Regulation of nucleolar signalling to p53 through NEDDylation of L11

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Supplementary Information

Methods

Plasmids

All plasmids used were described previously (Xirodimas et al., 2004; Xirodimas et al., 2008). For the NEDD8 siRNA rescue experiment, oligo 7 targets the NEDD8 sequence after the diglycine at the C-terminus of NEDD8. The siRNA insensitive NEDD8 construct ends at the diglycine in its processed form and was cloned untagged in pcDNA3. The L11 K0 mutant was created by site directed mutagenesis. Wild type L11 was cloned as N-terminal fusion to EGFP in the pEGFP-N1 vector (Clontech). H1299 cells stably expressing L11-EGFP were selected with 0.5mg/ml of G418. All sequences were confirmed by automated sequencing.

Antibodies and chemicals

Mdm2 and p53 were detected with 4B2 and DO-1 mouse monoclonal antibodies respectively. Rabbit anti-Ubc12 was purchased from ABGENT, mouse anti-APPBP1 (Abnova), mouse anti-ubiquitin (abcam), mouse anti-actin (Oncogene), mouse anti-cullin-1 (Zymed), rabbit anti-NEDD8 (in house), mouse anti-FLAG and anti-a-Tubulin (SIGMA), rabbit anti-p21 (Santa Cruz), sheep anti-NEDP1 (in house), mouse anti-IκBα (in house), mouse anti-Lamin A/C, goat anti-L11 (Santa Cruz) and mouse anti-His (Clontech). Actinomycin D, G418 and cycloheximide were purchased from SIGMA, MG132 (CalBiochem), TNF-α (Insight Biotechnology). The BCA protein assay kit was purchased from Pierce. All siRNAs were purchased from Dharmacon as On Target plus pools or individual duplexes. The control siRNA is a non-target pool.

Cell culture and transfections

H1299 cells were grown in RPMI, whereas MCF7 and U2OS in DMEM, all supplemented with 10% fetal calf serum. All transfections in H1299 cells were performed with calcium phosphate in 10 cm dishes. The total amount of CMV transfected plasmids was normalised with empty vector. Fugene 6 (Roche) was used for MCF7 and U2OS transfections. For siRNA transfections 30 nM of oligos were transfected with oligofectamine (Invitrogen). For the Mdm2 siRNA experiment, the method described in (Xirodimas et al., 2004) was used.

Gene expression

MCF7 cells were seeded in 6 cm dishes and transfected with 30 nM siRNAs. 48 hrs post transfection cells were treated with 5 nM ActD. RNA was isolated using the Promega SV Total RNA Isolation system. cDNA was made using the Invitrogen SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR with 300 ng of RNA. The cDNA was diluted with H_2O 20x and 2 μl were used in a 12 μl reaction. Real-time PCR was carried out in an ABI 7500 system using ABI PCR master mix according to the manufacture's instructions. Primers for mdm2 (P2) promoter, p21 and bax were previously described (Saville et al., 2004). Primers for actin, L11 and nedd8 were purchased from ABI.

Immunoprecipitations

Cells after 2x wash with PBS were harvested in PBS and the pellet was lysed in GE buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 10 μM $ZnCl_2$, 10 % glycerol, 1 % Triton X-100) containing Roche cocktail protease inhibitors. Cell lysis was performed for 30 min on ice before centrifugation at 4 ^{o}C for 15 min. Equal amount of total protein was incubated with the appropriate antibody

overnight before addition of 20 μl of protein G beads and 1 hr rotation at 4 ^{o}C. Beads were washed 3x with GE buffer, 50 μl of 2xSDS loading buffer was added and boiled for 5 min. Eluates were then used for western blot analysis .

Subcellular fractionation

The method described in (Lee et al., 1994) was used. Cells were washed and harvested in PBS. The pellet was resuspended in 1 packed cell volume of Buffer A (10 mM Hepes, pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF). Cells were allowed to swell on ice for 15 min, before lysed through a 26-gauge needle (5 strokes). Cells were centrifuged for 20 sec at 13,000 rpm at 4 ^{o}C and the supernatant (cytoplasmic fraction) was transferred to a fresh tube for further analysis (Ni^{2+} pull-down or direct western blotting). The pellet (nuclear fraction) was either lysed directly in 8 M urea, pH 8 for Ni^{2+} pull-downs or in 2xSDS loading buffer for western blotting.

Immunofluorescence and FRAP analysis

MCF7 cells were transfected with Fugene 6 with wild type Flag-L11 or the K0 mutant. 48 hrs post transfection cells were fixed with paraformaldehyde and stained with anti-FLAG antibodies (1:3000). Jackson FITC anti-mouse was used at 1:80 dilution before mounting. Between steps cells were washed with PBS for 5 min. For FRAP analysis H1299 cells stably expressing L11-EGFP were seeded in glass-bottom dishes (WILCO, Intracel) and transfected with 30 nM siRNAs. 48 hrs post-transfection, ActD was applied at 10 nM 1 hr prior to the experiment. FRAP measurements were recorded on a DeltaVision Core (Applied Precision, Seattle)

mounted on an Olympus IX71 equipped with a cooled CCD camera (Coolsnap HQ, Photometrics, Tuscon, MA) in a temperature controlled chamber at 37 ^{o}C (Solent Scientific, UK). Photobleaching was performed with a 488 nm, 20 mW laser at 10% power, which was focussed through a 60x oil immersion objective. Nucleoli were photobleached for 0.1 sec, which provided approximately 60% reduction in the EGFP signal. 32 images from each of 15 randomly selected cells were captured within 15 sec. Data analysis was done with SoftWorx (Applied Precision, Seattle).

Legends to figures

S1

(A) Transfections with control or NEDD8 siRNAs were performed in MCF7 cells. 48 hrs post-transfections WCL were analysed by western blotting as indicated. **(B)** H1299 cells were transfected with control or NEDD8 siRNAs and the next day cells were reseeded and the day after transfected with Flag-L11 (5 μg). Next day cycloheximide (30 μg/ml) was applied for the indicated time points and cells were lysed directly in 2x SDS LB. Equal amounts of protein were analysed by western blotting with the indicated antibodies. Signals were quantified with Image Gauge and presented as percentage of remaining protein. Data presented as mean from 3 independent experiments +/- SEM. **(C)** MCF7 cells were transfected with the indicated siRNAs. 48 hrs post-transfection cells were harvested and 20% were used for western blotting whereas the rest for RNA isolation and monitoring of L11 gene expression by TaqMan RT-PCR. Data presented as mean from 2 independent experiments +/- SEM. **(D)** MCF7 cells were transfected as in (A) in duplicates and TNF was applied as indicated to induce degradation of IkBa. WCL were used for western blotting. (**E)** MCF7 cells were transfected in duplicates with control or siRNAs targeting both the regulatory (APPBP1) and the catalytic (Uba3) subunits of the NEDD8 E1 enzyme. 48 hrs post-transfection cells were treated with ActD as indicated and western blotting was performed on WCL.

S2

2x 10 cm dishes of H1299 cells were transfected with 1μg wt Flag-L11 or 4 μg of K0 mutant using Fugene. 36 hrs post-transfection cells were harvested and ribosome profiling was performed as described in (Xirodimas et al., 2008). 1 ml fractions were collected and TCA precipitated before western blotting with anti-Flag antibody.

S3

Representative data for the FRAP analysis using control or NEDD8 siRNAs in the presence or absence of ActD. MF: Mobile Fraction, Half-life of recovery, T(1/2) in seconds.

S4

H1299 cells were transfected with 5 μg of NEDP1 construct. 48 hrs post-transfection cells were treated with ActD for the indicated times and WCL were used for western blotting.

S5

H1299 cells were transfected in 6 well plates with the indicated siRNAs. Next day were reseeded in 10 cm dishes and the day after were transfected with Flag-L11 (5 μg) and His_6-ubiquitin (2 μg) as indicated. MG132 (30 μM for 4 hrs) was applied as indicated. Ubiquitinated proteins and WCL were analysed by western blotting with the appropriate antibodies. Equal loading and transfection efficiency was monitored with β-gal (3 μg).

S6

H1299 cells were transfected with 3.5 μg of wild type Flag-L11 or with the indicated L11 lysine to arginine mutants, 2 μg of His_6-NEDD8 (ND8) or His_6-ubiquitin (ub) as indicated. Modified proteins were purified with Ni^{2+}-agarose and eluates or WCL were analysed by western blotting as before. Expression of His_6-NEDD8 or His_6-ubiquitin was monitored with anti-His antibody. Equal loading and transfection efficiency was monitored with β-gal (3 μg). (Bottom panel) The L11 3CKR mutant retains nucleolar localisation. Immunofluorescence was performed as in Fig. 2C.

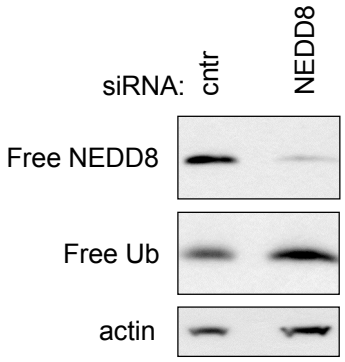
References

Lee, K.A.W., Zerivitz, K. and Akusjärvi, G. (1994). Small-scale preparation of nuclear extracts from mammalian cells. In *Cell Biology: A laboratory handbook* (Ed. J.E. Celis) pp. 668-673. Vol. I. Academic Press.

Saville, M.K., Sparks, A., X irodimas, D.P., Wardrop, J., Stevenson, L.F., Bourdon, J.C., Woods, Y.L. and Lane, D.P. (2004) Regulation of p53 by the ubiquitin-conjugating enzymes UbcH5B/C in vivo. *J Biol Chem*, **279**, 42169-42181.

S1

A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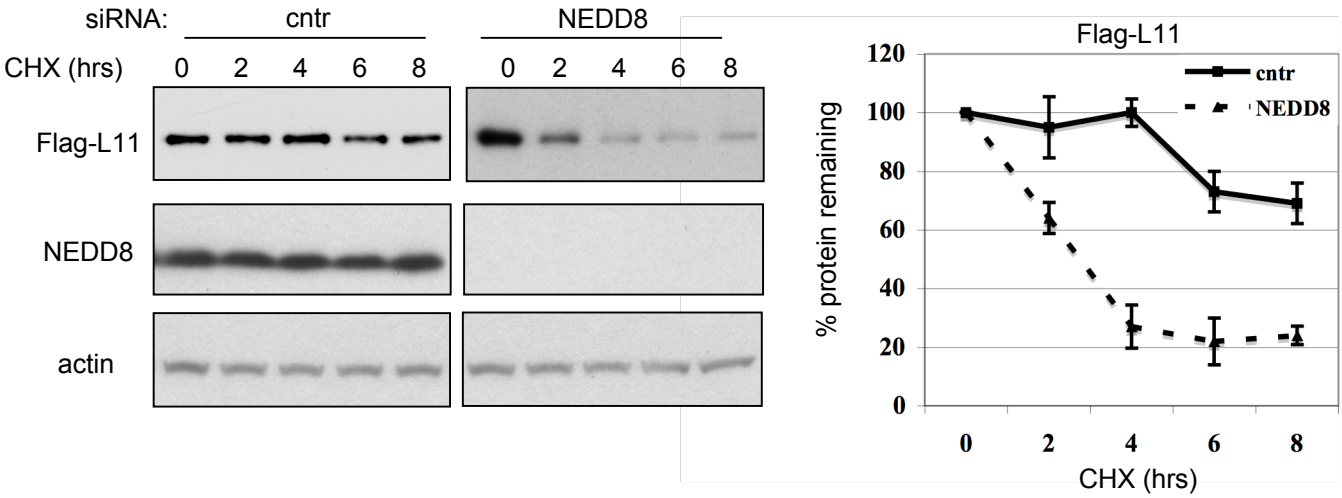
cntr
NEDD8
Free NEDD8
Free Ub
actin

B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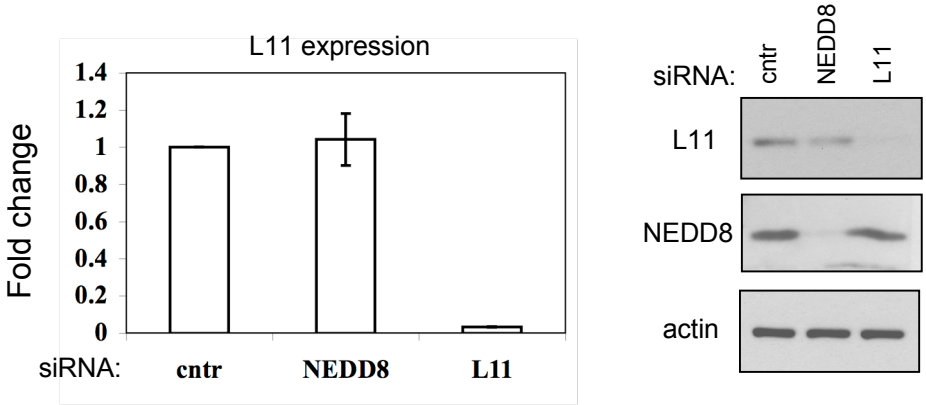
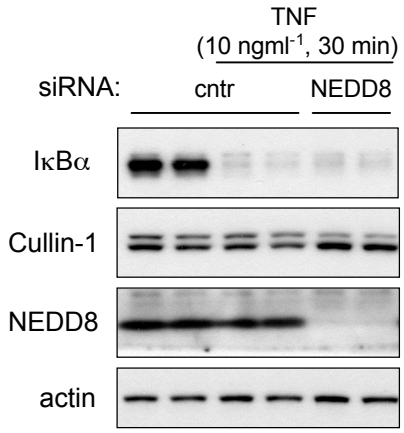
cntr
NEDD8
CHX (hrs)
0 2 4 6 8
0 2 4 6 8
Flag-L11
NEDD8
actin
Flag-L11
cntr
NEDD8
% protein remaining
120
100
80
60
40
20
0
0 2 4 6 8
CHX (hrs)

C


L11 expression
Fold change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
siRNA:
cntr
NEDD8
L11
siRNA:
cntr
NEDD8
L11
L11
NEDD8
actin

S1

D


TNF
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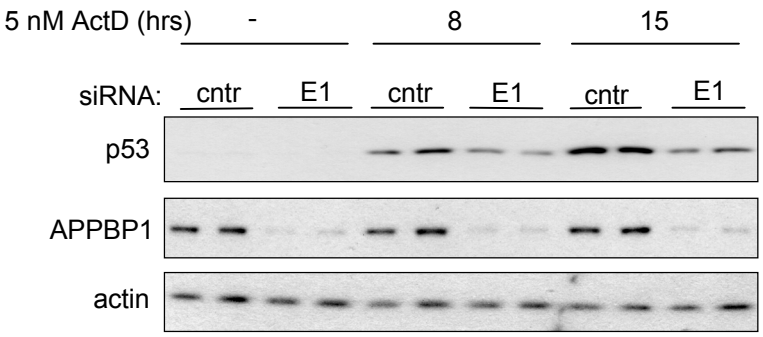
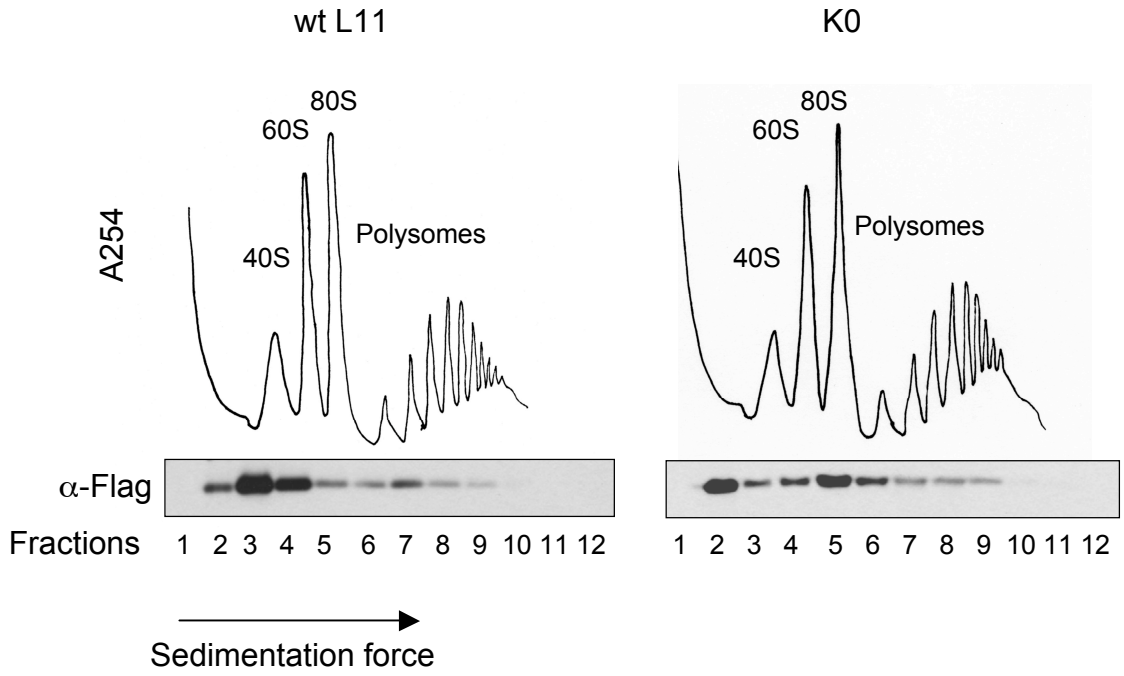
cntr
NEDD8
IκBα
Cullin-1
NEDD8
actin

E


5 nM ActD (hrs)
-
8
15
siRNA:
cntr
E1
cntr
E1
cntr
E1
p53
APPBP1
actin

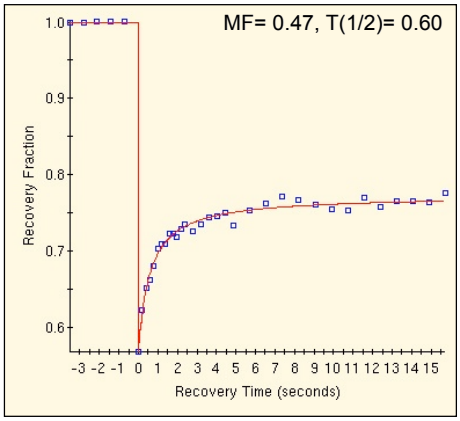
S2


wt L11
K0
80S
60S
40S
Polysomes
A254
α-Flag
Fractions
1 2 3 4 5 6 7 8 9 10 11 12
Sedimentation force

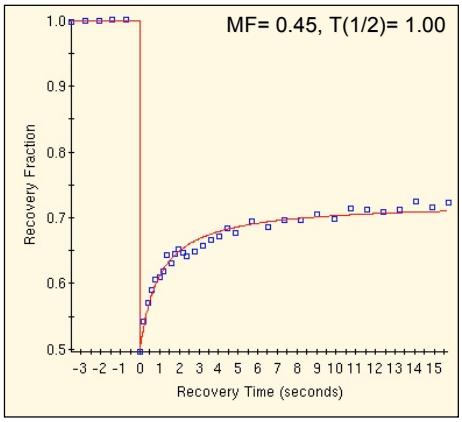
S3

siRNA:

cntr

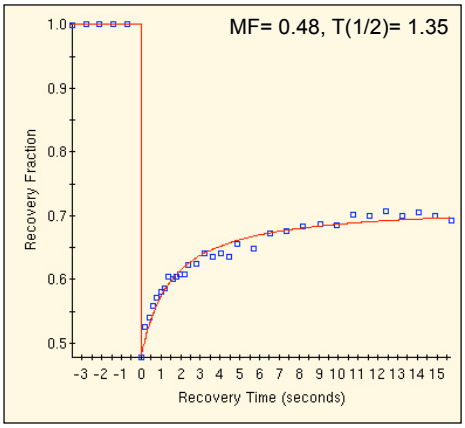


NEDD8

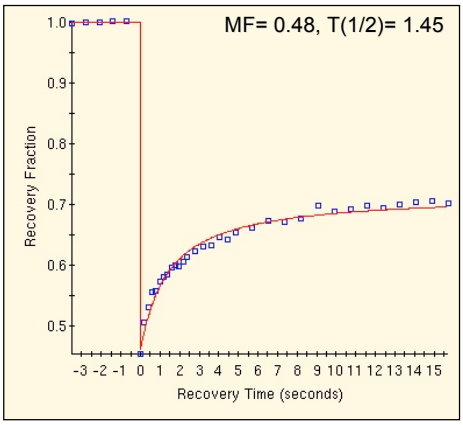


siRNA:

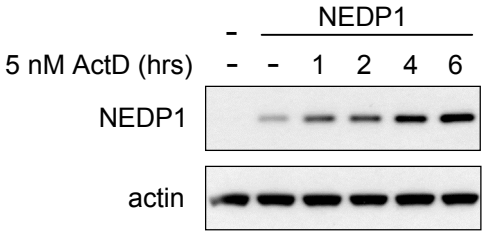
cntr + ActD



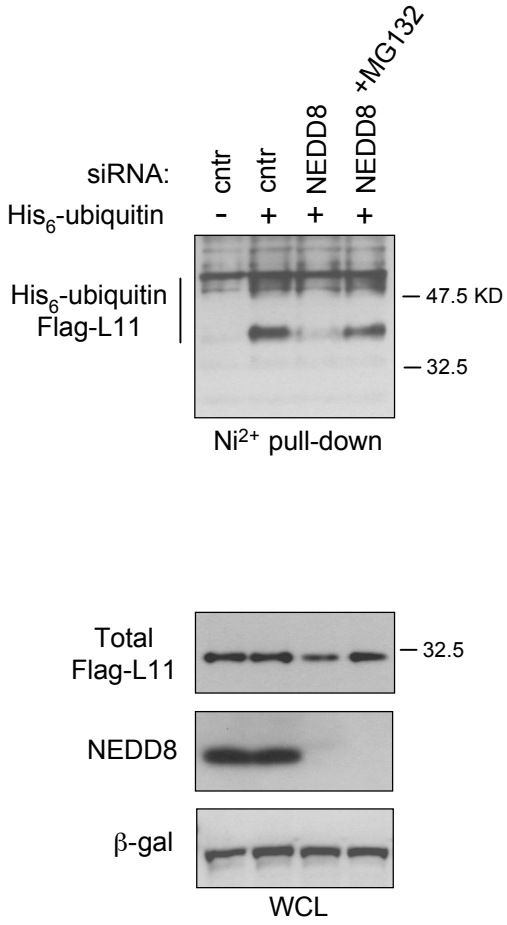
NEDD8 + ActD



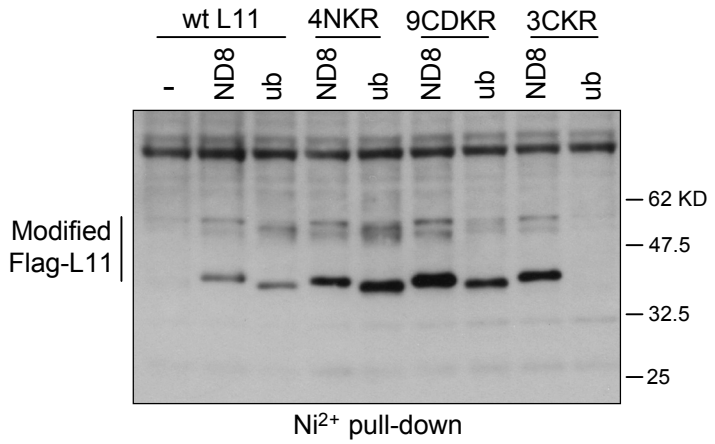
S4

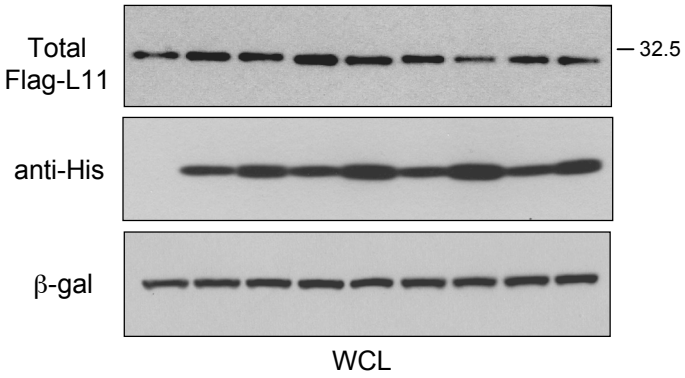


S5


siRNA:
cntr
cntr
NEDD8
NEDD8
+MG132
His6-ubiquitin
-
+
+
+
His6-ubiquitin
Flag-L11
47.5 KD
32.5
Ni2+ pull-down
Total
Flag-L11
32.5
NEDD8
β-gal
WCL

S6





4NKR: K8R, K19R, K38R, K52R

9CDKR: K67R, K78R, K85R, K88R, K96R, K118R, K144R, K145R, K154R,

3CKR: K159R, K169R, K178R