cells corresponding to (F). *** $p<0.001$ (unpaired t test with Welch's correction).

**A**

| | ALS - 1 | ALS - 2 | ALS - 3 | ALS - 4 | Ctrl - 1 | Ctrl - 2 | Ctrl - 3 | Ctrl - 4 |
|---|---|---|---|---|---|---|---|---|
| Male/female (M/F) | M | F | M | M | M | F | M | F |
| Age (y) | 64 | 76 | 64 | 51 | 61 | 78 | 72 | 64 |
| Clinical diagnosis | ALS with FTLD | ALS | ALS | ALS | Early-onset AD | Probable AD | Lewy Body Dementia | HD |
| Neuropathological diagnosis | ALS<br>PSP | ALS | ALS | ALS | ADNC, high | ADNC, int | ADNC, high<br>LATE-NC, stage 1 | HD<br>ADNC, low |



**Supplemental Figure 6. NLK/TDP43 dual immunohistochemistry in patient tissue.** (**A**) Patient characteristics. Ctrl: control. ALS: amyotrophic lateral sclerosis. HD: Huntington's disease. AD: Alzheimer's disease. ADNC: Alzheimer disease neuropathologic change. LATE-NC: Limbic predominant age-related TDP43 encephalopathy neuropathologic change. (**B**) Schematic showing workflow for quantification of dual immunohistochemistry. (**C**) Density plot depicting the change in NLK immunoreactivity in motor neurons with and without TDP43 pathology, corresponding to images in 7E. * $p<0.05$ by 2-sided Kolmogorov Smirnov test. (**D**) Dual immunohistochemistry for NLK and TDP43, performed on spinal cord tissue from four control patients without spinal cord pathology. Scale bar = 100 μm.



**Supplemental Figure 7. Supporting quantification and data for Figure 7.** (**A-C**) Cumulative hazard plots showing the relative risk of death in neurons from three independent C9orf72 patient-derived iPSC lines (C9a, C9b, and C9c), following transduction with lentivirus encoding either non-targeting (scramble) or NLK-directed shRNA. Each is compared to neurons from two non-disease (ND) control iPSC lines. (**D**) Western blot analysis of lysates from ND, C9a, and C9b iNeuron cultures showing no consistent changes in LAMP1, p62, or LC3B levels following NLK knockdown. (**E**) Western blot analysis of HEK293 cells stably expressing either non-targeting (NT) or NLK-directed shRNA showing no changes in LAMP1, p62, or LC3B upon NLK knockdown. (**F**) qPCR showing reduced NLK mRNA levels in HEK293 cells stably expressing NLK shRNA compared to NT controls. (**G,H**) Poly-GP and poly-GR ELISA measurements in C9b neurons after transduction with shNT or shNLK, showing no significant change in dipeptide repeat levels following NLK knockdown.

## Supplemental Methods:

### iPSC lines:

|  | ID | Onset age | Biopsy age | Gender | Onset | Notes |
|---|---|---|---|---|---|---|
| **C9orf72-fALS** | C9b (883) | 49 | 51 | M | Lumbar | C9orf72 mutation + |
|  | C9a (312) | 52 | 54 | M | Lumbar | C9orf72 mutation + |
|  | C9c (CS52) | 46 | 47 | M | Lumbar | C9orf72 mutation + |
| **TDP43-fALS** | TDP43 (M337V) |  | 54 | F |  | M337V and C-terminal Dendra2 inserted into ND1 (1021) by CRISPR/Cas9 |
|  | TDP43 (WT) |  | 54 | F |  | C-terminal Dendra2 inserted intoND1 (1021) by CRISPR/Cas9 |
| **Control** | ND1 (1021) |  | 54 | F |  | Healthy |
|  | ND2 (746) |  | 58 | M |  | Healthy |
|  | C9c-corrected | 46 | 47 | M | Lumbar | C9orf72 mutation removed from CS52i by CRISPR/Cas9 |

137 **Antibodies:**

| | Vendor | Catalog # | Concentration for ICC | Conc. for WB |
|---|---|---|---|---|
| Flag | AbCam | ab1170 | 1:200 | 1:1000 |
| TDP43 | R&D | MAB7778 | 1:200 | 1:1000 |
| TDP43 | Proteintech | 10782-2-AP | 1:200 | |
| FUS | Sigma | HPA008784 | 1:200 | |
| HNRNPA2B1 | Proteintech | 14813-1-AP | 1:100 | |
| MATR3 | Abcam | ab281927 | 1:200 | |
| V5 | Abcam | ab53418 | 1:200 | |
| KPNA2 | Proteintech | 10819-1-AP | 1:200 | 1:500 |
| KPNB1 | Abcam | AB2811 | 1:200 | 1:500 |
| Ran | BD Transduction labs | 610340 | 1:400 | |
| RanGAP | Abcam | AB92360 | 1:200 | 1:500 |
| RanBP2 | Abcam | AB64276 | 1:200 | 1:1000 |
| Mab414 | Abcam | AB24609 | 1:200 | |

| | | | | |
|---|---|---|---|---|
| NXF1 | Abcam | AB129160 | 1:200 | |
| SC-35 | Sigma | S4045-.2ML | 1:200 | |
| SFPQ | Proteintech | 15585-1-AP | 1:200 | |
| Nucleophosmin | Millipore | MABE937 | 1:200 | |
| NLK | Abcam | Ab97642 | 1:200 | |
| UPF1 | Abcam | Ab133564 | 1:200 | |
| GAPDH | Millipore | MAB374 | | 1:1000 |

## Plasmids

| Plasmid | Source |
|---|---|
| FLAG-NLK-WT | Gift from T. Ishitani (66) |
| FLAG-NLK KN (K155M) | Gift from T. Ishitani (66) |
| EYFP2-SV40NLS-NES | Gift from Yuh Min Chook (67) |
| EYFP2-SV40NLS | This paper |
| EYFP2-TDPNLS | This paper |
| EYFP2-FUSNLS | This paper |
| EYFP-MATR3NLS | This paper |

| | |
|---|---|
| pGW1-TDP43(2F/L)-EGFP | Flores et al 2019 (36) |
| FUGW-SNAP-FLAG | This paper |
| FUGW-SNAP-FLAG-NLK | This paper |
| DLK-GFP | This paper |

## Primers

| | |
|---|---|
| Amplify TDP43 NLS | For: ctcagatctcgaAAAAGAAAAATGGAT<br>Rev: ttatctagagatTCTTTTCACTTTCAC |
| Amplify FUS NLS | For: tcagatctCGAGGGGGCCGGGGT<br>Rev: tatctagaATACGGCCTCTCCCT |
| Mutagenesis: introduce stop codon into EYFP2-SV40NLS-NES | For:<br>ctcgaccaaaaaagaagagaaaggtataagctttagccttgaaa<br>Rev: tttcaaggctaaagcttatacctttctcttcttttttggtcgag |
| Primers used to clone pGW1 DLK-GFP | Forward primer to amplify FLAG-DLK:<br>atcatgacatcgattacaaggatgacgatgacaagctcGCCTGCCTCCATGAAACCCGAA |

| | |
|---|---|
| | Forward primer to amplify Kpn1-FLAG:<br>ggataggtacCATGgactacaaagaccatgacggtgattataaagatcatgacatcgatt<br>Reverse primer to amplify Sal1-EGFP:<br>tgcaggtcgaCTTACTTGTACAGCTCGTCCATGCCGAG |
| qPCR NLK | For: GGGTCCTCATAAACAGCCATC<br>Rev: AGACCAACATCCTGCAAAGG |
| qPCR GAPDH | For: GGG GTC ATT GAT GGC AAC AAT A<br>Rev: ATG GGC AAG GTG AAG GTC G |