DOI: 10.1038/ncb2651

nature cell biology

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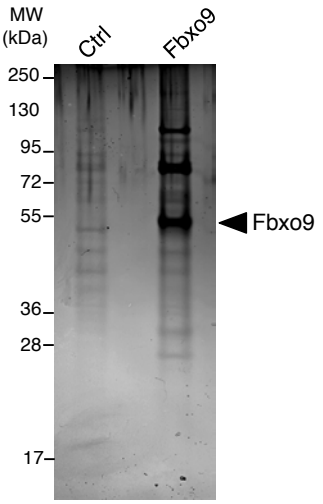

b

Telomere Length regulation protein Tel2 homolog IPI00016868

Sequence	Mascot Ion score	Modifications	Start	Stop
(R)QGLLSAVSSVLLSLPAAR(L)	72.1		768	785
(R)EVSVELAK(V)	26.45		551	558
(R)LVEQVPDR(A)	28.05		255	262
(R)QGLLSAVSSVLLSLPAAR(L)	34.48		768	785
(R)AmEAVLTGLVEAALGPEVLSR(L)	25.89	Oxidation (+16)	263	283
(R)LLEDLMDELLEAR(S)	90.83		786	798
(R)LLEDLmDELLEAR(S)	73.31	Oxidation (+16)	786	798
(K)AVLIcLAQLGEPELR(D)	63.76	Carbamidomethyl (+57)	363	377
(R)QGLLSAVSSVLLSLPAAR(L)	94.96		768	785
(R)LQQENLAEFFPQNYFR(L)	111.71		171	186
(R)AmEAVLTGLVEAALGPEVLSR(L)	90.93	Oxidation (+16)	263	283

Tel2-Interacting protein 1 homolog IPI 00011702

Sequence	Mascot Ion score	Modifications	Start	Stop
(K)FSTLSLLLGYLK(L)	41.07		398	409
(K)ALADILSESLHSLATSLPR(L)	47.69		369	387
(K)FSTLSLLLGYLK(L)	92.03		398	409
(K)ALIQVLELDVADIK(I)	76.35		431	444
(K)QLGDLFASFLPGISTALTR(L)	71.94		201	219

Figure S1 Purification of the Fbxo9 complex. **a,** Tandem-Strep-single-FLAG-tagged (SF-TAP)-Fbxo9 was expressed in HEK293 cells ($2x10^9$ cells) and subjected to a sequential immunopurification approach comprising immobilization on Strep-Tactin beads and elution with desthiobiotin in the first step, and immobilization on anti-FLAG resin and elution with 3x-FLAG-octapeptide in the second step. The FLAG eluate was separated by SDS-PAGE and analyzed by mass spectrometry. As a control, a purification was performed from HEK293 cells ($5x10^8$ cells) transfected with an empty vector. The depicted gel corresponds to 5% of the final FLAG-eluates visualized by silver staining. **b**, Sequences of identified peptides corresponding to Tel2 and Tti1.

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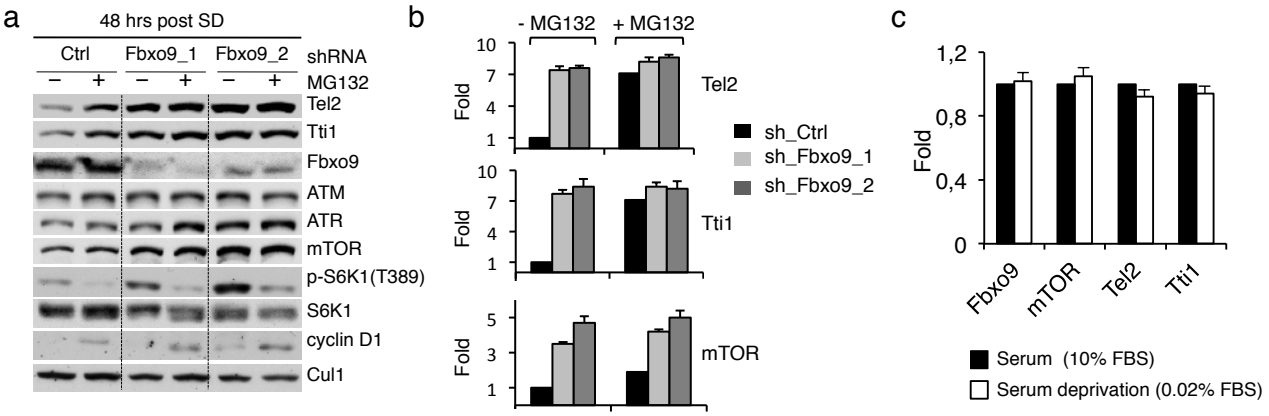

Figure S2 Fbxo9 mediates ubiquitin-proteasome dependent degradation of Tel2 and Tti1. **a,** T98G cells were infected with shRNA constructs directed against a nonrelevant mRNA or against two distinct sequences of Fbxo9 mRNA. Cells were deprived of serum (SD: 0.02% FBS) for the indicated times and during the last 4 hrs before harvesting, cells were treated with MG132 where indicated. Protein extracts were analyzed by immunoblotting with antibodies to the indicated proteins. **b**, Quantification of proteins shown in (a) averaged with two additional, independent experiments. The value given for the amount of protein present in the control sample (-MG132) was set as 1. Error bars represent s.d., n = 3. **c**, mRNA levels of the indicated genes were analyzed in serum treated or serum deprived (48hrs) T98G cells using real-time PCR. Error bars represent s.d., n = 3. The value given for the amount of mRNA in the serum treated sample of each gene was set as 1.

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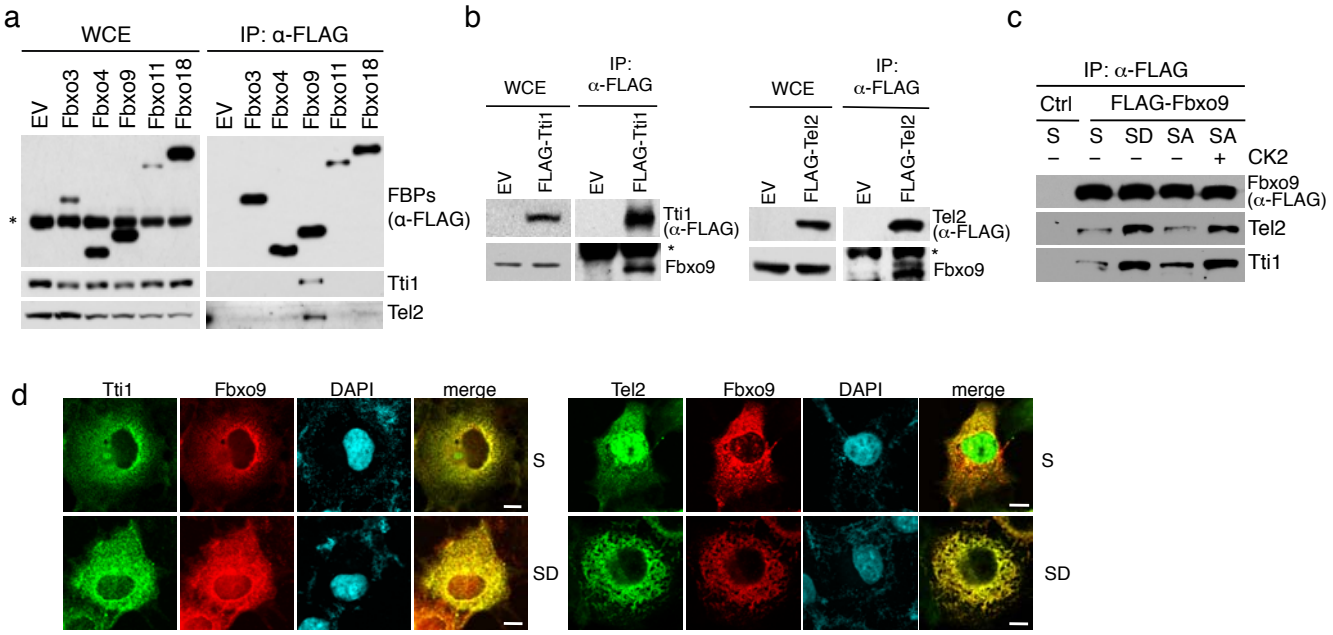

Figure S3 Fbxo9 interacts with Tel2 and Tti1 proteins. **a,** HEK293 cells were transfected with empty vector (EV) or constructs of the indicated FLAG-tagged F-box protein (FBPs). Whole cell extracts (WCE) were immunoprecipitated (IP) with anti-FLAG beads, and immunoprecipitates subsequently probed with antibodies to the indicated proteins. **b,** HEK293 cells were transfected with empty vector or FLAG-tagged Tel2 or Tti1 constructs. WCE were immunoprecipitated with anti-FLAG beads as in (**a**), and probed with the indicated antibodies. Asterisks denote nonspecific bands. **c,** U2OS cells, infected with a retrovirus expressing FLAG-tagged Fbxo9 or control virus and transfected with a CK2 expression plasmid as indicated, were either grown in normal serum (S: 10% FBS) or deprived of serum for 48 hrs (SD: 0.1% FBS) and then activated with serum for 2 hrs (SA:10% FBS). Thereafter, WCE were prepared, subjected to immunoprecipitations with anti-FLAG beads as described in (**a**), and immunoblotted with antibodies to the indicated proteins. **d,** Colocalization of Fbxo9 and Tel2/Tti1. Cos7 cells were transiently transfected with plasmids encoding HA-tagged Fbxo9 and either FLAG-tagged Tel2 or Tti1. Cells were then cultured in normal serum (S: 10% FBS) or deprived of serum for 5 hrs (SD: 0.02% FBS). Thereafter, cells were fixed with methanol and incubated with anti-FLAG antibody (green) and anti-HA antibody (red). DNA was stained with DAPI (blue). Scale bar, 10 μm.

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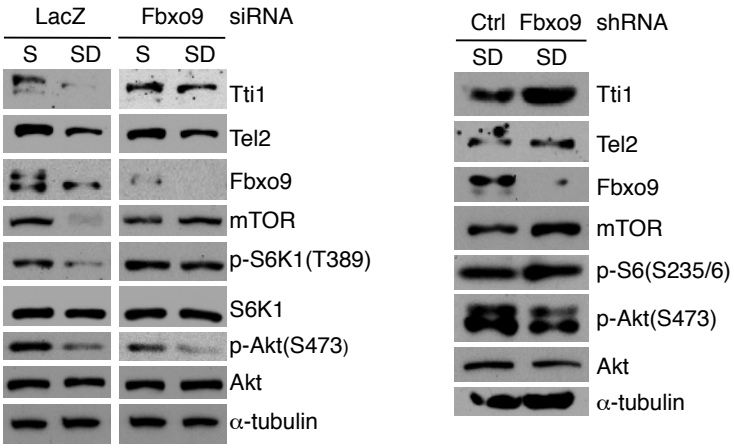

Figure S4. Fbxo9 promotes degradation of Tel2 and Tti1 proteins in response to serum withdrawal in primary IMR90 cells. IMR90 cells were treated with siRNAs directed against Fbxo9 or LacZ (left panel) or infected twice with shRNA constructs directed against Fbxo9 mRNA or against a nonrelevant mRNA (right panel). Cells were grown in normal medium (S:10% FBS) or deprived of serum (SD: 0.2% FBS) for 48 hrs. Protein extracts were then prepared and probed with antibodies as specified.

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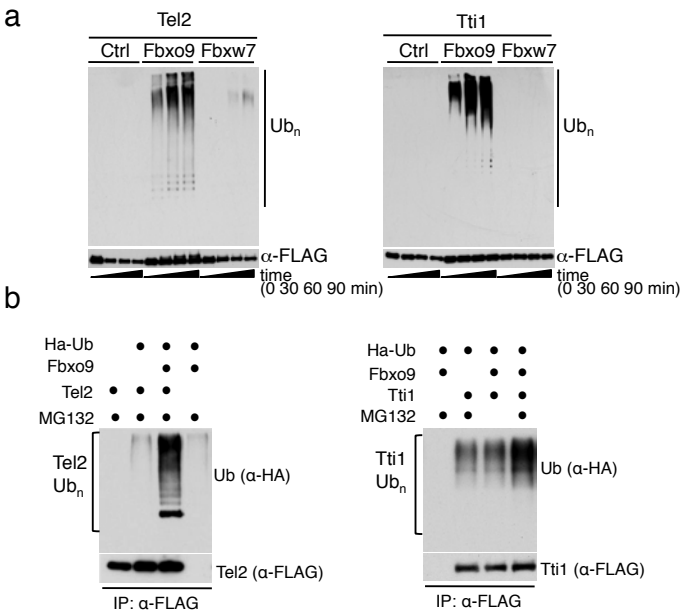

Figure S5 *In vitro* **and** *in vivo* ubiquitylation of Tel2/Tti1 proteins via Fbxo9. **a,** *In vitro* ubiquitylation assays of Tel2 and Tti1 proteins. SCF^{Fbxo9} and SCF^{Fbxw7} were expressed using baculoviruses and purified from Sf21 insect cells. FLAG-tagged Tel2 and Tti1 were immunoprecipitated from HEK293 cells. Samples were supplemented with ubiquitin, E1, and E2, incubated as specified, and subjected to immunoblotting with the indicated antibodies. The brackets on the right side mark polyubiquitylated Tel2 or Tti1. **b,** *In vivo* ubiquitylation assays of Tel2 and Tti1 proteins. HEK293 cells were transfected with plasmids expressing FLAG-tagged Tel2 or Tti1, HA-ubiquitin and Fbxo9 as indicated. Following treatment with MG132, extracts were prepared and subjected to anti-FLAG-immunoprecipitations using denaturing conditions.

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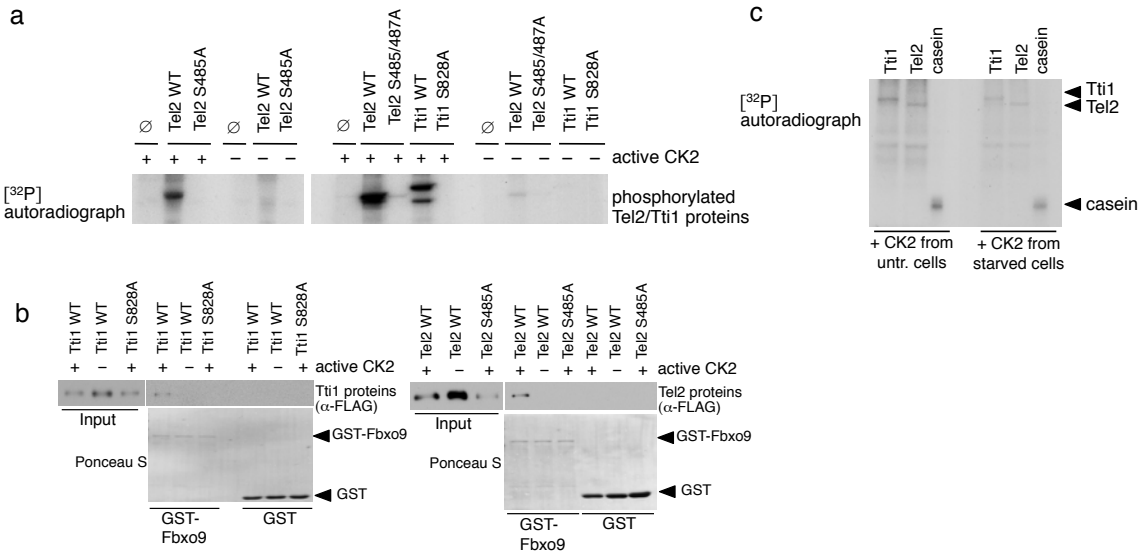

Figure S6 CK2 phosphorylates Tel2 and Tti1 proteins and mediates binding to Fbxo9 *in vitro*. **a,** Equal amounts of the indicated purified forms of Tel2 and Tti1 were transferred to an *in vitro* kinase reaction to test for phosphorylation by purified active CK2 in the presence of [^{32}P]ATP. Phosphorylated proteins were visualized by autoradiography. **b,** The indicated forms of purified Tel2 and Tti1 were phosphorylated by CK2 *in vitro* as indicated, and tested for their binding to GST-tagged Fbxo9 using *in vitro* pull-down assays. **c,** FLAG-CK2α was immunoprecipitated from untreated or serum starved cells and subjected to an an *in vitro* kinase reaction using purified Tel2, Tti1 and casein as substrates. Phosphorylated proteins were visualized by autoradiography.

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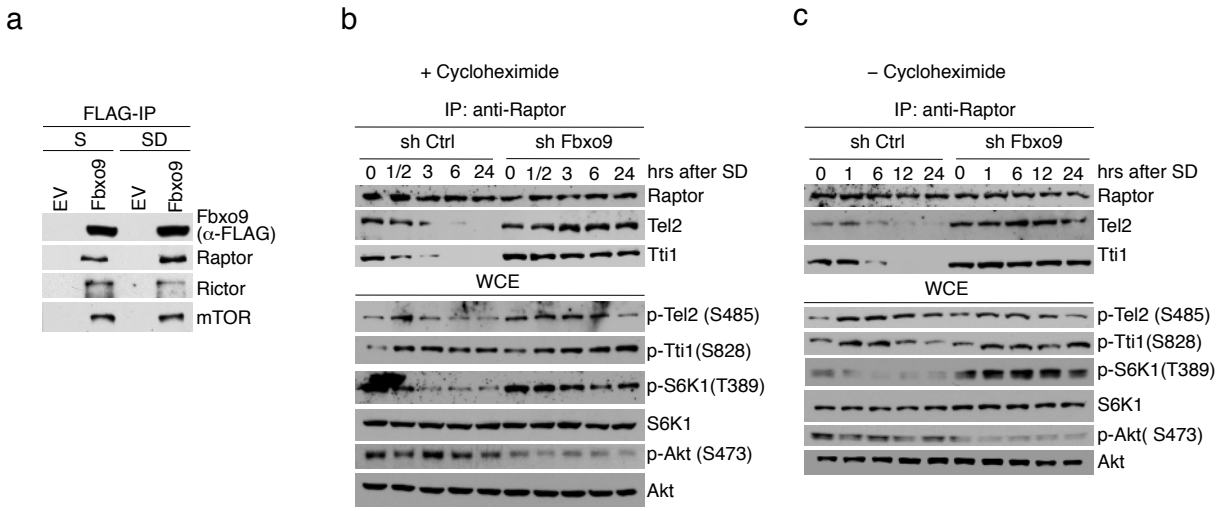

Figure S7 mTORC1 specific degradation of Tel2/Tti1 via Fbxo9 maintains the cellular response to growth factor withdrawal. **a,** T98G cells expressing FLAG-Fbxo9 from retroviral infection were grown in the presence of serum (S: 10 % FBS) or deprived of serum (SD: 0.02% FBS) for 48 hrs. Thereafter, anti-FLAG immunopreciptitations were performed and and probed with antibodies as specified. **b,** T98G cells treated to downregulate Fbxo9 or control cells were deprived of serum (SD: 0.02% FBS) in the presence of cycloheximide and harvested at the indicated timepoints. Endogenous Raptor was subsequently immunoprecipitated (IP) from whole cell extracts (WCE), and immunocomplexes and WCE were probed with antibodies to the indicated proteins. **c,** The experiment was essentially performed as in **(b)**, except that cycloheximide was omitted.

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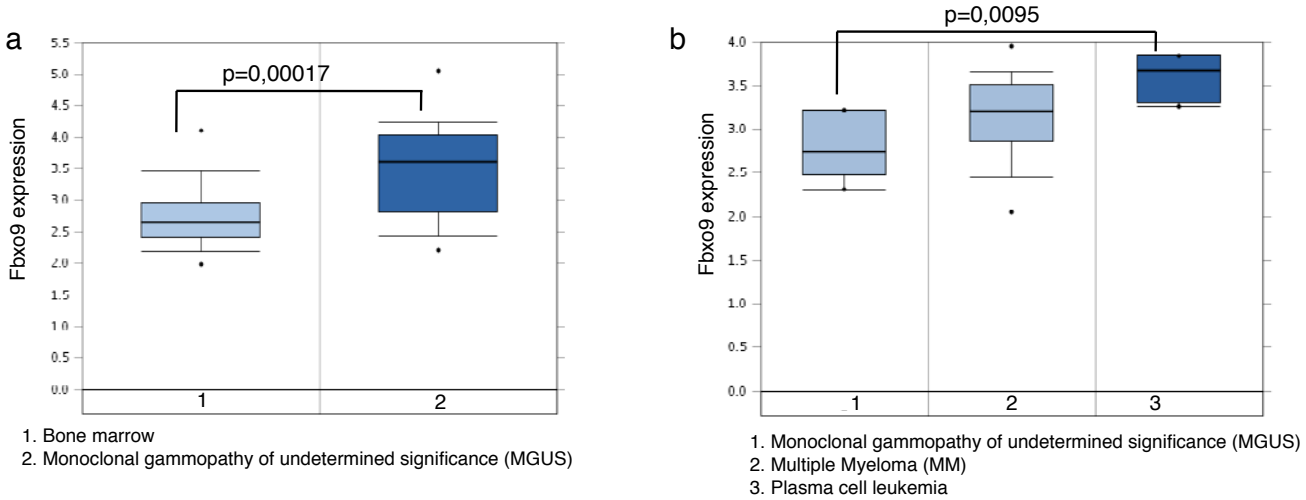

Figure S8 *Fbxo9* expression correlates with MM progression. All data was provided by the Oncomine database. The associated p values are shown for each study. **a,** Data from (23) reanalyzed to show expression levels of *Fbxo9* in normal bone marrow and monoclonal gammopathy of undetermined significance (MGUS) (n = 78). **b,** Data from (24) reanalyzed to show expression levels of *Fbxo9* in MGUS, MM, and plasma cell leukemia (n = 52). Both box-and-whisker plots show the upper and lower quartiles (25–75%) with a line at the median, whiskers extend from the 10th to the 90th percentile, and dots correspond to minimal and maximal values.

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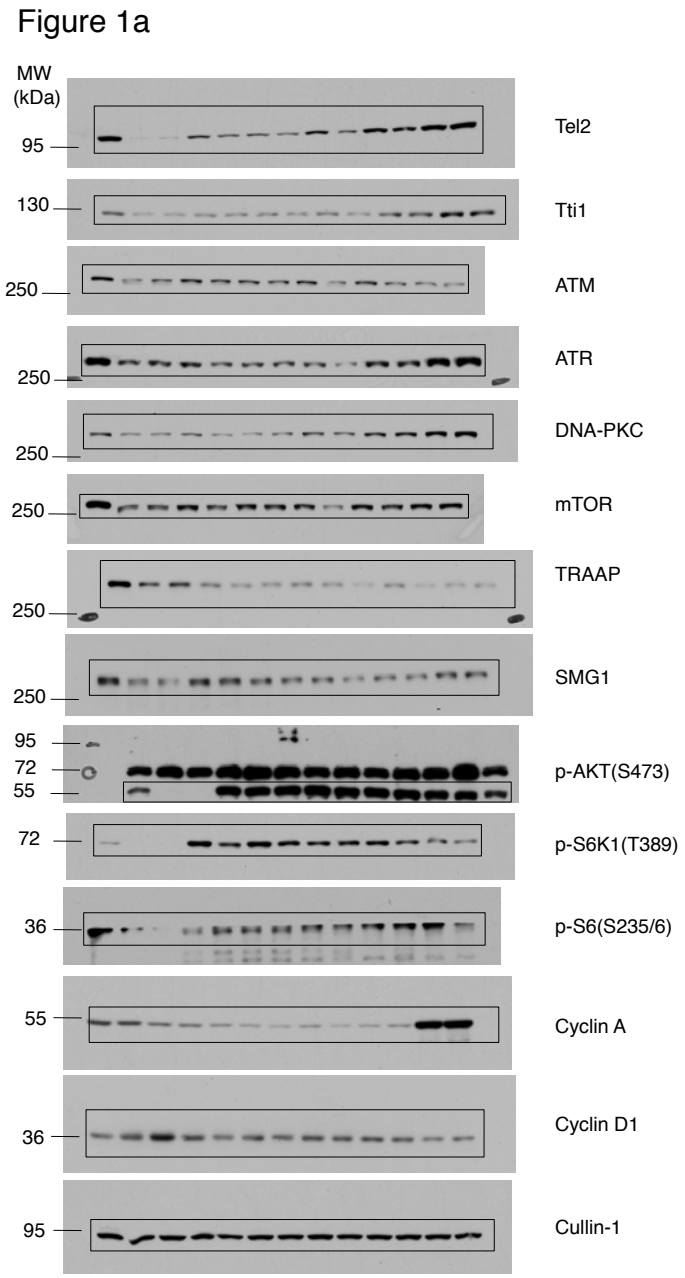

Figure S9 Full scans

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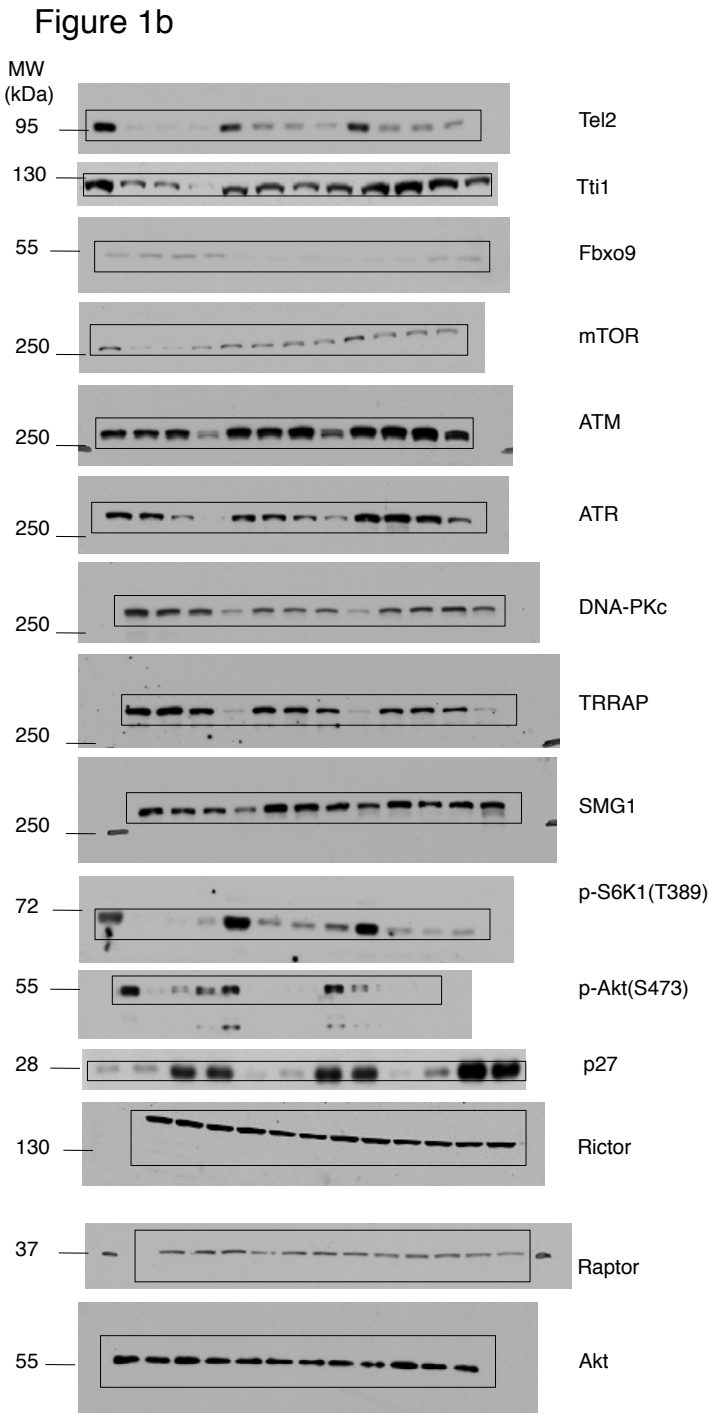

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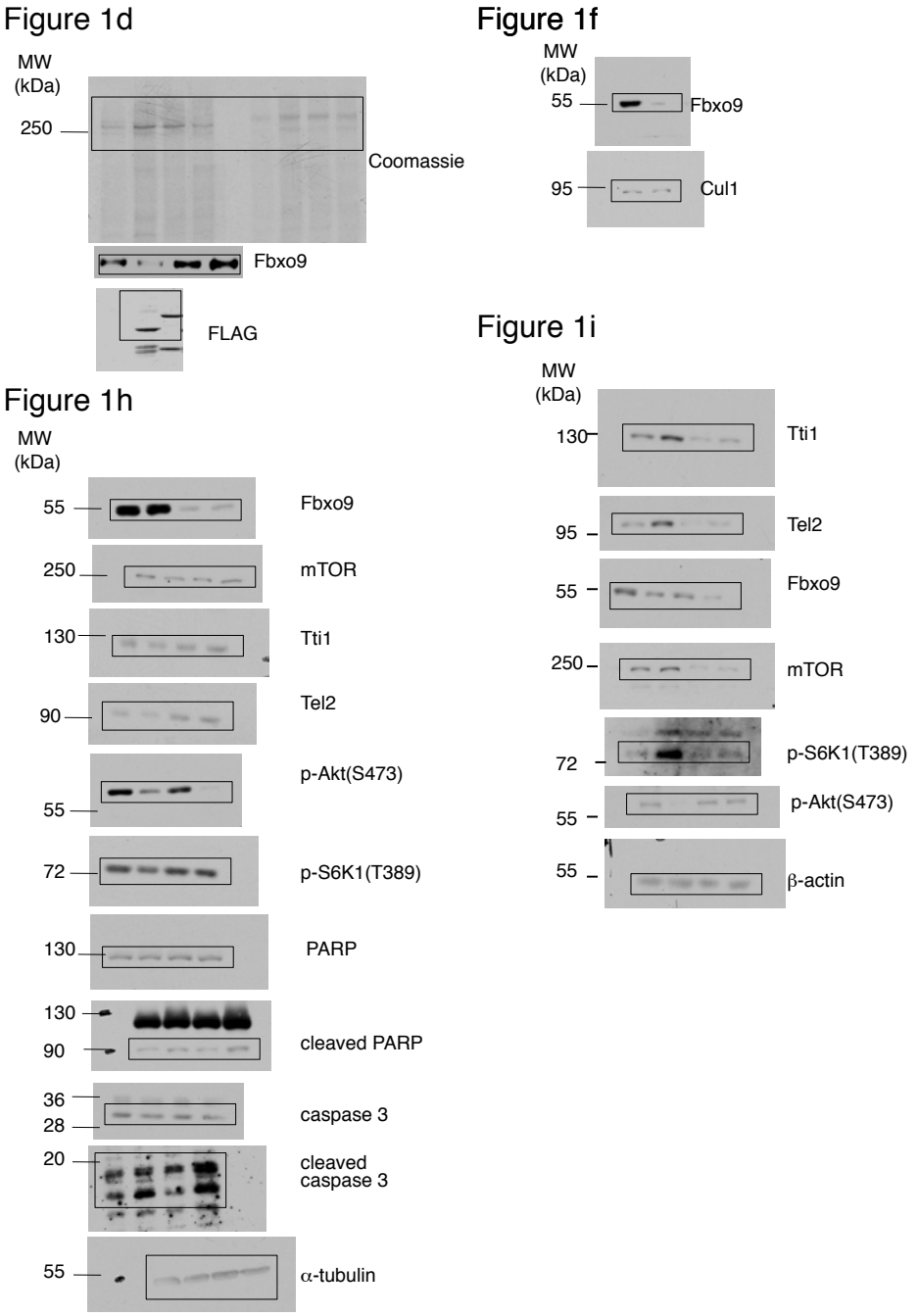

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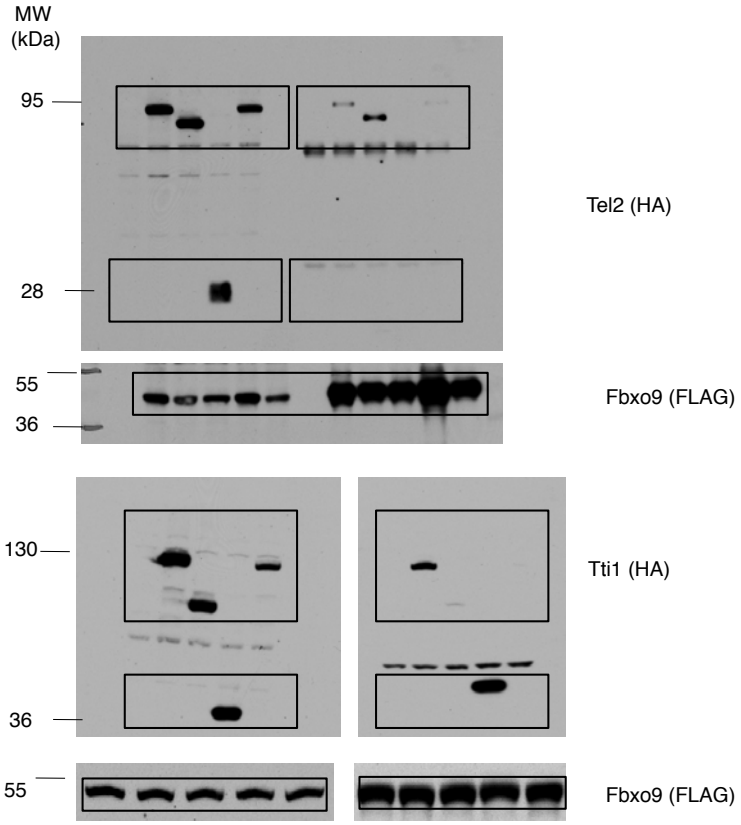

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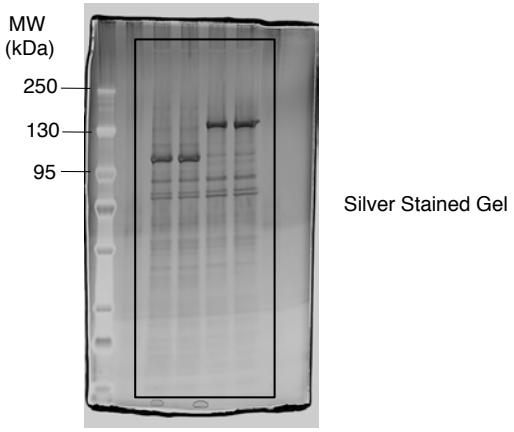

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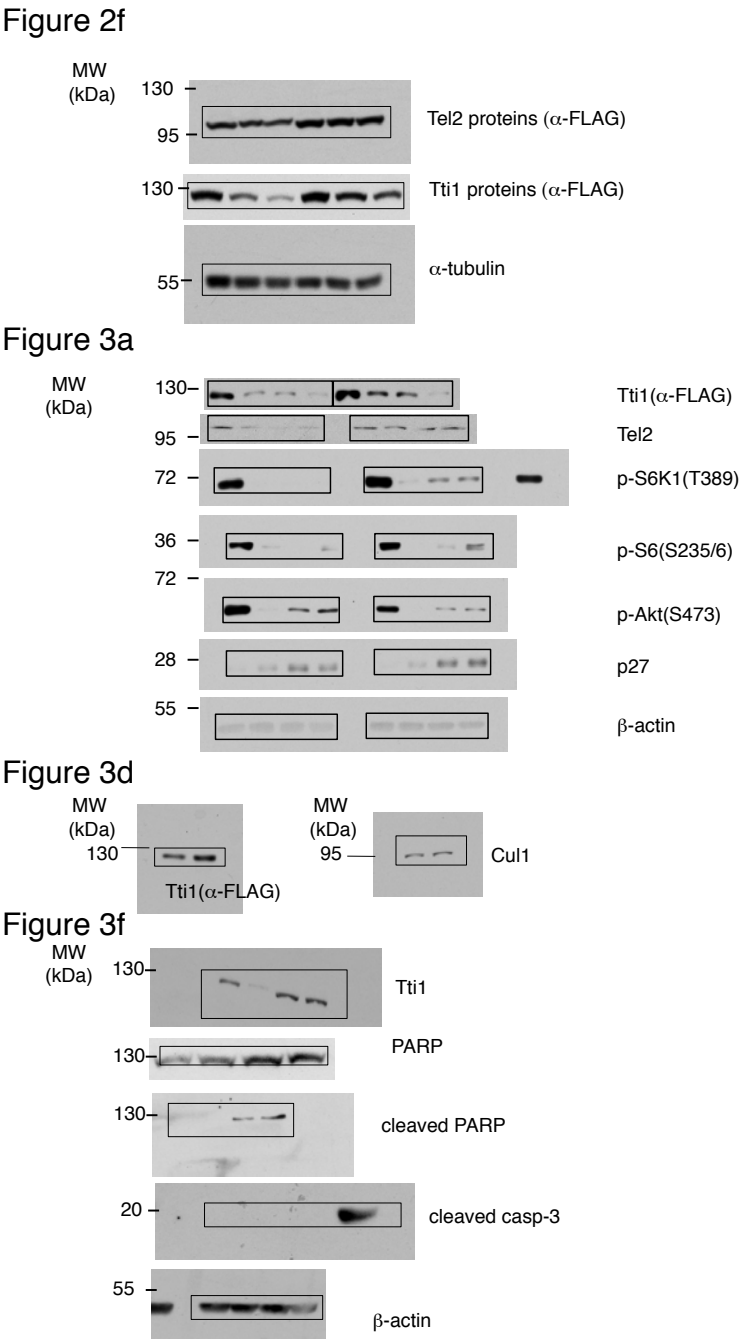

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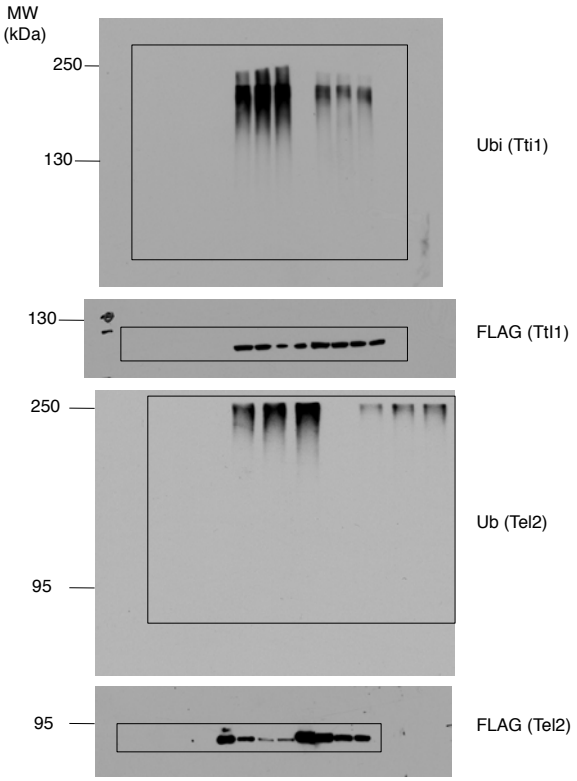
Figure 3g



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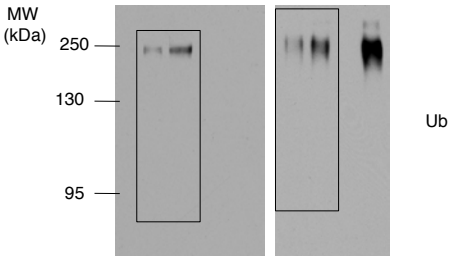

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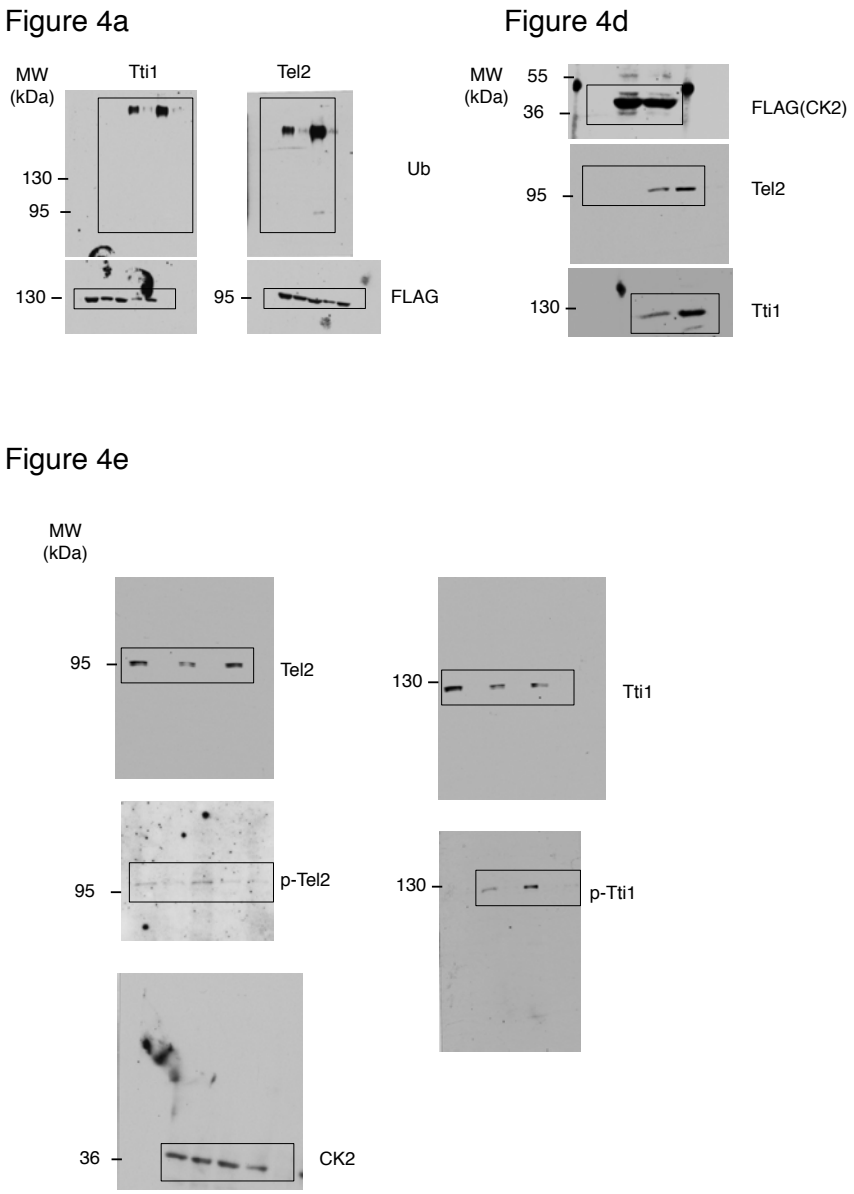

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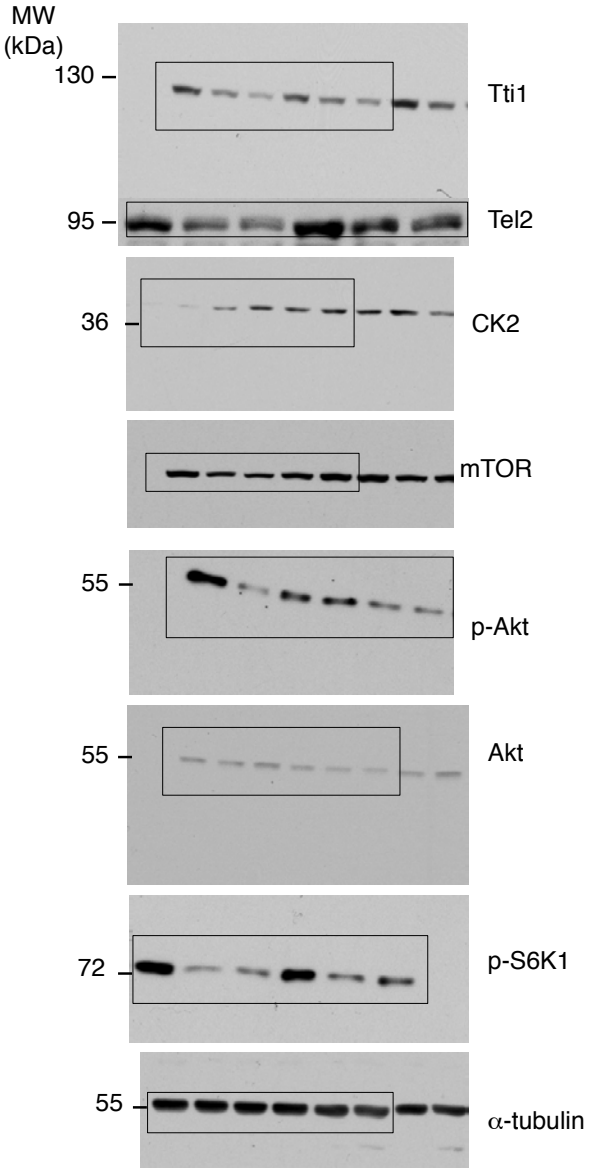

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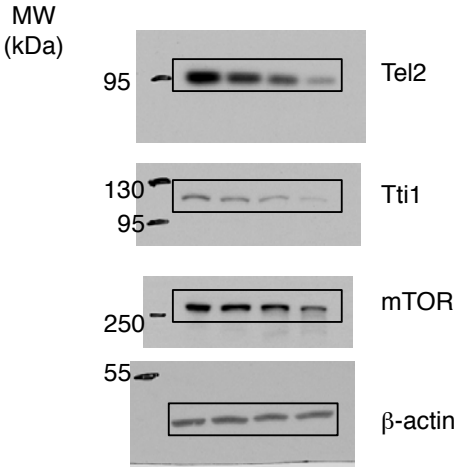

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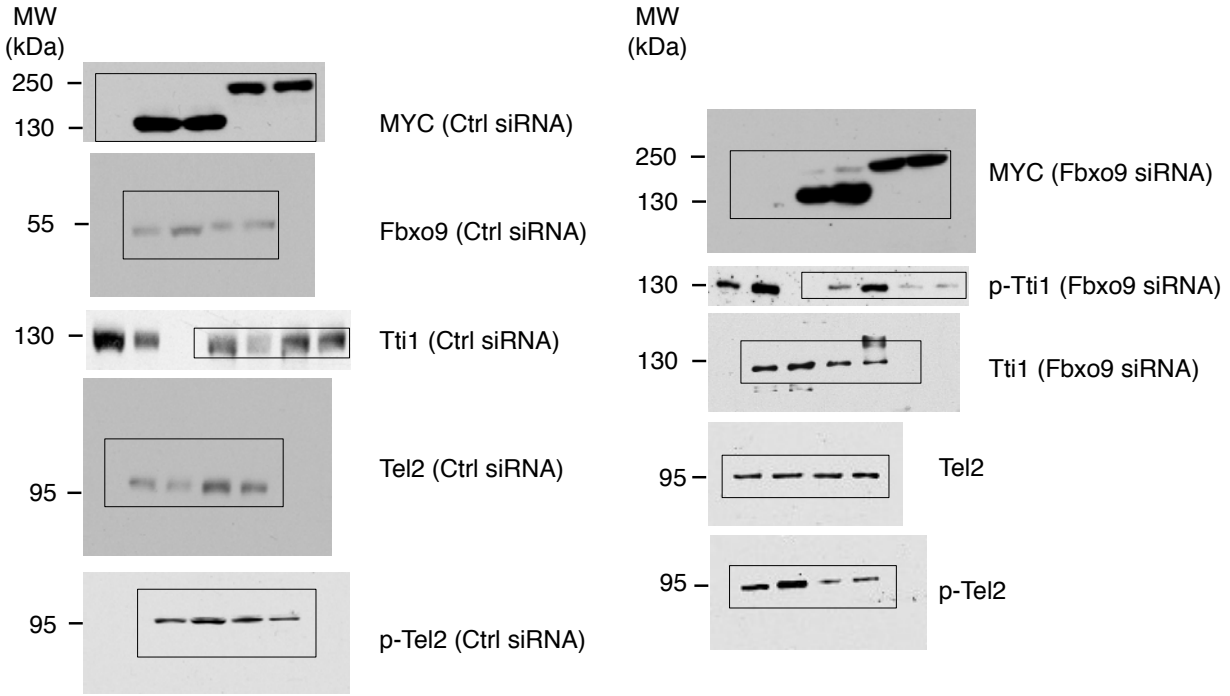

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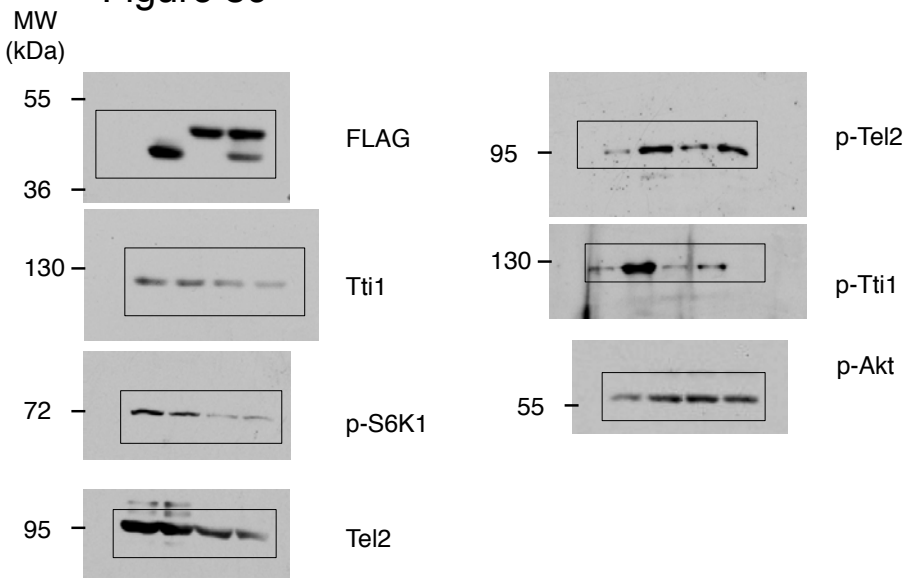

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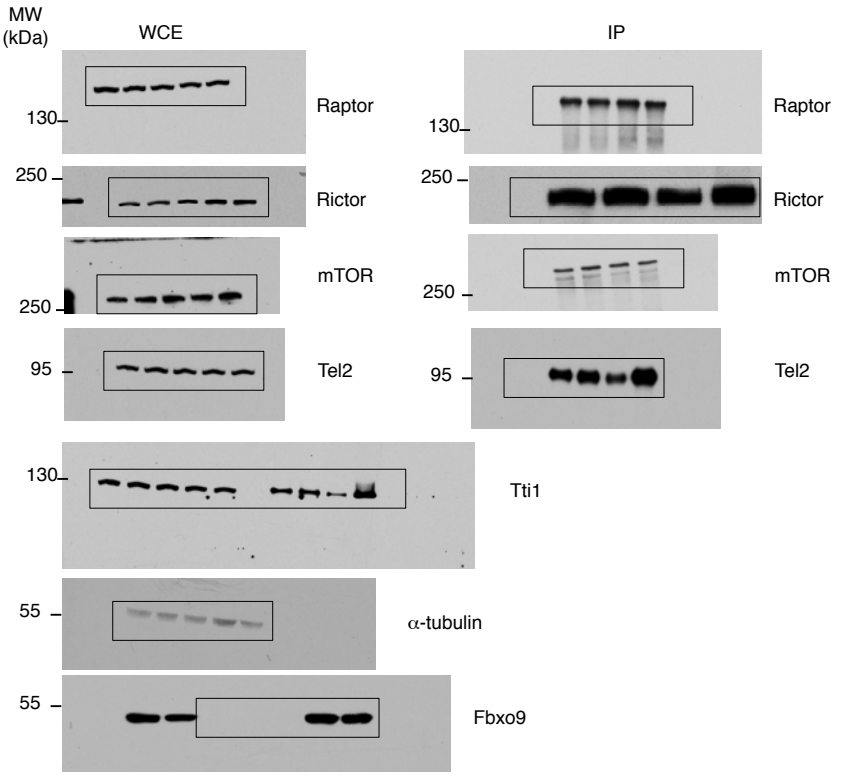

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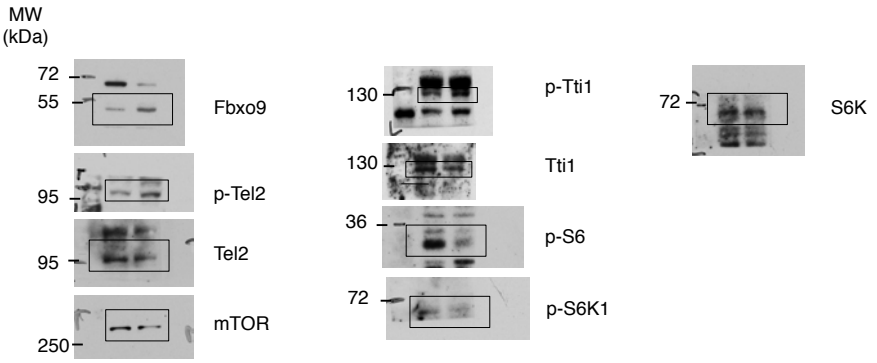

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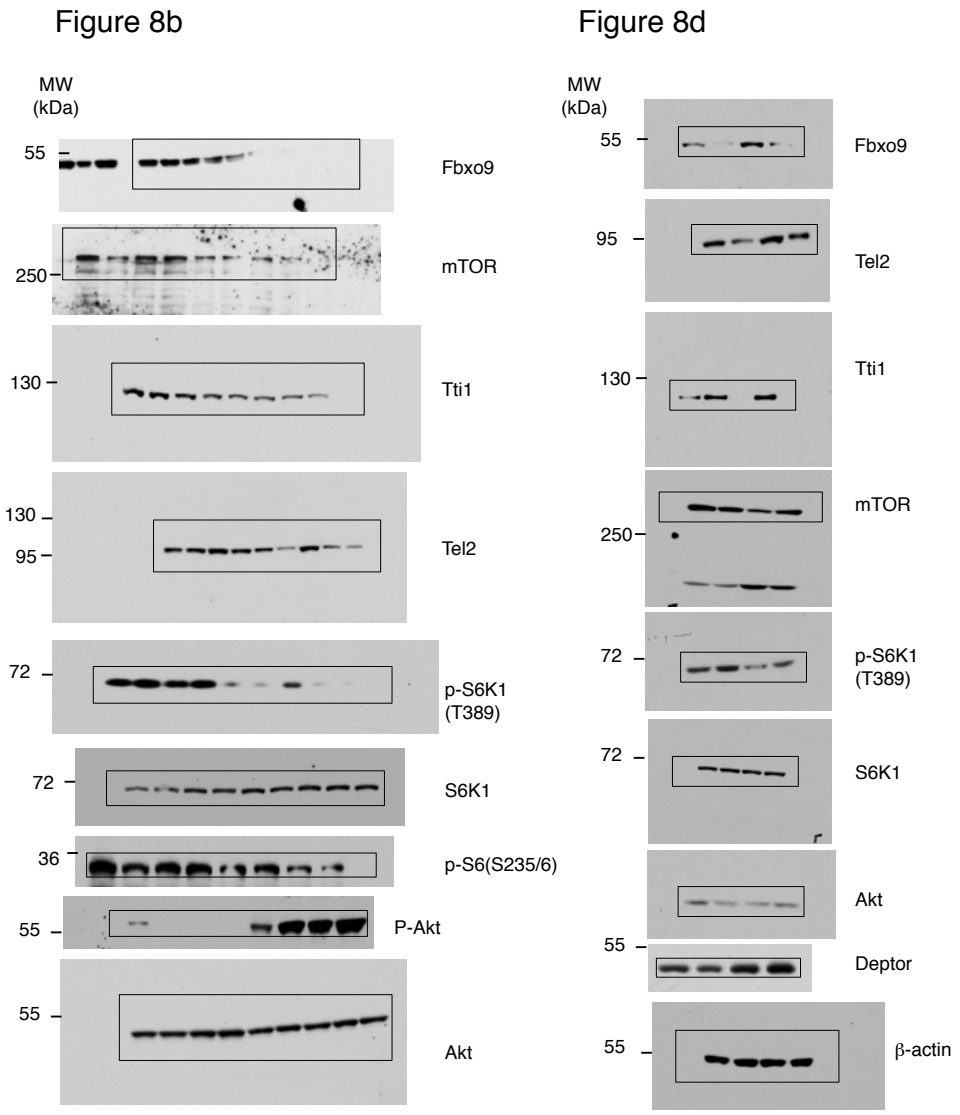

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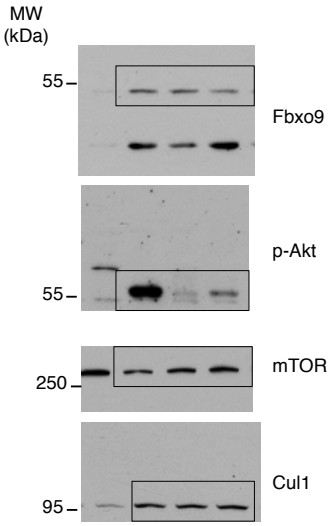

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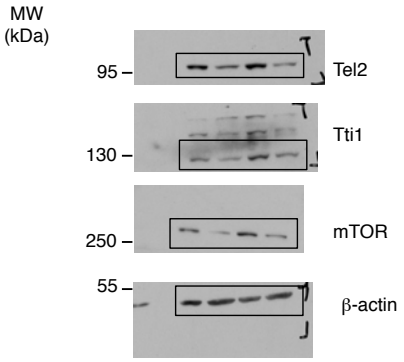

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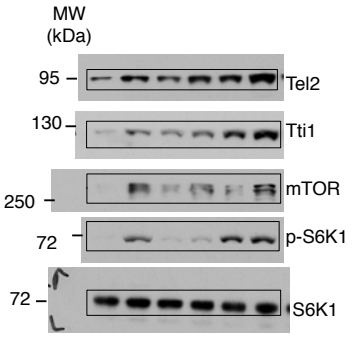

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