NEDD4 MEDIATES AGONIST-DEPENDENT UBIQUITINATION, LYSOSOMAL TARGETING AND DEGRADATION OF THE β_2 ADRENERGIC RECEPTOR

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Running Title: *Nedd4 regulates β_2AR lifespan*

Supplementary Information

Figure Legends:

S1. Distribution of β_2ARs at 18 and 24 hours of isoproterenol treatment:
Confocal panels display the subcellular distribution of β_2AR in 'green channel' and LAMP2 in 'red channel' in HEK-293 cells that have been stimulated with 1μM isoproterenol for 18h (top row) and 24h (bottom row). Cells in the right panels have also been treated with an inhibitor of lysosomal proteases, leupeptin along with the agonist. In the presence of leupeptin, receptors are stabilized in the lysosomes as indicated by more pronounced receptor and LAMP2 colocalization (yellow).

S2. Effect of isoproterenol on β-arrestin2-Nedd4 interaction in COS-7 cells: A) COS-7 cells were transfected with HA-Nedd4 alone (lanes 1 and 5) or with β-arrestin2-Flag. Twenty four hours post-transfection, cells were serum starved for 1h, stimulated with 1μM isoproterenol for indicated times and immunoprecipitated β-arrestin2 was probed for bound Nedd4 (WW2 domain Ab, Millipore) shown in the top panel. The blot was stripped with Restore stripping buffer (Pierce) and reprobed with Flag antibody (M1, Sigma) displayed in the lower panel. B) The Nedd4 bands in β-arrestin2 immunoprecipitate from three independent experiments were quantified and plotted as bar graphs * $p<0.05$, than non-stimulated condition.

S3. Nedd4 binds to both N and C domains of β-arrestin2:
A. A diagrammatic representation of β-arrestin2 to show the N and C domains and locations of poly-proline sequences.
B. COS-7 cells were transfected with HA-Nedd4 alone or along with β-arrestin2-Flag, β-arrestin2 ΔN-Flag or β-arrestin ΔC-Flag. Flag immunoprecipitates were probed for Nedd4 (top panel) and β-arrestin2 species (third panel). Nedd4 expression as detected in lysates is shown in the middle panel. This experiment is representative of three independent experiments with identical results.

S4. β-arrestin2 binds hNedd4 with mutations in all four WW domains:
A. A schematic showing the location of C2, WW and HECT domains in human-Nedd4. WW4 mutant carries point mutations within all the WW domains, which abolishes its interaction with the ENaC.
B. COS-7 cells were transfected with hNedd4, h-Nedd4 ΔC2 or hNedd4 ΔC2-WW4 alone or with β-arrestin2-Flag. Flag-β-arrestin2 IPs were probed for Nedd4 (top panel) and Flag (3rd panel). Lysate blots

for Nedd4 and β-arrestin2 expression are also depicted. The bracketed bands collectively represent exogenously expressed Nedd4 species. The hNedd4 ΔC2 WW4 mutant expressed at much lower levels than full length or C2 domain mutant did. The IP and Lysate blots were first probed for Nedd4 and later stripped and reprobed for β-arrestin. These results are representative of three independent experiments.

Supplementary Table 1

Receptor levels (femtomoles/mg of cellular protein) determined by [^{125}I]-(-)Iodocyanopindolol binding after 24h of isoproterenol or vehicle treatment:

	Vehicle	Isoproterenol
Vector	776 ± 39	538 ± 23
Mdm2-DN	1181 ± 76	637 ± 94
Nedd4-DN	767 ± 50	704 ± 37
AIP4-DN	1444 ± 248	876 ± 35

Fig S1

Distribution of β_2ARs at 18 and 24 h of isoproterenol treatment

Green: β_2AR

Red: LAMP2

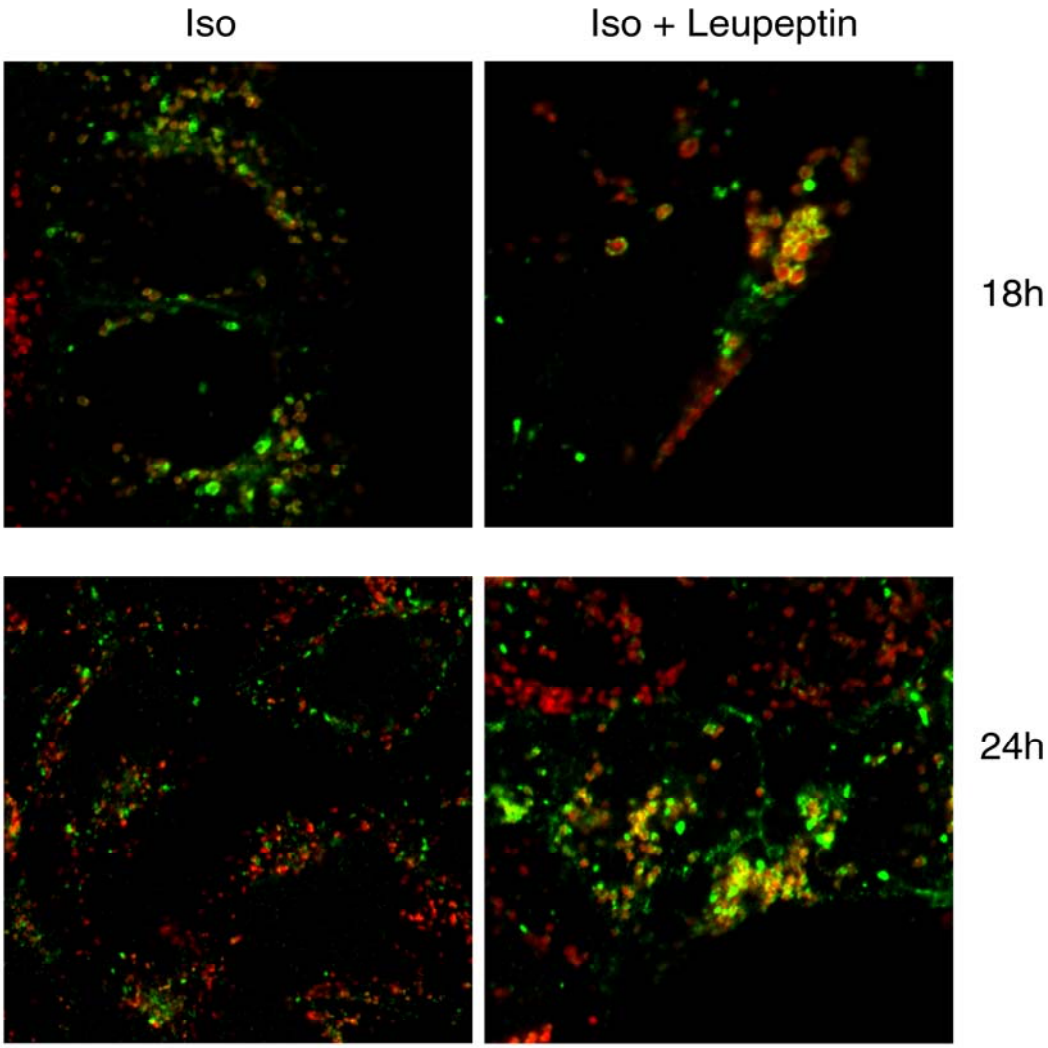

FIG S2

Effect of isoproterenol on β-arrestin2-Nedd4 interaction in COS-7 cells

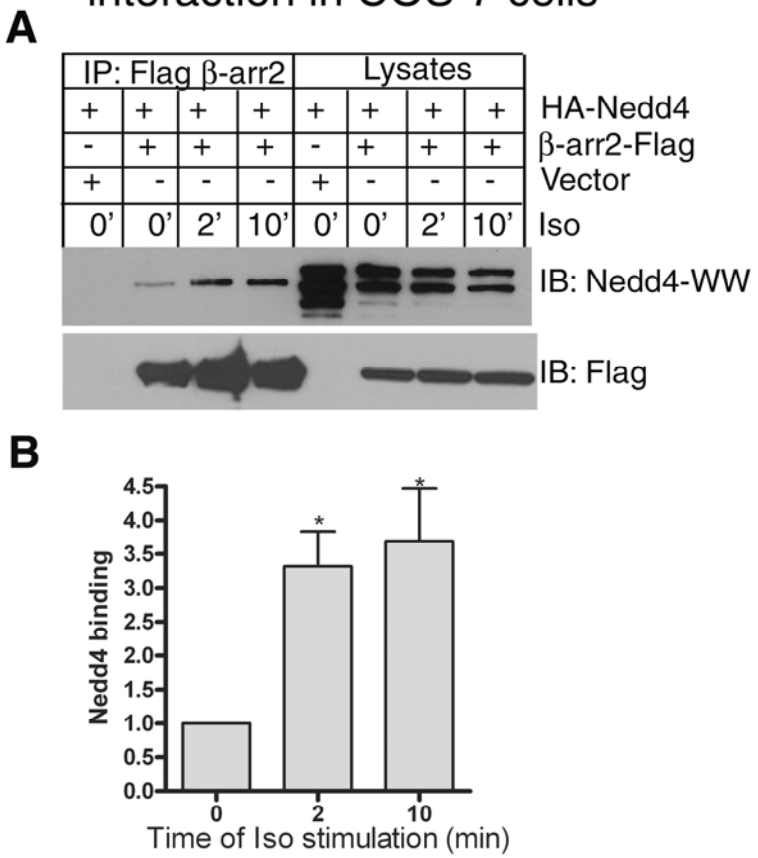

FIG S3

Nedd4 binds to both N and C domains of β-arrestin2

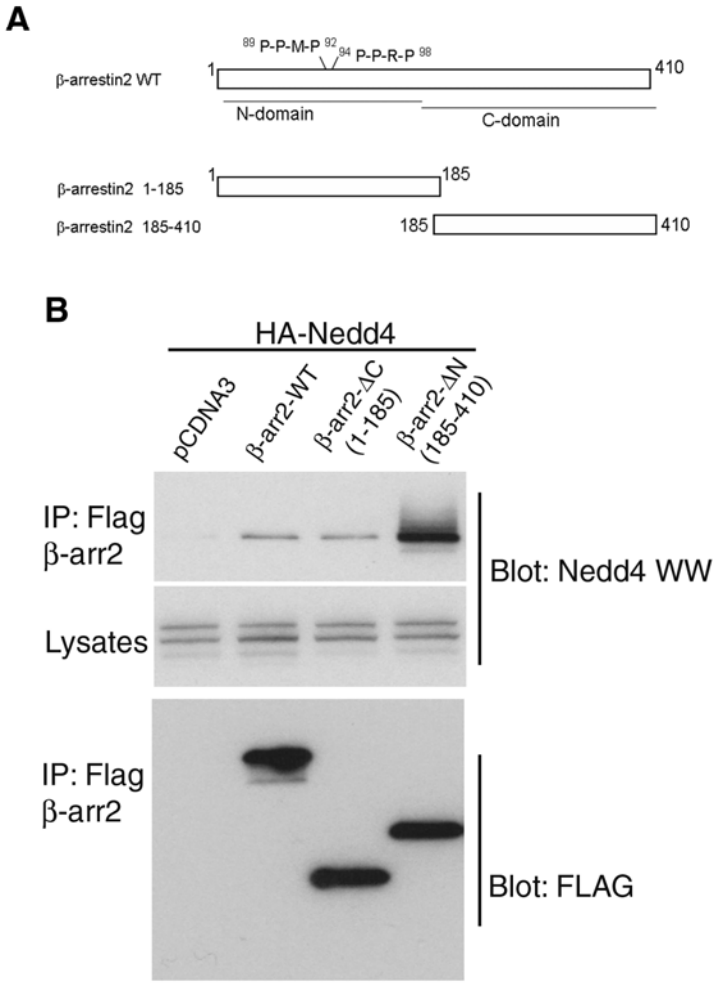

Fig S4

β-arrestin2 binds hNedd4 with mutations in all four WW domains