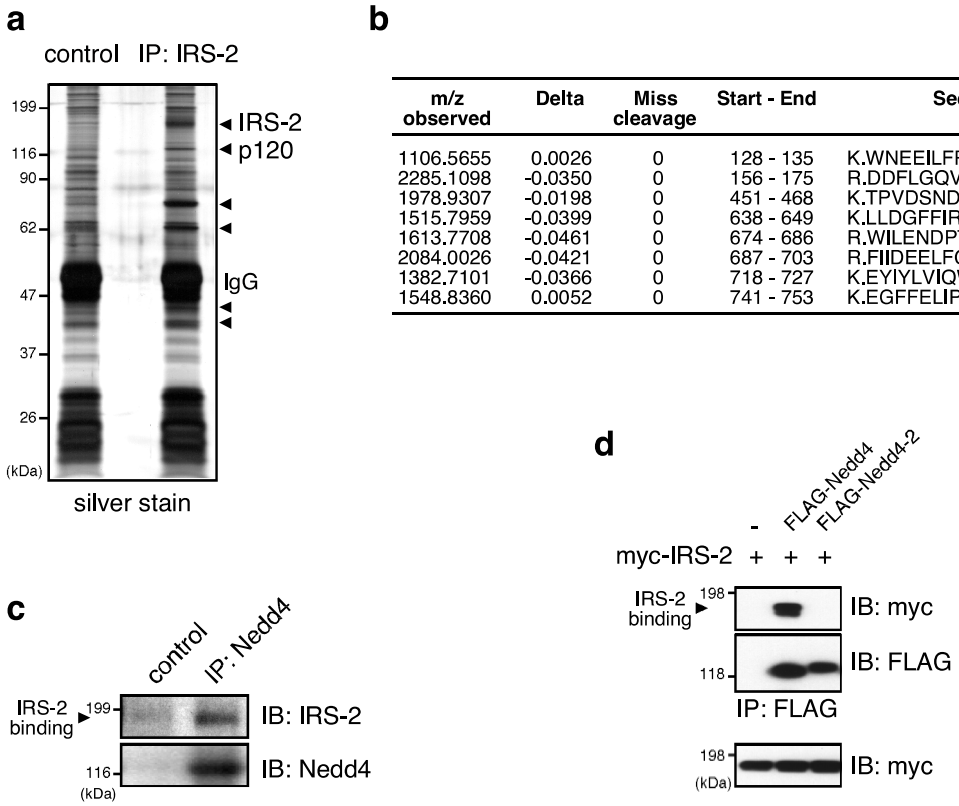
Nedd4-induced mono-ubiquitination of IRS-2 enhances IGF-I signaling and mitogenic activity

(by Fukushima et al.)

Supplementary Figures and Legends

Supplementary Fig. 1

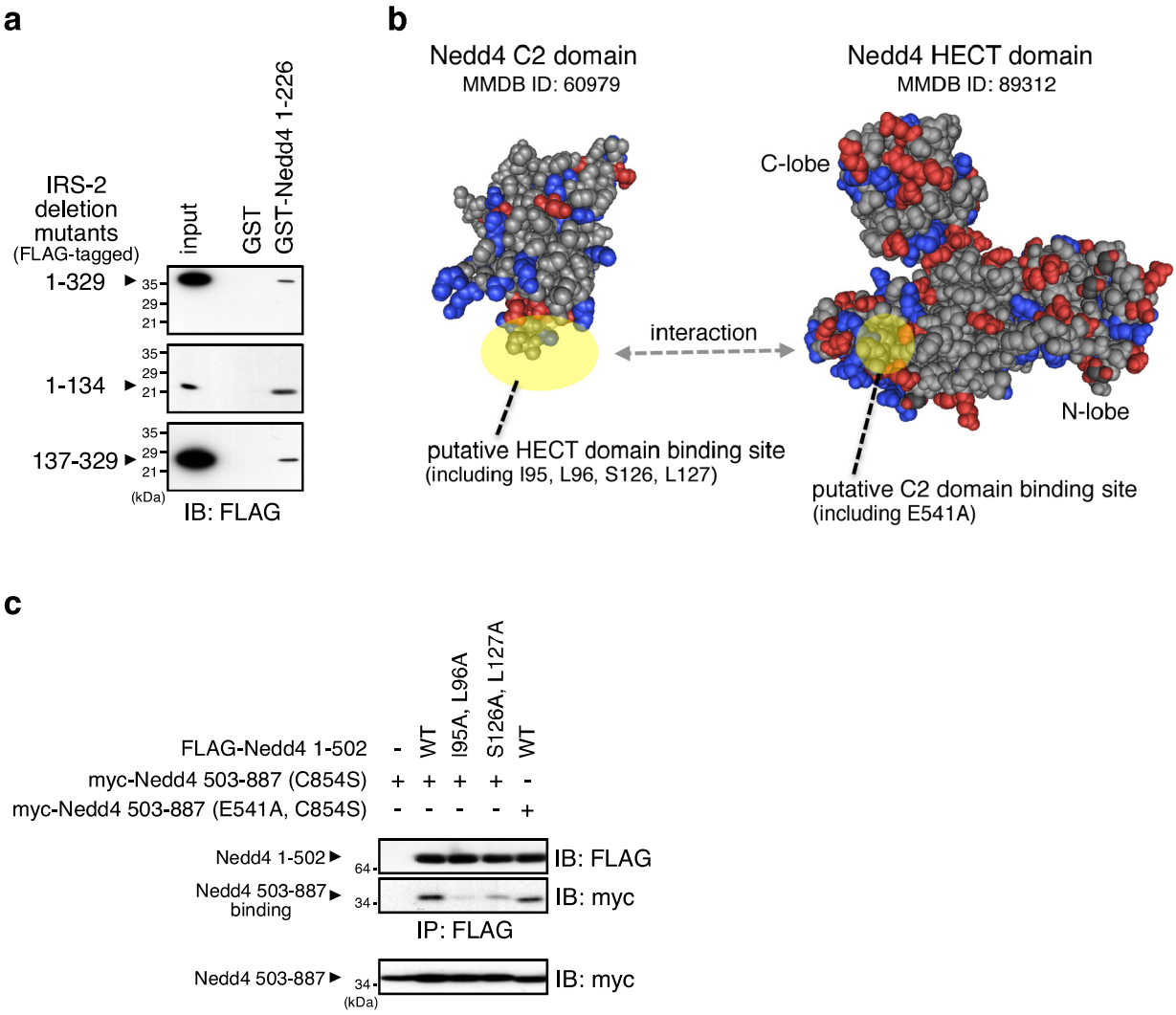


m/z observed	Delta	Miss cleavage	Start - End	Sequence
1106.5655	0.0026	0	128 - 135	K.WNEEILFR.V
2285.1098	-0.0350	0	156 - 175	R.DDFLGQVDVPLYPLPTENPR.M
1978.9307	-0.0198	0	451 - 468	K.TPVDSNDLGPLPPGWEER.T
1515.7959	-0.0399	0	638 - 649	K.LLDGFFIRPFYK.M
1613.7708	-0.0461	0	674 - 686	R.WILENDPTELDLR.F
2084.0026	-0.0421	0	687 - 703	R.FIIDEELFGQTHQHELK.T
1382.7101	-0.0366	0	718 - 727	K.EYIYLVIQWR.F
1548.8360	0.0052	0	741 - 753	K.EGFFELIPQDLIK.I

Supplementary Fig. 1 Identification of Nedd4 as an IRS-2-associated protein in cAMP-treated FRTL-5 cells.

(a) FRTL-5 cells were treated with 1 mM dibutyryl cAMP for 24 h, and the lysates were subjected to immunoprecipitation with anti-IRS-2 antibody. As a negative control, the antigen peptide was mixed with anti-IRS-2 antibody prior to immunoprecipitation. Immunoprecipitates were subjected to SDS-PAGE and silver stain. Representative data is shown, and arrowheads indicate bands of IRS-2-associated proteins. (b) p120 was subjected to "in gel" digestion with trypsin, and the peptides were analyzed with MALDI-TOF MS. Peptides attributed to E3 ubiquitin ligase Nedd4 are shown (#matched peaks/#subjected peaks, 8/24; sequence cover 12%). (c) Lysates of cAMP-treated FRTL-5 cells were subjected to immunoprecipitation and immunoblotting using the indicated antibodies. Non-specific IgG was used as a negative control. (d) HEK293 cells overexpressing indicated proteins were serum starved. Cell lysates were subjected to immunoprecipitation and immunoblotting.

Supplementary Fig. 2

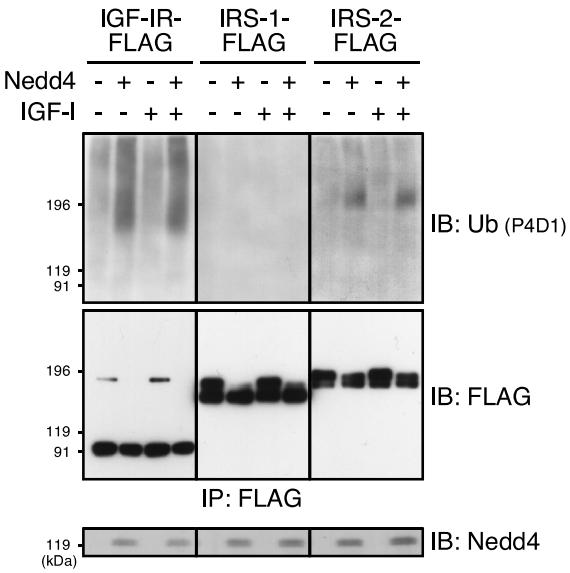


Supplementary Fig. 2 The association of Nedd4 with IRS-2 (related to Fig. 1)

(a) The association of the Nedd4 N-terminal region and the IRS-2 PH/PTB domain in cell-free binding assay. HEK293T cells overexpressing IRS-2 deletion mutants were serum-starved, and the lysates were subjected to pull-down analysis using GST-Nedd4 1-226. (b) Putative intramolecular binding sites in the Nedd4 C2 domain and HECT domain. Blue, basic residues; red, acidic residues. (c) Determination of the intramolecular binding sites in Nedd4 C2 domain and HECT domain. HEK293T cells overexpressing indicated proteins were serum-starved. Cell lysates were subjected to immunoprecipitation and immunoblotting. Nedd4 503-887 (C854S), a mutant of the ubiquitin ligase active site, was used because intact Nedd4 503-887 was unstable.

Supplementary Fig. 3

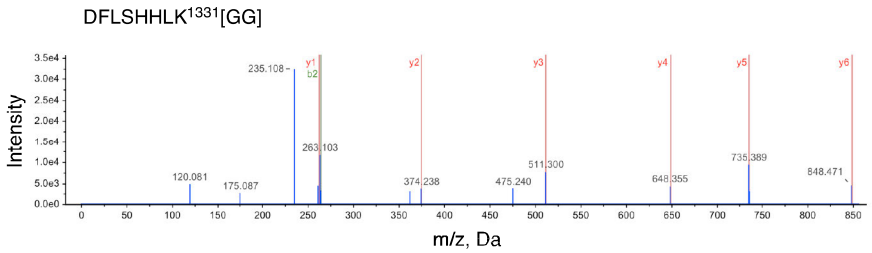
a



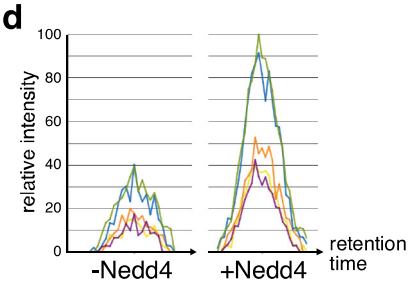
b

Region	Ubiquitination site	trypsin		trypsin and Asp-N		trypsin and chymotrypsin	
		Detected Peptide	Confidence	Detected Peptide	Confidence	Detected Peptide	Confidence
PH	K80	LEYYESEkK	99	LEYYESEkK	99		
	K81			LEYYESEKk	99		
	K90					AGAPkR	44.7
	K103			DCCLNINkR	99		
	K110	HkYLIALYTK	58.4	HkYLIALYTK	86.6		
	K118			YLIALYTk	88		
PTB	K203	EVWQVNLkPK	99	EVWQVNLkPK	99	QVNLkPK	63.8
	K205	EVWQVNLKPkGLGQSK	87.4	EVWQVNLKPkGLGQSK	87.3	QVNLKPkGL	99
	K211	GLGQSkNLTGVYR	99	GLGQSkNLTGVYR	99	GLGQSkNLTGVY	96.3
	K230			TIGFVkLNCEQPSVTLQLMNIR	99	VkLNCEQPSVTL	94.1
	K292			DDSVVAQNIHETILEAMkALK	95.8		
	K295			DDSVVAQNIHETILEAMKALk	93		
	K305	SkSQSSGSSATHPISVPGAR	99	SkSQSSGSSATHPISVPGAR	99	SkSQSSGSSATHPISVPGAR	99
IPK	K355	TDSLAATPPAAkCSSCR	78.2	DSLAATPPAAkCSSCR	99	TDSLAATPPAAkC	98.3
	K415	GSkVALLPAGGALQH	81.8			GSkVALLPAGGAL	99
KRLB	K687			DDYMPMSPASVSAPkQILQPR	99	SPASVSAPkQILQPR	99
C1	K750	MWCGSkLSMEHADGK	10				
	K811	SYkAPYTCGGDSDQYVLMSSPVGR	96.5	SYkAPYTCGGDS	89.8		
C2	K1092					GVAATPPQPIAAPPkPEAAR	99
	K1106	VASPTSGVkR	15.3	VASPTSGVkR	23.5	VASPTSGVkR	22.9
C3	K1134			GAkVIR	21.8	GAkVIR	28.1
	K1184	kSSEGGVGVGPGGGDEPPTSPR	27.7				
	K1281	EEPGLPPQPQPPPPPLPQPGDkSSWGR	96.5	DkSSWGR	88.3	EEPGLPPQPQPPPPPLPQPGDkSSW	96.6
	K1331			DFLSHHLk	99	kEATIVK	17.5

c



d



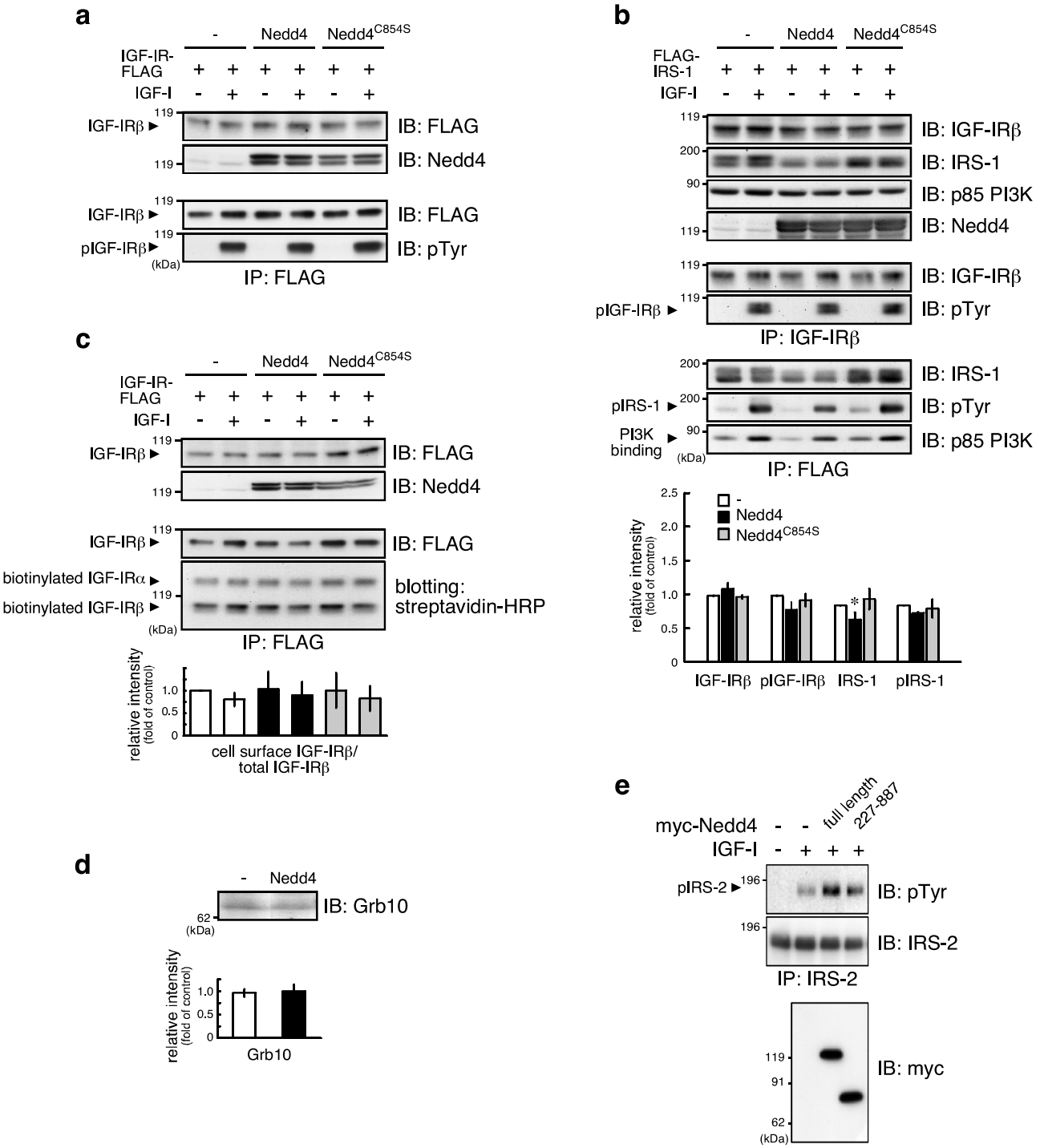
e

IRS-2 peptides	Sequence	Precursor m/z (charge state)	Fragment ions for PRM
K1106	VASPTSGVK[1106][GG]R	598.295 (+2)	$y_2^+, y_3^+, y_4^+, y_5^+, y_6^+, y_7^+, y_8^+$
K1134	GAK[1134][GG]VIR	379.238 (+2)	$y_2^+, y_3^+, y_4^+, y_5^+$
K1281	DK[1281][GG]SSWGR	475.228 (+2)	$y_2^+, y_3^+, y_4^+, y_5^+, y_6^+$
K1331	DFLSHHLK[1331][GG]	555.788 (+2)	$y_2^+, y_3^+, y_4^+, y_5^+, y_6^+$

Supplementary Fig. 3 Ubiquitination of IRS-2 by Nedd4 (related to Fig. 2)

(a) Effects of Nedd4 overexpression on IGF-IR, IRS-1 and IRS-2 ubiquitination. HEK293T expressing indicated proteins were serum-starved, followed by IGF-I stimulation (100 ng/ml, 1 min). Cell lysates were denatured, followed by immunoprecipitation and immunoblotting using the indicated antibody. (b) IRS-2 peptides with ubiquitin remnant detected by LC-MS/MS analysis. IRS-2 immunoprecipitates derived from HEK293T cells expressing Nedd4 and IRS-2 were treated with indicated proteases, and the resulted peptides with ubiquitin remnant motif (K-ε-GG) were enriched using anti-ubiquitin remnant motif antibody-conjugated beads. The sample was subjected to LC-MS/MS analysis. Peptides attributed to IRS-2 are shown. Lower case letters "k" in peptide sequences indicate ubiquitinated Lys residues. (c) Representative MS/MS spectrum of the peptide "DFLSHHLK^{1331}[GG]". (d, e) Quantitative MS analysis results. IRS-2 immunoprecipitates derived from HEK293T cells expressing IRS-2 (-Nedd4) or IRS-2 and Nedd4 (+Nedd4) were treated with trypsin and Asp-N. Peptides with ubiquitin remnant motif were enriched, and subjected to LC-MS/MS analysis. Chromatograms of several product ions (different colors) derived from DFLSHHLK^{1331}[GG] are shown in (d). Fragment ions used for PRM are shown in (e). The area under the curves (AUCs) were summed and shown in Fig. 2i.

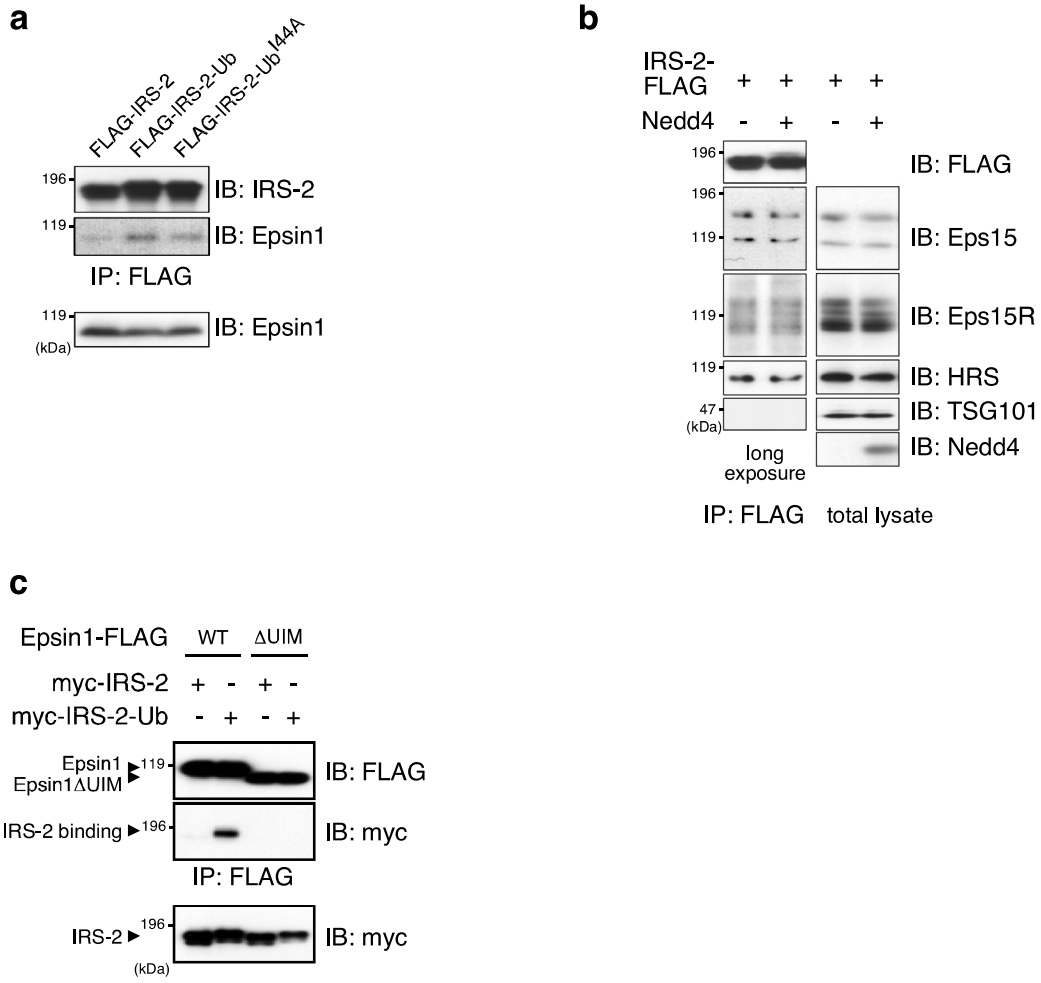
Supplementary Fig. 4



Supplementary Fig. 4 Regulation of IGF-I signaling by Nedd4 (related to Fig. 3)

(a, b) Effects of Nedd4 overexpression on IGF-I-dependent IGF-IR and IRS-1 tyrosine phosphorylation. HEK293 cells overexpressing Nedd4 or Nedd4^{C854S} together with IGF-IR-FLAG (a) or FLAG-IRS-1 (b) were serum-starved, followed by IGF-I stimulation (100 ng/ml, 1 min). Lysates were subjected to immunoprecipitation and immunoblotting as indicated. In (b), densitometric analyses were performed, and tyrosine phosphorylation levels of IGF-IRβ and IRS-1 were normalized to their protein levels in immunoprecipitates. p85 PI3K bound to IRS-1 was normalized to IRS-1 levels in immunoprecipitates. The graph shows means ± SD of three independent experiments. □, significant difference from control (P<0.05, one-way ANOVA followed by Tukey-Kramer test). (c) Effects of Nedd4 overexpression on cell surface IGF-IR levels. HEK293 cells overexpressing Nedd4 or Nedd4^{C854S} together with IGF-IR-FLAG were serum-starved, followed by IGF-I stimulation (100 ng/ml, 1 min). Cell surface proteins were biotinylated, followed by immunoprecipitation and blotting with indicated antibodies or streptavidin-HRP. Densitometric analyses were performed, and biotinylated IGF-IRβ □□□□□□ were normalized to IGF-IRβ levels in immunoprecipitates. The graph shows means ± SD of four independent experiments. (d) Effects of Nedd4 overexpression on Grb10 levels. HEK293 cells overexpressing IRS-2 alone, or IRS-2 and Nedd4 were serum-starved. Grb10 levels were measured by immunoblotting. The graph shows show means ± SD of three independent experiments. (e) Effects of the deletion of Nedd4 N-terminal region on IRS-2 tyrosine phosphorylation. HEK293 cells overexpressing IRS-2 and myc-Nedd4 (wild type or aa 227-887) were serum-starved, followed by IGF-I stimulation (100 ng/ml, 1 min). Cell lysates were subjected to immunoprecipitation and immunoblotting using the indicated antibodies.

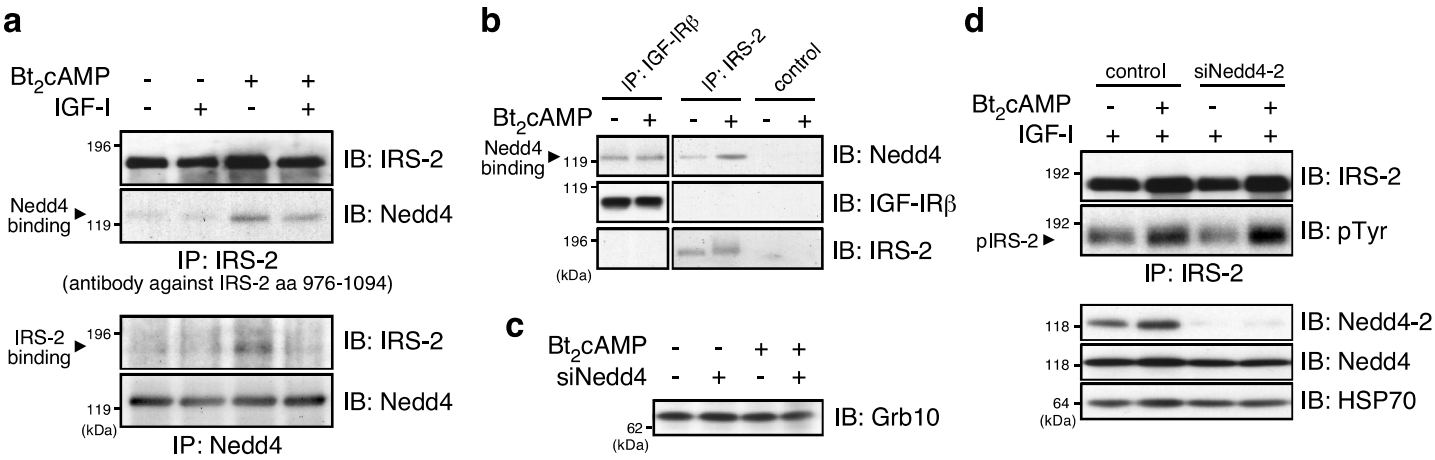
Supplementary Fig. 5



Supplementary Fig. 5 Association of IRS-2 with ubiquitin binding proteins (related to Fig. 4)

(a) Effects of the IRS-2-ubiquitin chimeric protein and its mutant, (b) Nedd4 overexpression, and (c) the deletion of Epsin1 UIM motifs on the association of IRS-2 with the indicated ubiquitin binding proteins. HEK293T cells overexpressing indicated proteins were serum-starved. Lysates were subjected to immunoprecipitation and immunoblotting as indicated.

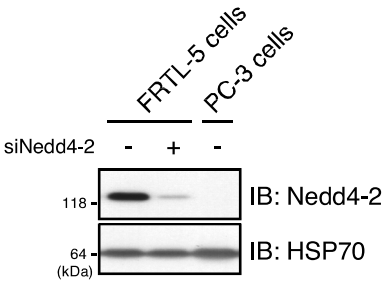
Supplementary Fig. 6



Supplementary Fig. 6 Regulation of IGF-I signaling by Nedd4 in thyrocytes (related to Fig. 5)

(a, b) The association of Nedd4 with IRS-2 or IGF-IR. FRTL-5 cells were treated with 1 mM dibutyryl cAMP for 24 h (a, b), and then treated with 100 ng/ml IGF-I for 1 min (a). Cell lysates were subjected to immunoprecipitation and immunoblotting as indicated. In (b), the antigen peptide was mixed with anti-IRS-2 antibody prior to immunoprecipitation as a negative control. (c) Effects of Nedd4 knockdown on Grb10 levels. Cells transfected with Nedd4 siRNAs were treated with dibutyryl cAMP. Grb10 levels were measured by immunoblotting. (d) Effects of Nedd4-2 knockdown on IGF-I-induced IRS-2 tyrosine phosphorylation. Cells transfected with Nedd4-2 siRNAs were treated with dibutyryl cAMP followed by IGF-I stimulation as described above. Cell lysates were subjected to immunoprecipitation and immunoblotting.

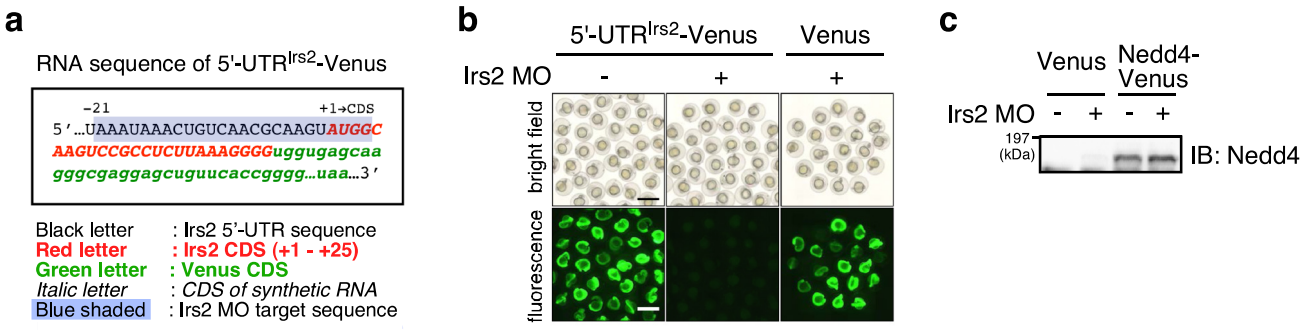
Supplementary Fig. 7



Supplementary Fig. 7 Lack of Nedd4-2 expression in PC-3 cells (related to Fig. 6)

FRTL-5 cells and PC-3 cells were transfected with Nedd4-2 siRNAs or control RNAs. Cell lysates were subjected to immunoblotting using the indicated antibodies.

Supplementary Fig. 8

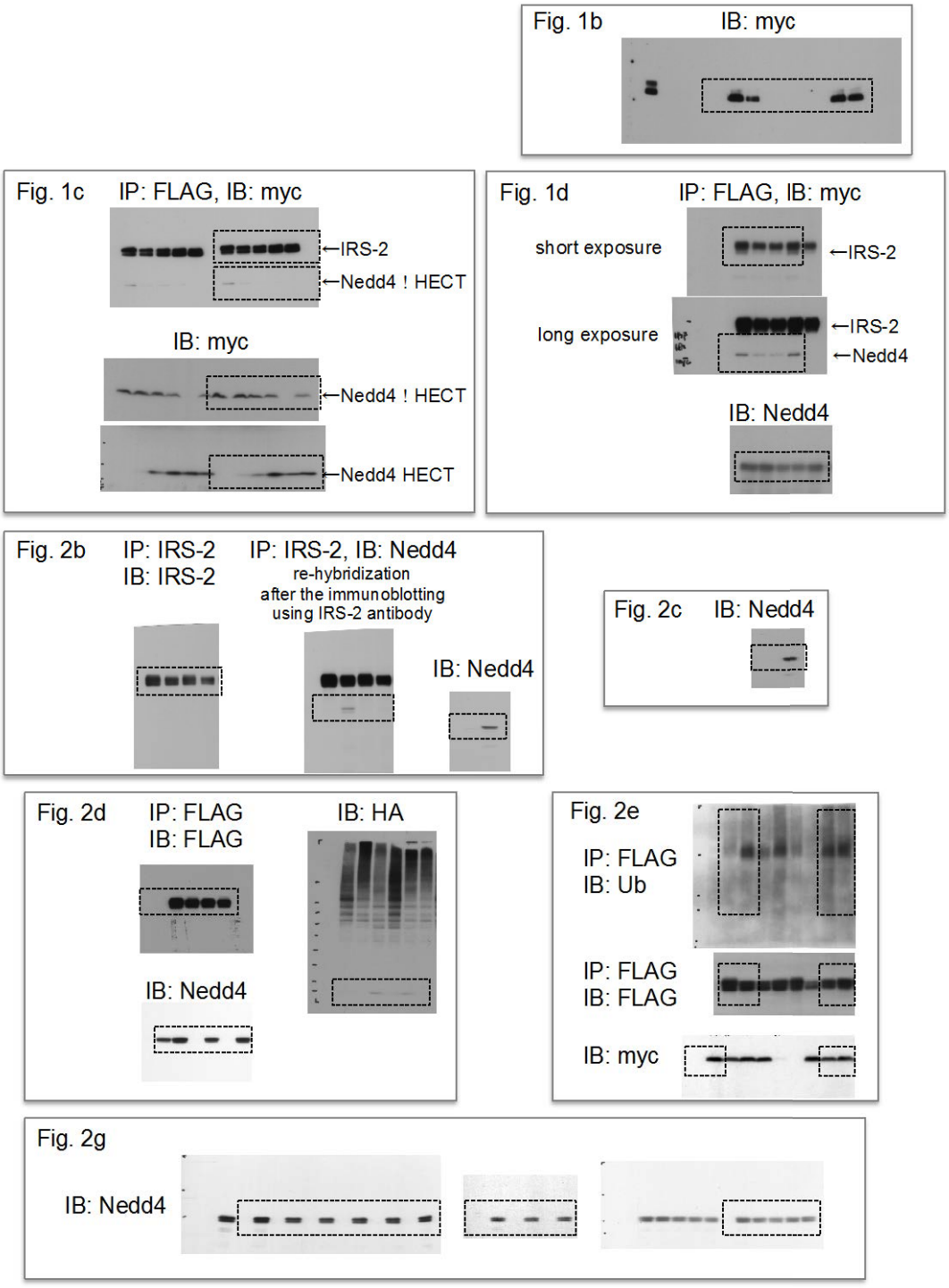


Supplementary Fig. 8 Confirmation of Irs2 knockdown and Nedd4 expression in zebrafish embryo (related to Fig. 7)

(a) Sequence information of the 5'-UTR^{Irs2}-Venus capped RNA used for the validation of zebrafish Irs2 MO. The RNA contains a sequence of zebrafish Irs2 mRNA 5'-UTR (black letter) and translation start site (25 base, red letter), fused with Venus fluorescent protein-cording sequence (green letter). Irs2 MO target sequence is shown as blue shaded.

(b) Efficient knockdown of Irs-2 by Irs2 MO. 5'-UTR^{Irs2}-Venus mRNA or Venus mRNA was co-injected with either Irs2 MO or control MO into 1-2 cell stage embryos. Bright field images and Venus fluorescence at 22 hpf are shown. bars, 1.0 mm. (c) Successful Nedd4 expression. Nedd4-Venus mRNA or Venus mRNA was co-injected with either Irs2 MO or control MO into 1-2 cell stage embryos. Cell lysate was prepared from 12 hpf embryos, and subjected to immunoblotting.

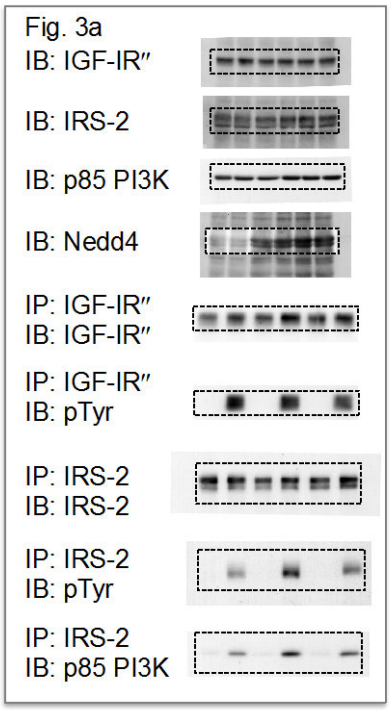
Supplementary Fig. 9

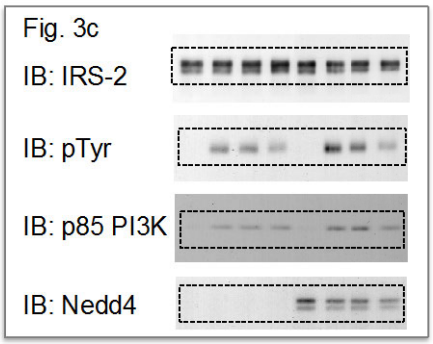


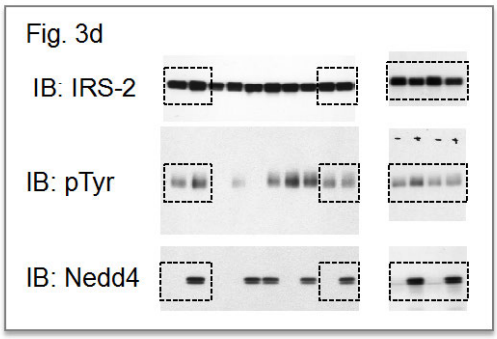
Supplementary Fig. 9 Uncropped scans of blots shown in Fig. 1b, 1c, 1d, 2b, 2c, 2d, 2e and 2g.

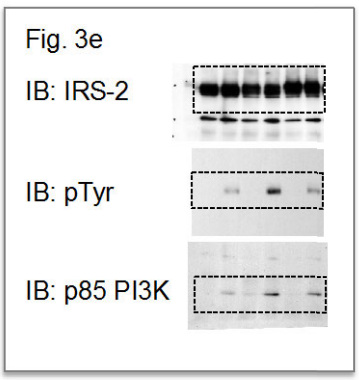
Rectangles delimit cropped areas used in the indicated panels.

Supplementary Fig. 10





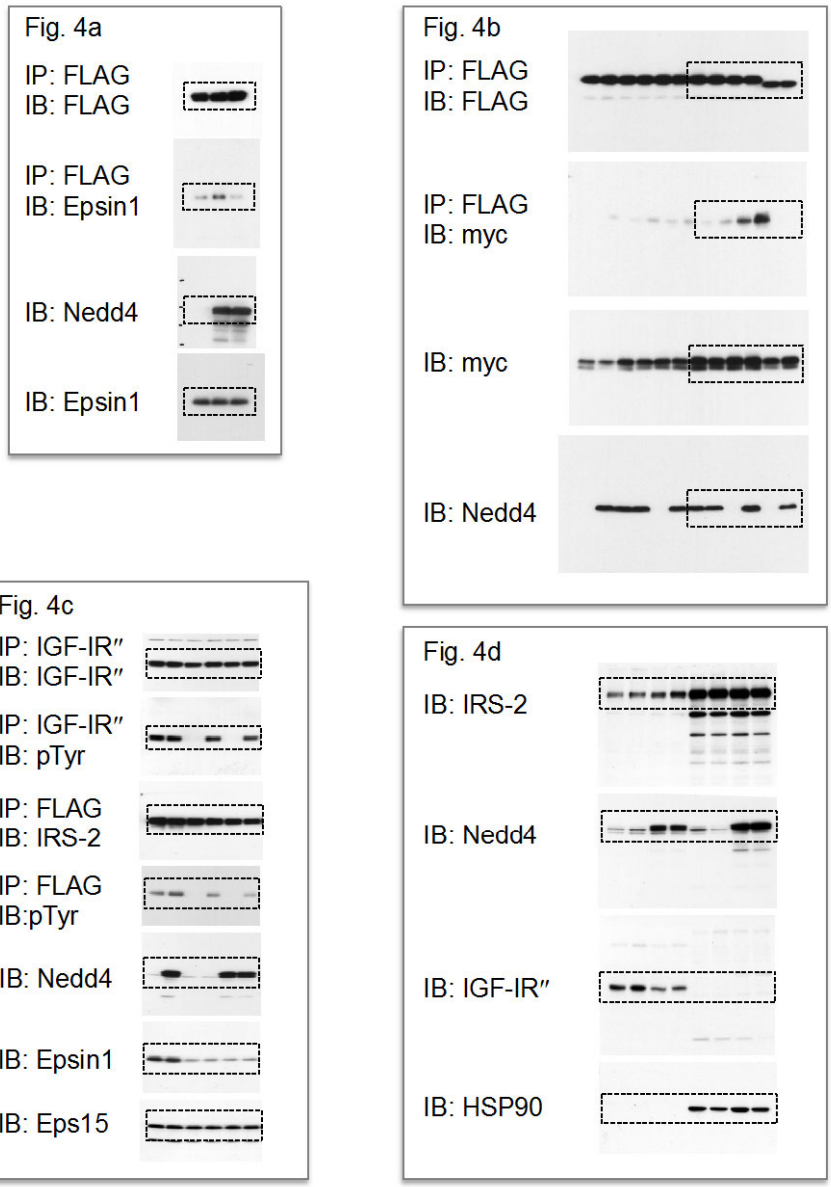




Supplementary Fig. 10 Uncropped scans of blots shown in Fig. 3a, 3c, 3d and 3e.

Rectangles delimit cropped areas used in the indicated panels.

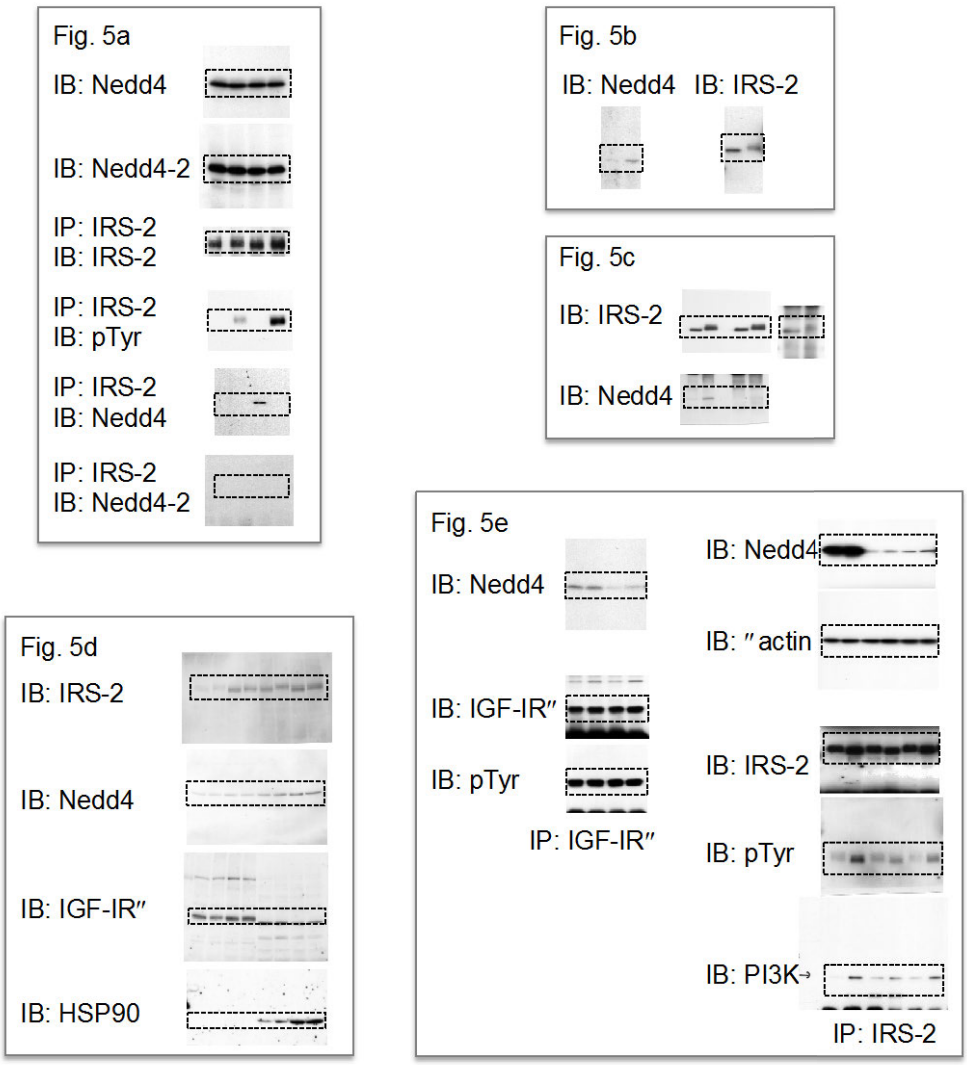
Supplementary Fig. 11



Supplementary Fig. 11 Uncropped scans of blots shown in Fig. 4a, 4b, 4c and 4d.

Rectangles delimit cropped areas used in the indicated panels.

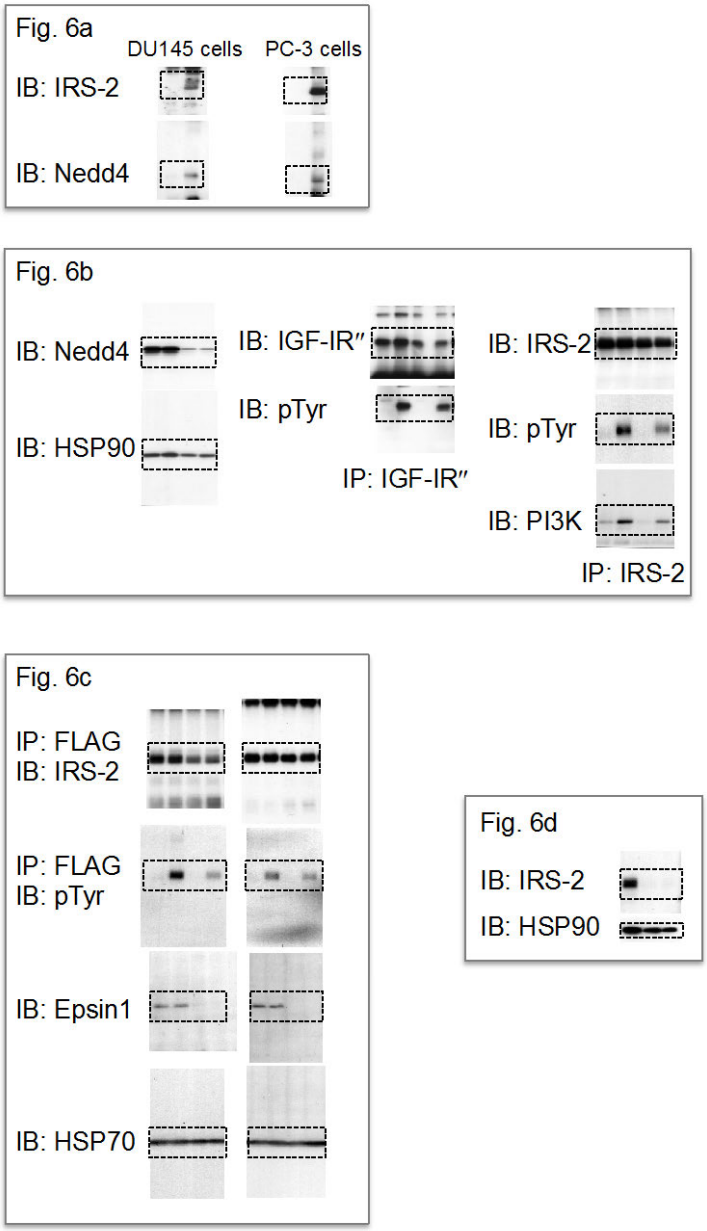
Supplementary Fig. 12



Supplementary Fig. 12 Uncropped scans of blots shown in Fig. 5a, 5b, 5c, 5d and 5e.

Rectangles delimit cropped areas used in the indicated panels.

Supplementary Fig. 13



Supplementary Fig. 13 Uncropped scans of blots shown in Fig. 6a, 6b, 6c and 6d.

Rectangles delimit cropped areas used in the indicated panels.