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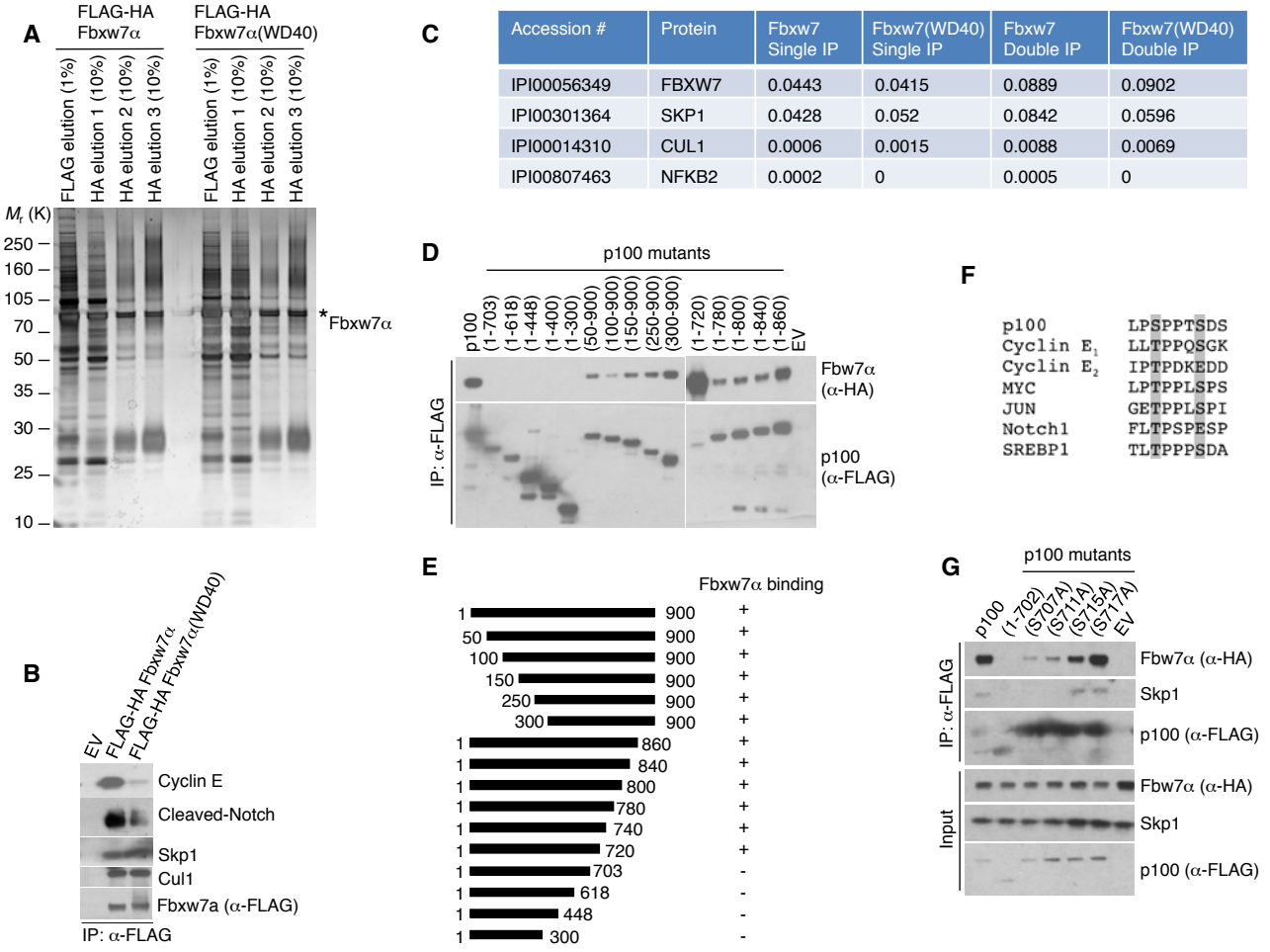
nature cell biology



Accession #	Protein	Fbxw7 Single IP	Fbxw7(WD40) Single IP	Fbxw7 Double IP	Fbxw7(WD40) Double IP
IPI00056349	FBXW7	0.0443	0.0415	0.0889	0.0902
IPI00301364	SKP1	0.0428	0.052	0.0842	0.0596
IPI00014310	CUL1	0.0006	0.0015	0.0088	0.0069
IPI00807463	NFKB2	0.0002	0	0.0005	0

Figure S1 Purification of the Fbxw7α complex and identification of the p100 degron. **(a)** Purification of the Fbxw7α complex. HEK293 cells were transfected with constructs encoding either FLAG-HA tagged Fbxw7α or FLAG-HA tagged Fbxw7α(WD40). Proteins were immunoprecipitated (IP) with anti-FLAG resin (α-FLAG), and a second immunoprecipitation was performed with an anti-HA antibody, as indicated. Immunocomplexes were eluted with 1% SDS, precipitated, and resolved by SDS-PAGE. The gel was then stained with silver stain for protein visualization. **(b)** Fbxw7α binds to substrates through specific residues in the WD40 domain. HEK293 cells were transfected with cDNAs encoding either FLAG-HA tagged Fbxw7α or FLAG-HA tagged Fbxw7α(WD40), a previously-established, substrate binding mutant (Hao *et al. Mol Cell* **26**, 131-143, 2007), in which three residues (Ser462, Thr463, and Arg465) within one of the seven WD40 repeats in Fbxw7α are changed to Ala. Proteins were immunoprecipitated (IP) from cell extracts with anti-FLAG resin (α-FLAG), and immunoblotting was performed for the indicated proteins. In contrast to WT Fbxw7α, Fbxw7α(WD40) was unable to bind its substrates (*e.g.*, cyclin E and intracellular Notch1), although it still interacted with core components of the SCF ligase, such as Skp1 and Cul1. **(c)** The table shows mass spectrometry analysis of four Fbxw7α immunoprecipitations, listing normalized spectral abundance factors for the proteins indicated. **(d)** p100 binds Fbxw7α through its C-terminal domain. HEK293 cells were transfected with constructs encoding HA-tagged Fbxw7α and FLAG-tagged p100 deletion mutants. p100 was immunoprecipitated from cell extracts with anti-FLAG resin (α-FLAG), and immunocomplexes were probed with antibodies to the indicated proteins. **(e)** Schematic representation of p100 mutants. p100 mutants that interact with Fbxw7α are designated with the symbol (+). **(f)** Alignment of the p100 degron with the degrons of other Fbxw7 substrates. Conserved serine, threonine or negatively charged residues are highlighted in gray. **(g)** p100 binds Fbxw7α through Ser707 and Ser711. HEK293 cells were transfected with constructs encoding HA-tagged Fbxw7α and FLAG-tagged p100 mutants. p100 was immunoprecipitated from cell extracts with anti-FLAG resin (α-FLAG), and immunocomplexes were probed with antibodies to the indicated proteins.

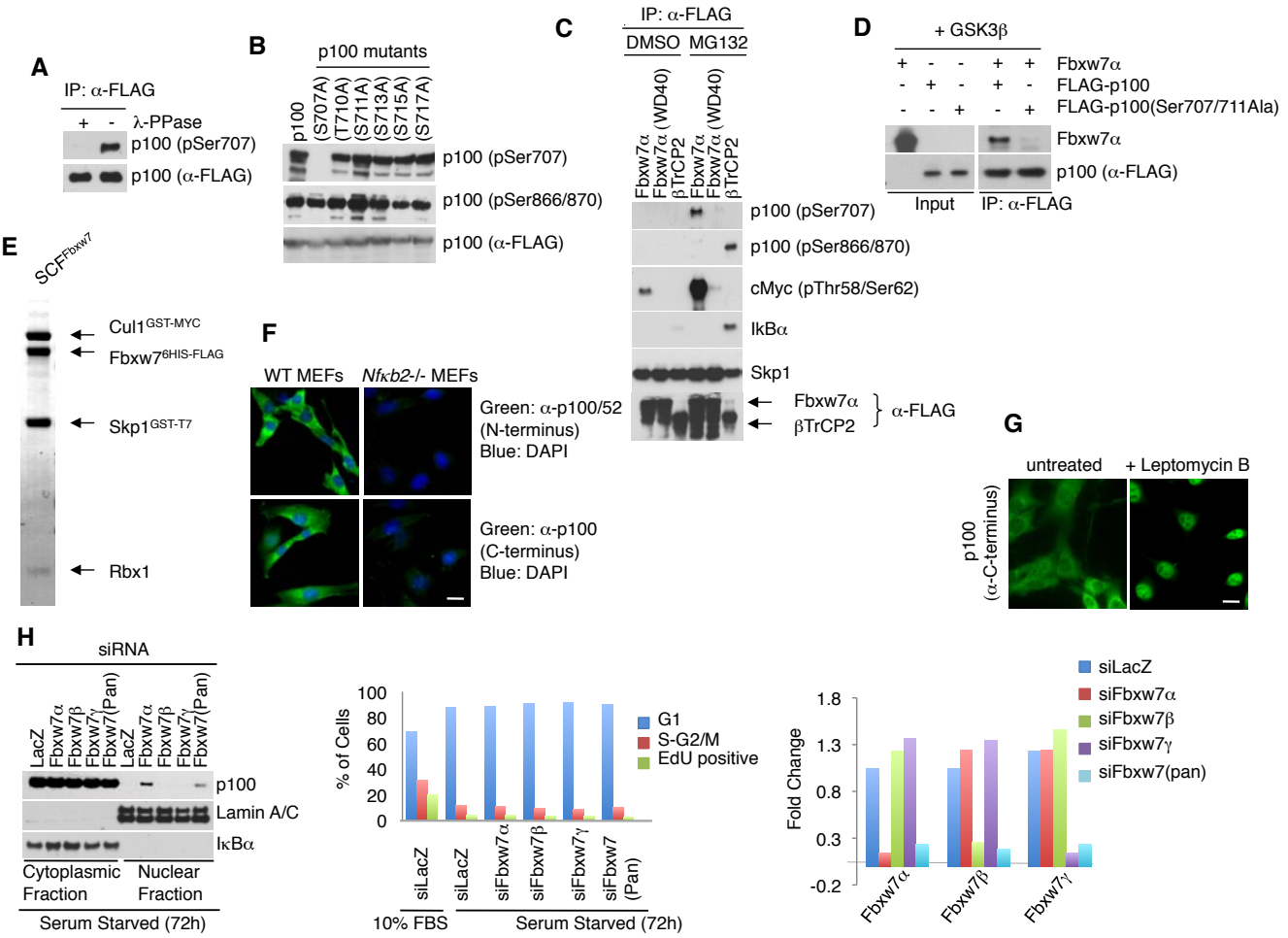


Figure S2 p100 is phosphorylated on Ser707 and Fbxw7α controls p100 stability in the nucleus. **(a)** The p100 (pSer707) antibody is phosphorylation specific. A phospho-specific antibody against the ^{704}PLP*p*SPPTSDSDSDSEGP^{720} peptide with a phospho-serine at position 707 was generated. HEK293 cells were transfected with a construct encoding FLAG-tagged p100. p100 was immunoprecipitated from cell extracts with anti-FLAG resin (α-FLAG). Immunocomplexes were incubated for 1 hour at 30°C with or without λ-phosphatase and blotted with the indicated antibodies. **(b)** Phosphorylation site specificity of the p100 (pSer707) antibody. HEK293 cells were transfected with constructs encoding different FLAG-tagged p100 mutants. Cell extracts were immunoblotted with the indicated antibodies. **(c)** Fbxw7α binds p100 phosphorylated on Ser707, but not Ser866 and Ser870. HEK293 cells were transfected with the indicated FLAG-tagged constructs and treated with MG132 (10 μM) or DMSO for 6 hours. FLAG-tagged proteins were immunoprecipitated from cell extracts and probed with antibodies to the indicated proteins. **(d)** GSK3-mediated phosphorylation of p100 is required for the *in vitro* binding of p100 to Fbxw7α. *In vitro*-translated, FLAG-tagged p100 or p100(Ser707/711Ala) was incubated with GSK3β before incubation with *in vitro*-translated Fbxw7α. Proteins were immunoprecipitated (IP) using anti-FLAG resin (α-FLAG), and immunoblotting for the indicated proteins was performed. 30% of inputs are also shown. **(e)** Insect cells (*Sf9*) were co-infected with baculoviruses encoding His-Fbxw7α, Skp1, Cul1, and Rbx1. Cell lysates were subjected to Ni-NTA affinity purification. An aliquot of the purified complex was analyzed on SDS-PAGE, and the gel was stained with Coomassie Blue. **(f)** Specificity of antibodies to the C-terminus or N-terminus of p100. WT and *Nfκb2 -/-* MEFs were fixed and stained with the indicated antibodies. DAPI is shown in blue. The antibody specifically recognizing a C-terminal epitope of p100 distinguishes p100 from p52 in immunofluorescence studies. Scale bar, 20 μm. **(g)** p100 shuttles between the cytoplasm and the nucleus. MEFs were treated with Leptomycin B (LMB) for 4 hours, fixed, and stained with an antibody against the C-terminus of p100 (green). Scale bar, 20 μm. **(h)** p100 is targeted by Fbxw7α in the nucleus of resting, primary human fibroblasts. IMR90 cells were transfected with either an siRNA targeting all Fbxw7 isoforms (pan;), or isoform-specific Fbxw7α/β/γ siRNAs. Cells were serum starved in 0.1% FBS for 72 hours. Sub-cellular fractionation was performed and lysates were subjected to immunoblotting for the indicated proteins (left panel). Growth arrest following serum starvation was confirmed by EdU incorporation and Propidium Iodide (PI) staining followed by FACS analysis (middle panel). The G1 bar indicates the percentage of cells with a 2N DNA content and the S-G2/M bar indicates the percentage of cells with a DNA content between >2N and <4N (as determined by PI stain). The percentage of actively replicating cells (positive for EdU staining) is shown in green. Isoform-specific knockdown of Fbxw7 was confirmed by qPCR (right panel) using amplification primers recognizing the unique 5′ exon of each isoform.

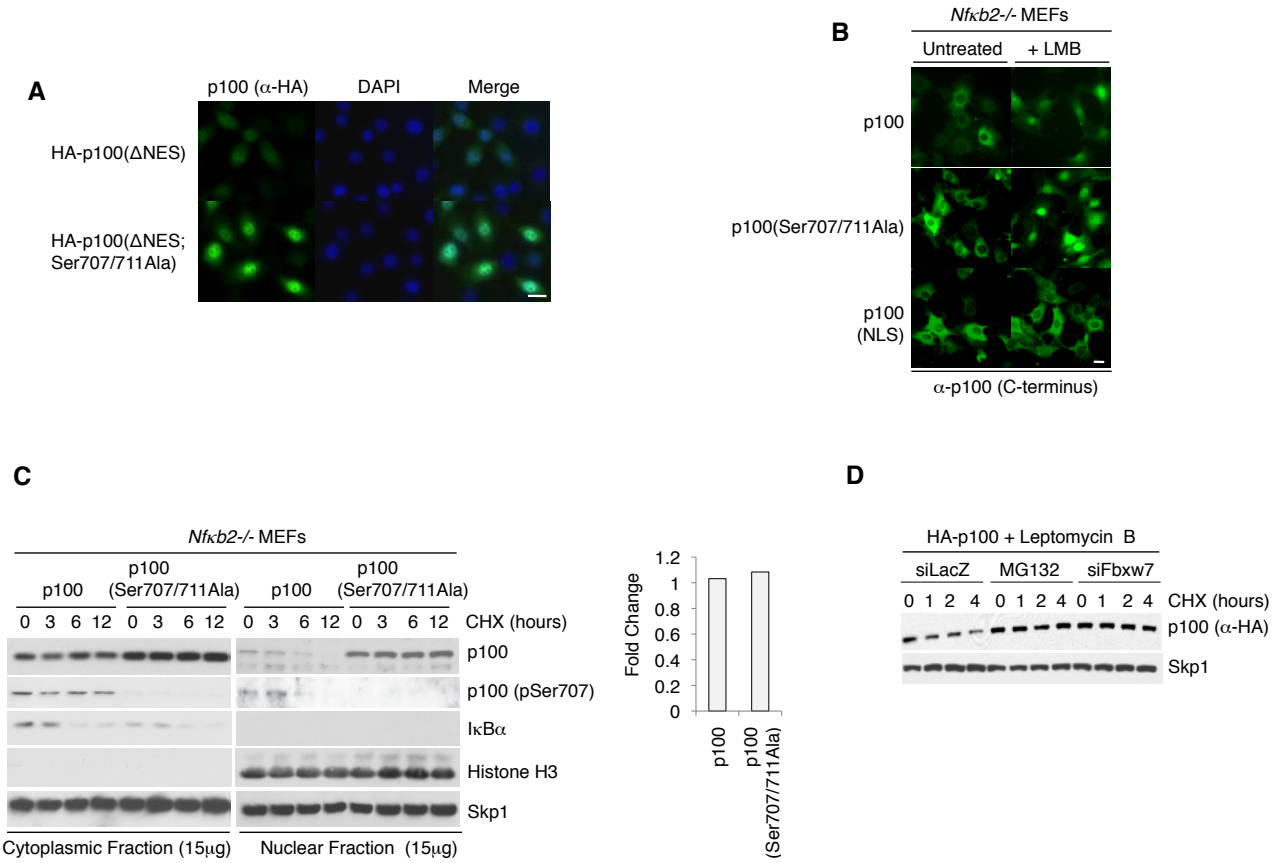


Figure S3 p100 stability is dependent on its cellular localization. **(a)** Mutation of Ser707 and Ser711 to Ala induces the accumulation of p100(ΔNES), a constitutively nuclear mutant of p100. HeLa cells were infected with retroviruses expressing either HA-tagged p100(ΔNES) or HA-tagged p100(ΔNES;Ser707/711Ala). Cells were fixed and stained with an antibody against the HA tag (α-HA). Scale bar, 20 μm**. (b)** p100 requires an intact NLS for nuclear-cytoplasmic shuttling. *Nfkb2-/-* MEFs were infected with retroviruses expressing either p100, p100(Ser707/711Ala), or p100(NLS). Cells were treated with Leptomycin B (LMB) for four hours, fixed, and stained with an antibody against the C-terminus of p100 (green). Scale bar, 20 μm. **(c)** Ser707 and Ser711 are required for the degradation of nuclear p100. *Nfαb2 -/-* MEFs were infected with retroviruses expressing either p100 or p100(Ser707/711Ala). Cells were treated with cycloheximide (CHX) for the indicated times. Cytoplasmic and nuclear fractions were isolated and analyzed by immunoblotting for the indicated proteins. mRNA levels of retrovirally-expressed p100 were analyzed by qPCR (right panel). In the nucleus, p100 half-life was approximately three hours, whereas in the cytoplasm p100 remained stable after 12 hours of CHX treatment. A similar difference was observed for p100 phosphorylated on Ser707, showing that, in contrast to nuclear p100, cytoplasmic p100 is stable even when phosphorylated on the Fbxw7α degron. The Ser707/711Ala mutations increased both the steady state level (time 0) and the stability of nuclear p100. IκBα half-life is included as control for CHX. IκBα and Histone H3 are used as controls of fractionation. **(d)** The half-life of nuclear p100 is regulated by Fbxw7α and the proteasome. HeLa cells were infected with retroviruses expressing HA-tagged p100 2. Cells were treated with Leptomycin B (LMB) for four hours, and cycloheximide (CHX) was then added for the indicated times. Cell extracts were analyzed by immunoblotting for the indicated proteins.

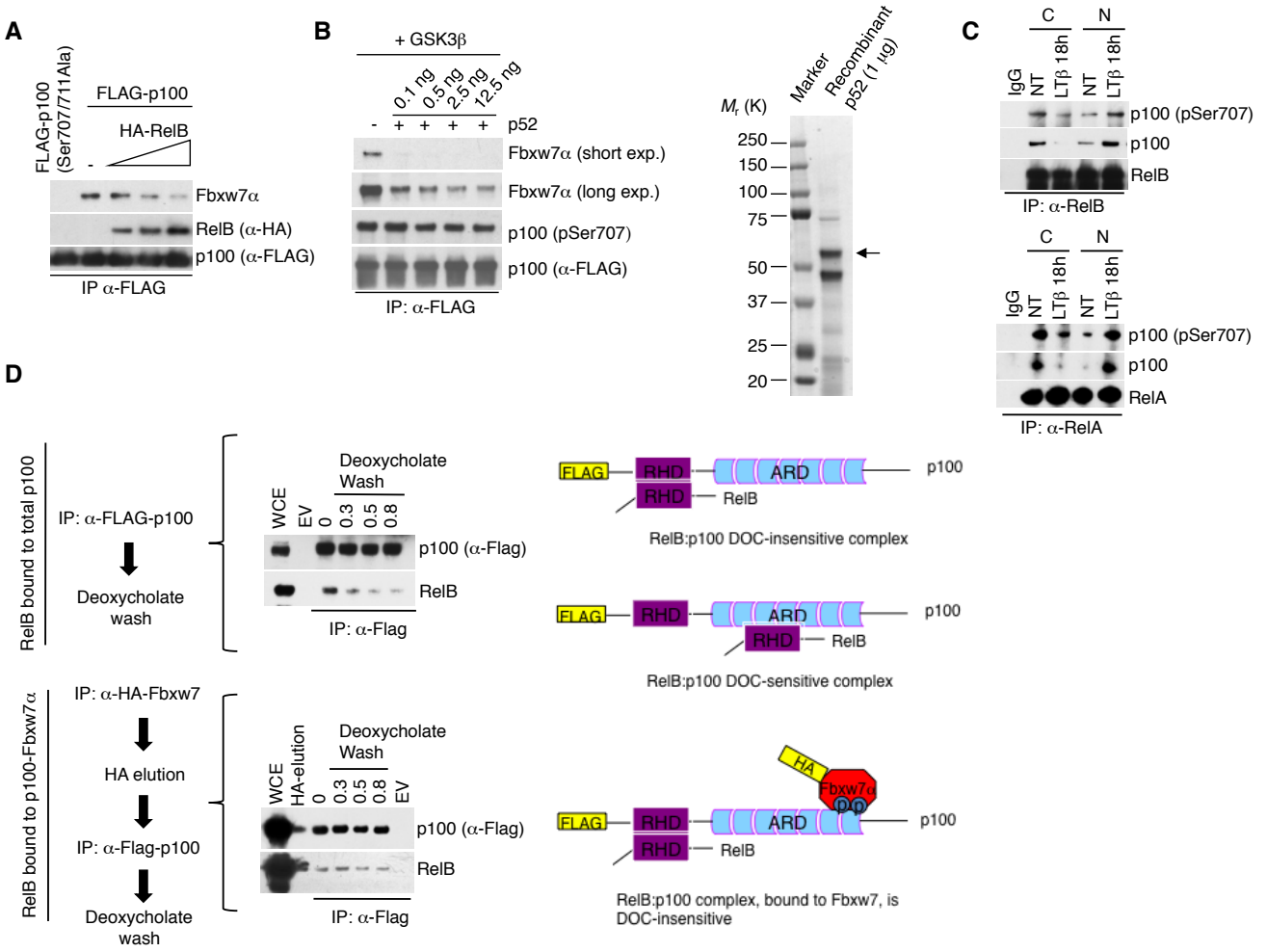


Figure S4 p52 and RelB compete with Fbxw7α for binding to p100. (a) RelB competes with Fbxw7α for binding to p100 *in vivo.* HEK293 cells were transfected with cDNAs encoding either FLAG-tagged p100 or FLAG-tagged p100(Ser707/711Ala). Increasing amounts of a plasmid encoding HA-tagged RelB were also transfected as indicated. Proteins were immunoprecipitated (IP) from cell extracts with anti-FLAG resin (α-FLAG), and immunoblotting was performed for the indicated proteins. The first lane shows cells transfected with an empty vector (EV). (b) p52 competes with Fbxw7α for binding to p100 *in vitro. In vitro* translated, FLAG-tagged p100 was immunoprecipitated and incubated at 30°C with purified, recombinant GSK3β. The indicated amounts of purified, recombinant p52 were added and allowed to bind immunoprecipitated p100. Unbound p52 was washed off, and *in vitro* translated Fbxw7α was incubated with the p100-p52 complexes. After washing, immunoblotting for the indicated proteins was performed. In the right panel the purified, recombinant p52 is shown. Recombinant p52 was expressed as GST-p52 in *E. coli.* Purified GST-p52 was cleaved with PreScission protease, and an aliquot of the eluted, purified protein was analyzed by SDS-PAGE and Coomassie Blue staining. Arrow indicates the expected size of full-length p52.**(c)** Following LTαR activation, p100 binding to NF-κB proteins decreases in the cytoplasm and increases in the nucleus. MEFs were treated with α-LTbR for 18 hours. Cells were harvested, fractionated, and endogenous RelA and RelB were immunoprecipitated from both cytoplasmic and nuclear fractions. IgG was used as a negative control. Immunocomplexes were blotted for the indicated proteins. Following α-LTbR ligation, p100 binding to RelA and RelB was decreased in cytoplasmic fractions, whereas more p100 associated with RelA and RelB in the nucleus. A similar result was observed for the fraction of p100 phosphorylated on Ser707. These data indicate a distinct regulation of the cytoplasmic and nuclear pools of p100 with regard to its binding to NF-κB proteins. **(d)** p100 bound to Fbxw7α associates to NF-κB proteins via RHD:RHD interactions. HEK293 cells were transfected with FLAG-tagged p100 or an empty vector (EV) either alone or in combination with HA-tagged Fbxw7α. To analyze total RelB-p100 complexes (top panel), FLAG-p100 was immunoprecipitated with an α-FLAG resin. The IP was divided in four tubes and then washed with lysis buffer containing the indicated concentrations of deoxycholate (DOC). To analyze RelB-p100 complexes bound to Fbxw7α (bottom panel), HA-tagged Fbxw7α was immunoprecipitated with an α-HA resin. The IP was washed and eluted using the HA peptide. 5% of the eluate was resolved by SDS-PAGE (designated "HA-elution"). The remaining eluate was subjected to a second round of immunoprecipitation with an α-FLAG resin to isolate FLAG-p100 complexes. The IP was divided in four tubes and then washed with lysis buffer containing the indicated concentrations of DOC. IPs were resolved by SDS-PAGE and immunoblotted for the indicated proteins. The presence of DOC in wash buffer has been demonstrated to interfere with RHD:ARD interactions, but not with stronger RHD:RHD interactions. The RHDs of NF-κB proteins bind p100 either through its RHD (i.e. forming a complex that is DOC-insensitive) or its ARD (i.e. forming an inactive complex that is DOC-sensitive). We found that, in contrast to total p100, which forms both DOC-sensitive and DOC-insensitive complexes, the fraction of p100 bound to Fbxw7α only forms DOC-insensitive complexes with RelB, indicating that the latter binds the RHD of p100 and not its ARD. A schematic representation of RelB:p100 complexes is shown in the right panels.

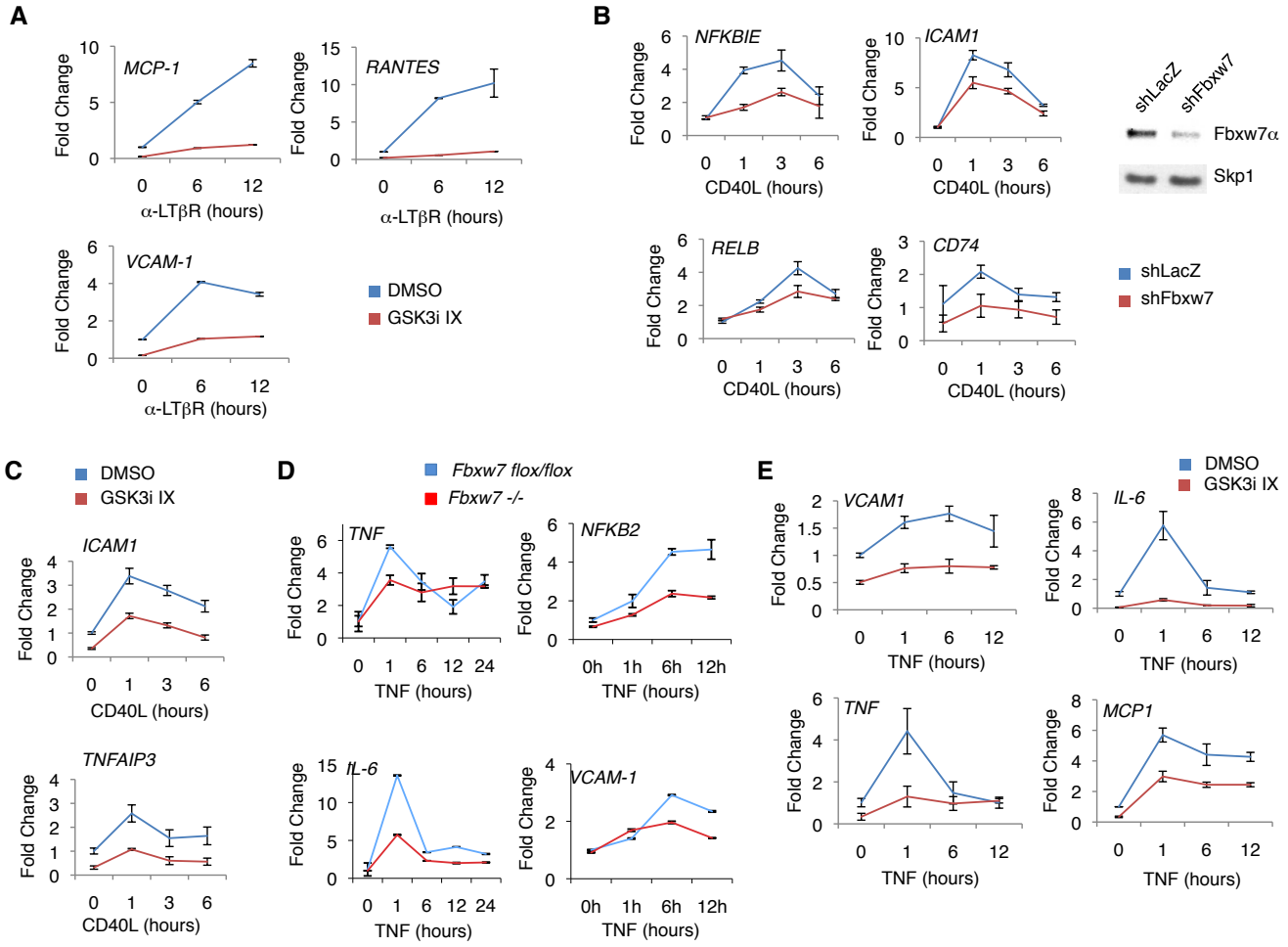


Figure S5 Clearance of nuclear p100 is required for efficient activation of NF-κB signaling pathways. **(a)** LTbR-dependent gene transcription is impaired upon GSK3 inhibition. MEFs were treated with GSK3i IX (5 μM), stimulated with agonistic α-LTbR antibodies, and harvested at the indicated times. The levels of the indicated mRNAs were determined by quantitative real time PCR (± s.d., n=3). The value given for the amount of PCR product present in DMSO treated MEFs at time 0 was set to 1. **(b)** CD40-dependent gene transcription is impaired upon Fbxw7 silencing. RAMOS cells were infected with lentivirus encoding shRNA (targeting either *LacZ* or *Fbxw7*) and a GFP reporter. GFP-positive cells were sorted, stimulated with recombinant human CD40L (50 nM), and finally harvested at the indicated times. The levels of the indicated mRNAs were determined by quantitative real time PCR (± s.d., n=3). The value given for the amount of PCR product present in LacZ at time 0 was set to 1. Knockdown of Fbxw7α was confirmed by immunoblot (right panel). **(c)** CD40-dependent gene transcription is impaired upon GSK3 inhibition. RAMOS cells were treated with GSK3i IX (5 μM) stimulated with recombinant CD40L (50 nM), and processed as in *(b)*. The levels of the indicated mRNAs were determined by quantitative real time PCR (± s.d., n=3). The value given for the amount of PCR product present in DMSO treated cells at time 0 was set to 1. **(d)** TNF-dependent gene transcription is inhibited by loss of Fbxw7. *Fbxw7 flox/flox* and *Fbxw7 -/-* MEFs were stimulated with purified, recombinant murine TNF (10ng/ml) and harvested at the indicated times. The levels of the indicated mRNAs were determined by quantitative real time PCR (± s.d., n=3). The value given for the amount of PCR product present in *Fbxw7 flox/flox* MEFs at time 0 was set to 1. **(e)** TNF-dependent gene transcription is impaired upon GSK3 inhibition. MEFs were treated with GSK3i IX (5 μM), stimulated with recombinant TNF, and harvested at the indicated times. The levels of the indicated mRNAs were determined by quantitative real time PCR (± s.d., n=3). The value given for the amount of PCR product present in DMSO treated cells at time 0 was set to 1.

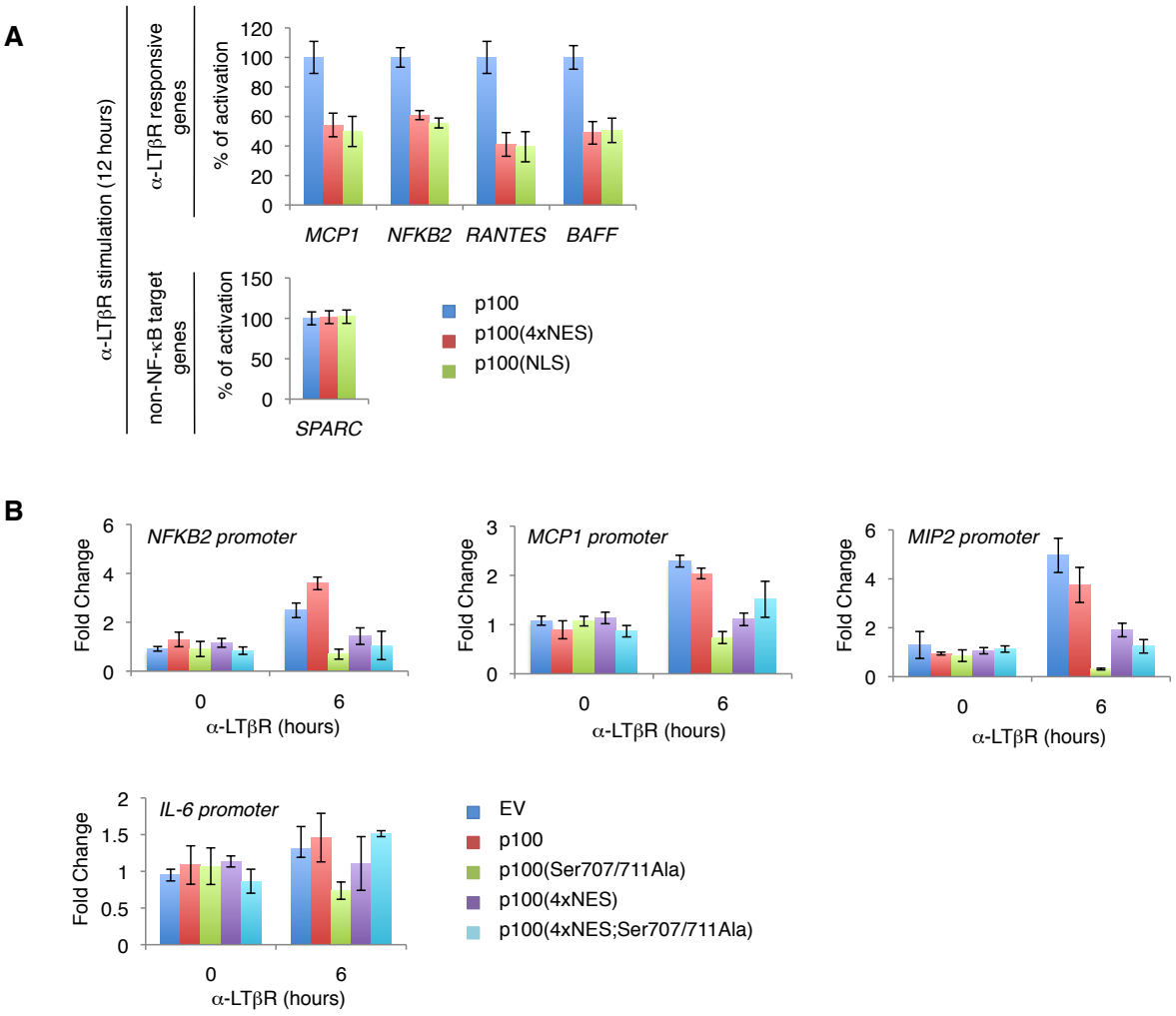


Figure S6 Stabilization or mislocalization of p100 inhibits NF-κB signaling. **(a)** Forced localization of p100 in either the nucleus or cytoplasm inhibits LTbR-dependent NF-κB target gene transcription. MEFs retrovirally expressing p100, p100(4xNES), or p100(NLS) were stimulated with agonistic α-LTbR antibodies and harvested 12 hours later. The levels of the indicated mRNAs were determined by quantitative real time PCR (± s.d., n=3) and normalized to percent of activation for p100 WT infected cells. **(b)** Stabilization or mislocalization of p100 in the nucleus compromises RelB association with NF-κB target gene promoters upon LTbR stimulation. MEFs retrovirally expressing empty vector (EV), p100, p100(Ser707/711Ala), p100(4xNES), or p100(4xNES;Ser707/711Ala) were stimulated for six hours with agonistic α-LTbR antibodies. Chromatin immunoprecipitation (ChIP) was performed using an anti-RelB antibody and immunoprecipitated DNA was amplified by real-time PCR with primers flanking NF-κB elements in the indicated gene promoters (± s.d., n=3). The LTbR unresponsive *IL-6* promoter was used as negative control. The value given for the amount of PCR product present in empty vector infected MEF cells was set to 1.

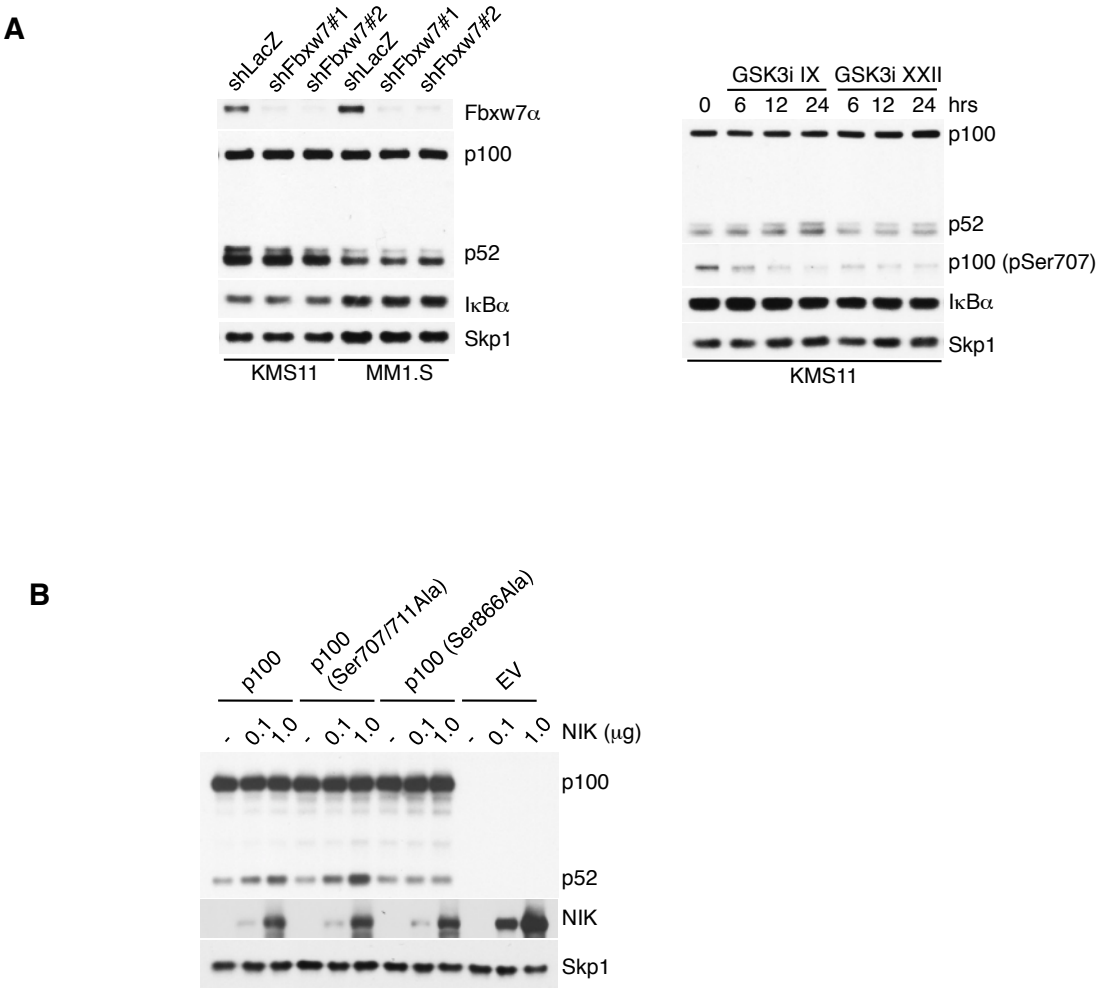


Figure S7 Processing of p100 to p52 is independent of the Fbxw7α-GSK3 axis. **(a)** Processing of p100 to p52 in HMMCLs is unaffected by knockdown of Fbxw7 or inhibition of GSK3. Left panel: KMS11 and MM1.S cells were infected with lentiviruses encoding shRNAs targeting LacZ or *FBXW7* (two different targeting sequences). After 72 hours, cells were harvested and analyzed by immunoblot for the indicated antibodies. Right panel: KMS11 were treated with either GSK3i IX or GSK3i XXII (2 μM and 1 μM, respectively). Cells were harvested at the indicated times, and subjected to immunoblot analysis for the indicated proteins. **(b)** Mutation of the Fbxw7α phospho-degron does not affect NIK-inducible processing of p100 to p52. HEK293 cells were transfected with p100, p100(Ser707/711Ala), p100(Ser866Ala), or an empty vector (EV), along with the indicated amounts of NIK cDNA. Cells were harvested at 24 hours after transfection and western blot analysis was performed for the indicated proteins. The results show that p100 and p100(Ser707/711Ala) are processed to a similar extent upon NIK expression. A p100 mutant defective for inducible processing, p100(Ser866Ala), is shown as a control.

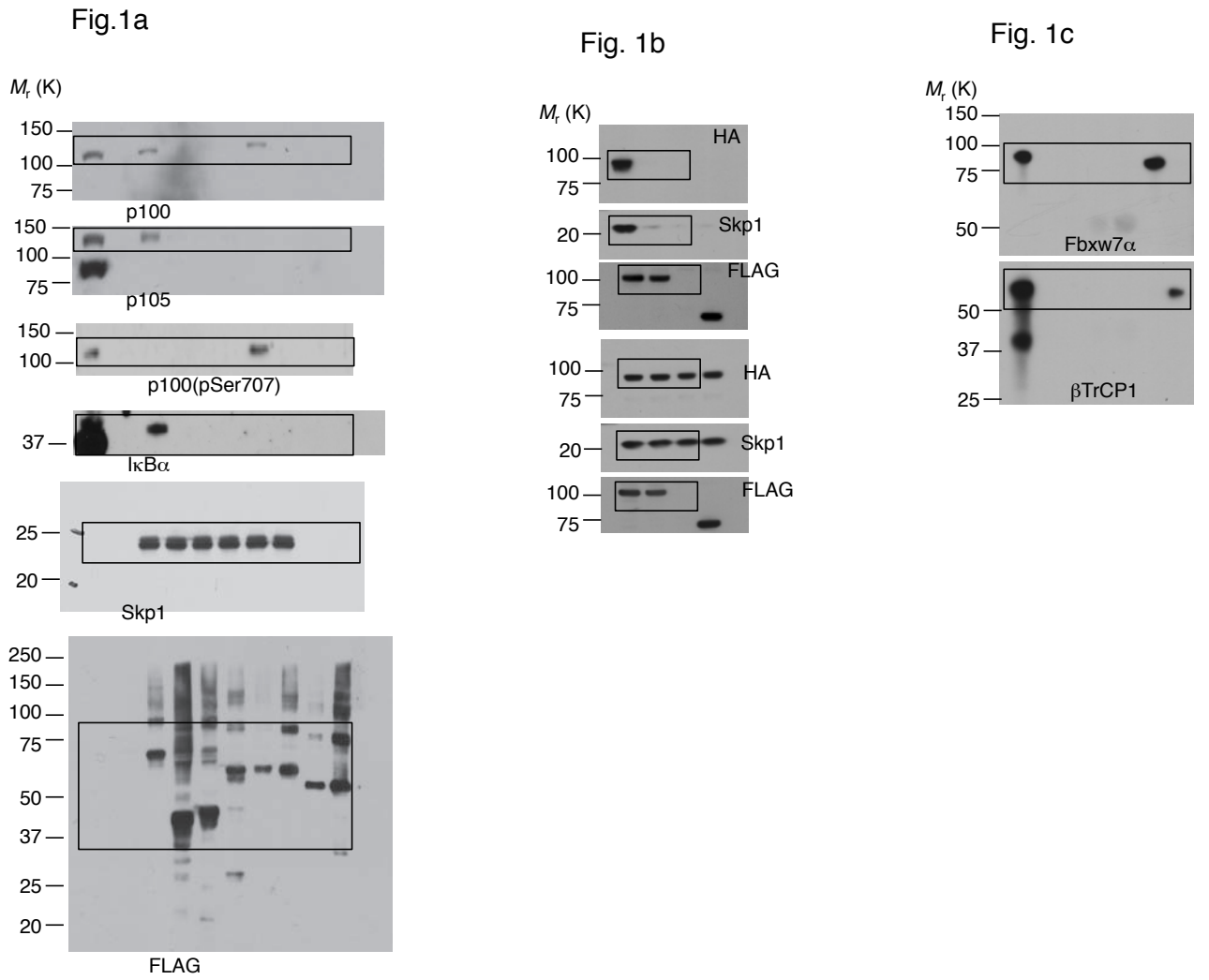


Figure S8 Full scans of the key immunoblots. Boxes indicate cropped images used in the figures and numbers indicate the molecular weight (KDa).

Fig. 1d

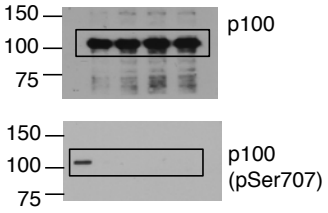


Fig. 1e

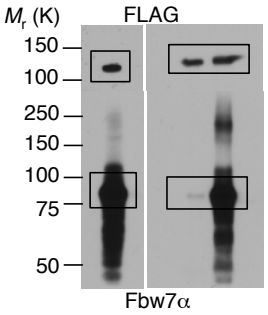


Fig. 1f

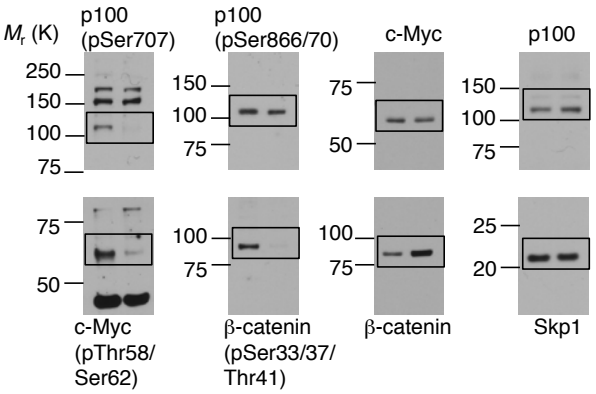


Fig. 1g

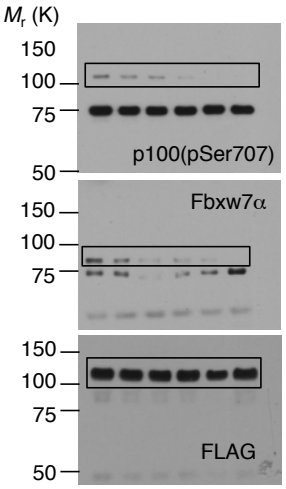


Figure S8 continued

Fig.1h

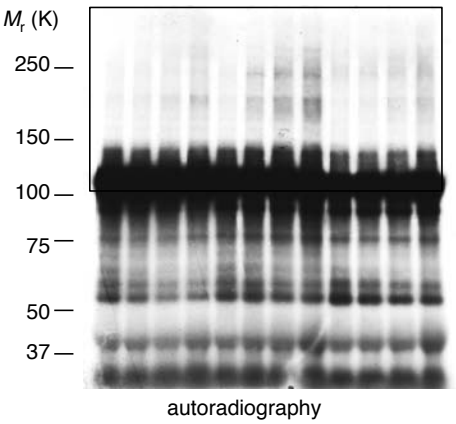
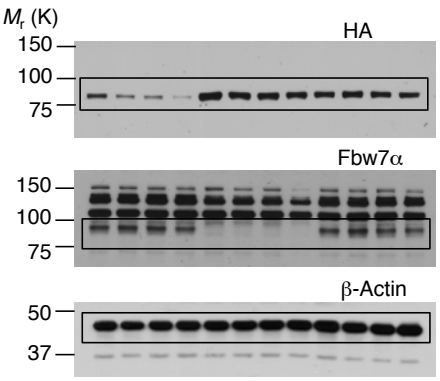


Fig. 2a



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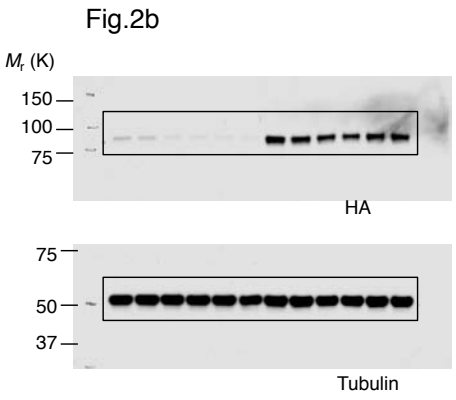

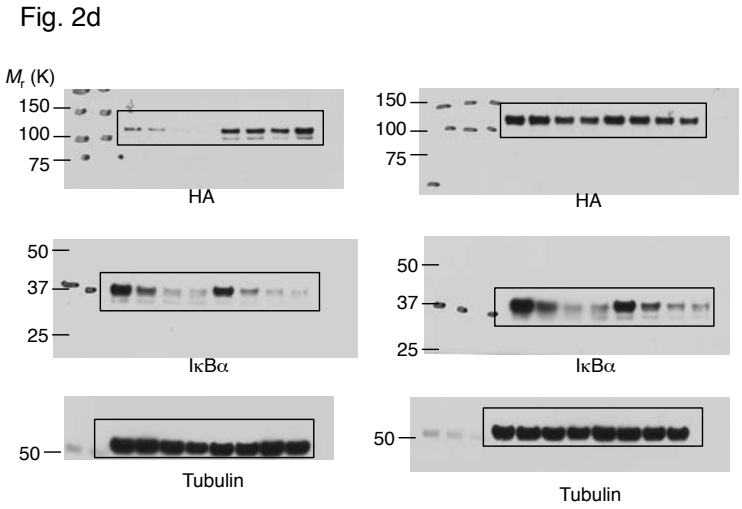

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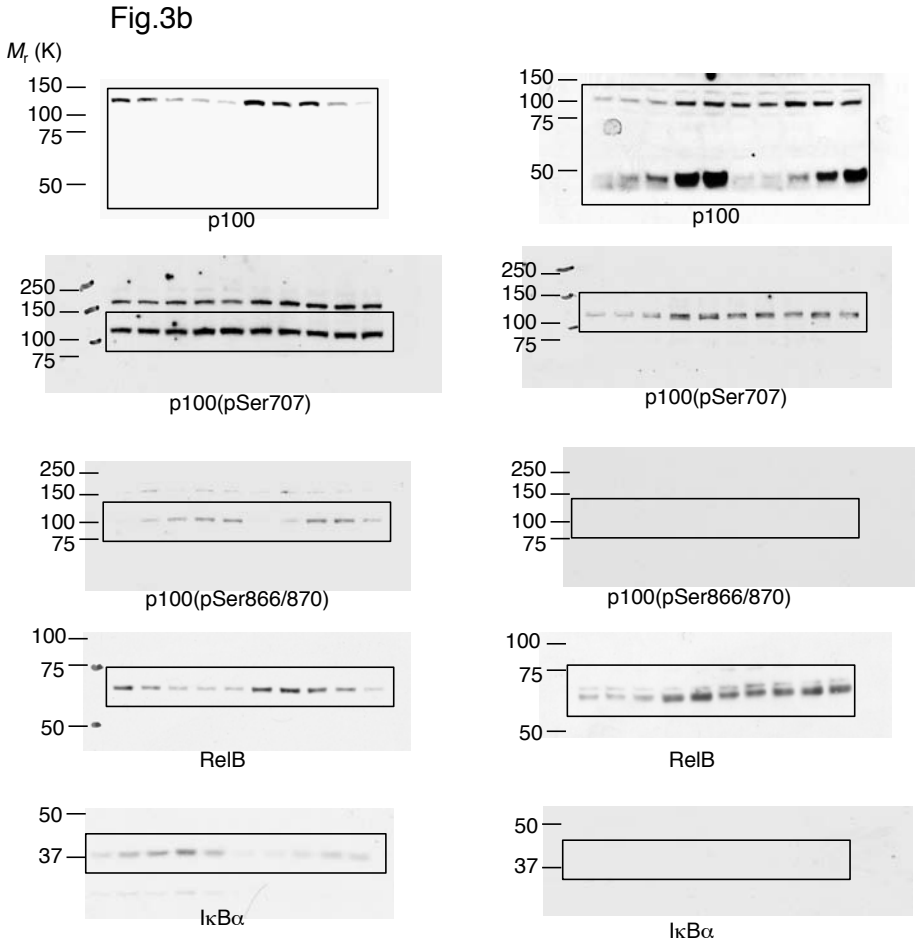

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Fig. 3c

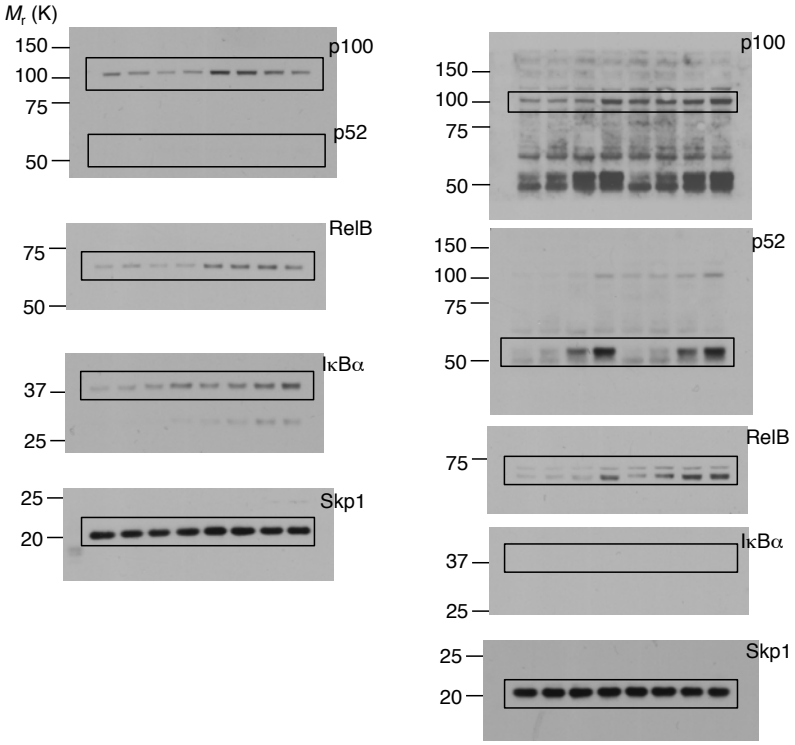
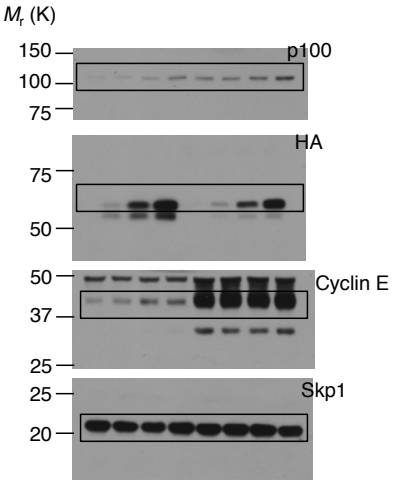


Fig. 3e



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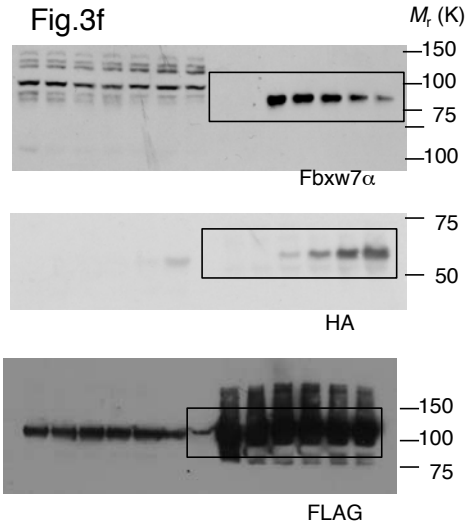

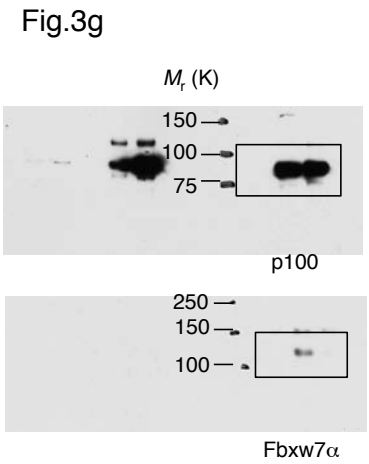

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Fig. 5a

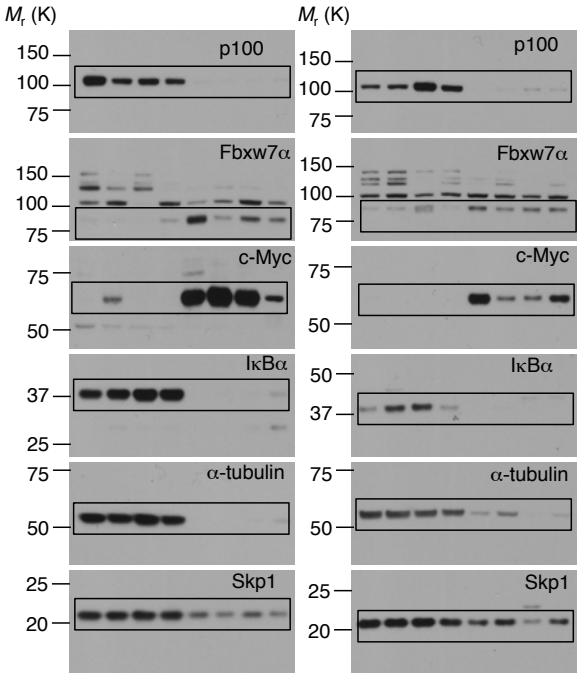

Fig. 5b

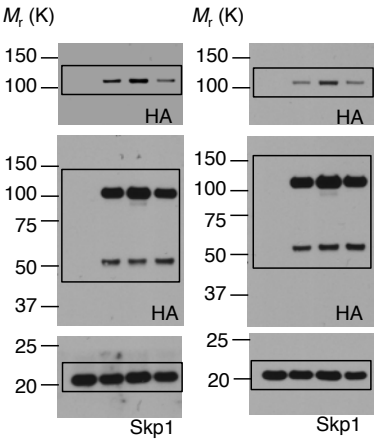

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Fig. 5g

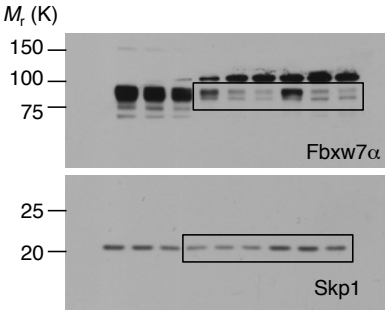

Fig. 5h

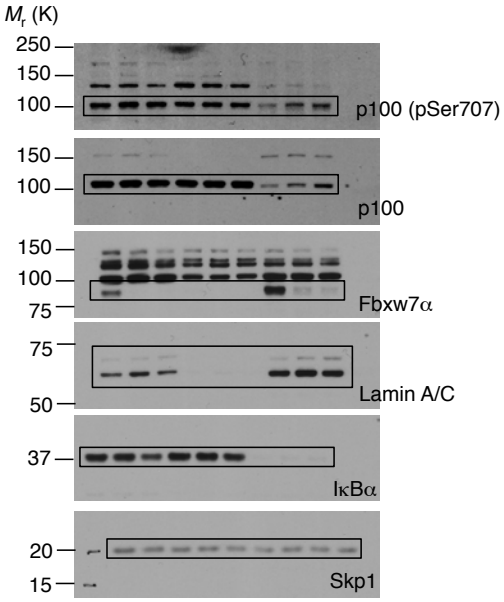

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Fig. 7a

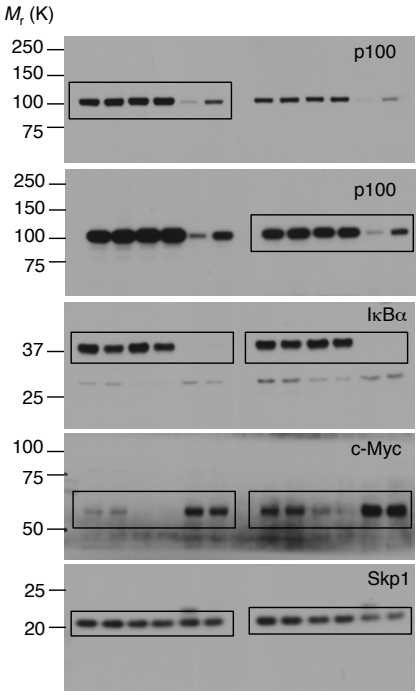

Fig. 7b

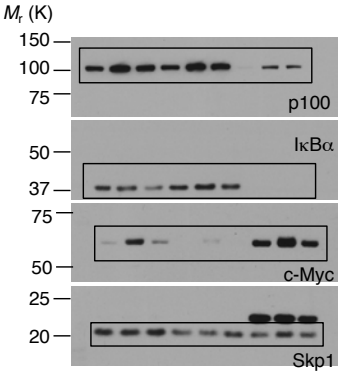

Fig. 7e

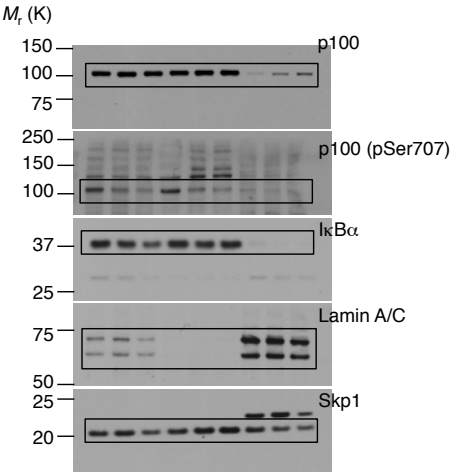

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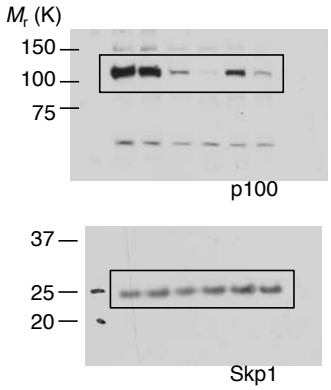
Fig.8a



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