

Identification of F-box only protein 7 as a negative regulator of NF-kappaB signaling

Running Title:

FBXO7 is a negative regulator of NF- κ B signaling

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Abstract

The Nuclear factor κ B (NF- κ B) signaling pathway controls important cellular events such as cell proliferation, apoptosis and immune responses. Pathway activation occurs rapidly upon TNF α stimulation and is highly dependent on ubiquitination. Using cytoplasmic to nuclear translocation of the NF- κ B family member p65 as a read-out, we screened a synthetic siRNA library targeting enzymes involved in ubiquitin (Ub) conjugation and de-conjugation. We identified F-Box protein only 7 (FBXO7), a component of SCF-Ub ligase complexes as a negative regulator of NF- κ B signaling. FBXO7 binds to, and mediates ubiquitination of cIAP1 and TRAF2, resulting in decreased RIP1 ubiquitination and NF- κ B signaling activity.

Keywords

FBXO7, NF- κ B signaling, ubiquitination, cIAP1

Introduction

The Nuclear factor κ B (NF- κ B) signaling pathway controls important cellular events such as cell proliferation, apoptosis and immune responses (Chen & Goeddel, 2002). Binding of TNF α to TNF-Receptor 1 (TNF-R1) promotes rapid recruitment of a multi-protein complex known as the TNF-R1 signaling complex (TNF-RSC), consisting of TRADD, TRAF2/5, cIAP1/2, LUBAC and RIP1. TRADD functions as an adaptor protein recruiting TRAF2, which through constitutive association co-recruits cIAP1 and 2 (cIAP1/2) (Chan, 2007).

NF- κ B signaling relies heavily on ubiquitination. Both TRAF2 and cIAP1/2 are ubiquitin (Ub) ligases proposed to attach K63-linked Ub chains to RIP1. However, RIP1 K63-ubiquitination and NF- κ B signaling activity are restored upon reconstitution with an Ub-ligase activity-deficient TRAF2 mutant in TRAF2^{-/-} MEFs (Vince et al, 2009), suggesting that TRAF2 serves as a scaffold enabling cIAP1/2-mediated ubiquitination of RIP1 (Mahoney et al, 2008). This in turn leads to recruitment and activation of the TAK/TAB kinase complex and the Inhibitor of κ B Kinase (IKK) complex, consisting of IKK α , - β and NEMO, by binding to the K63-linked Ub chains on RIP1 (Kanayama et al, 2004; Wu et al, 2006). Moreover, K63-linked ubiquitination of TRAF2 has been reported to aid in recruitment of the IKK complex (Li et al, 2009). Recently it was also found that linear Ub chains formed by the linear Ub chain assembly complex enhance recruitment of NEMO to RIP1, thus increasing NF- κ B signaling activity (Haas et al, 2009). Once the IKK complex is active, it phosphorylates I κ B α , targeting this inhibitor for ubiquitination by SCF ^{β TRCP} and proteasomal degradation (Yaron et al, 1998). Subsequently, the NF- κ B

transcription factor hetero-dimer (p50/p65) translocates from the cytoplasm to the nucleus and induces target gene transcription (Baldwin, 1996).

In addition to being the likely candidate as the K63-Ub ligase for RIP1, cIAPs have several other targets within the NF- κ B signaling pathway including NEMO, TRAF2 and themselves by auto-ubiquitination (Samuel et al, 2006; Tang et al, 2003; Varfolomeev et al, 2007). However little is known about how cIAP Ub ligase activity and substrate specificity is regulated.

A number of de-ubiquitinating enzymes (DUBs) have been firmly placed as negative regulators of NF- κ B signaling. The Cylindroma tumor suppressor protein (CYLD) de-ubiquitinates NEMO and TRAF2 (Brummelkamp et al, 2003; Trompouli et al, 2003), while USP15 reverses β TRCP-mediated ubiquitination of I κ B α (Schweitzer et al, 2007). Moreover, the dual-activity protein A20 removes K63-Ub chains from RIP1, and targets it for proteasomal degradation (Wertz et al, 2004). Finally a number of other DUBs influence NF- κ B signaling upstream of, or at the level of I κ B α (Harhaj & Dixit, 2011).

Recruitment of the TNF-RSC and the ensuing ubiquitination events triggering signaling activation occur quite rapidly. In fact, within minutes of TNF α stimulation target gene activation can be measured. In the context of this biological timeframe we set out to identify new modulators of regulatory ubiquitination within NF- κ B signaling. We performed a siRNA screen based on cellular redistribution of p65 upon TNF α stimulation, using a library targeting DUBs and proteins involved in Ub conjugation. We identified several negative regulators of NF- κ B signaling and focused on the F-box only protein 7 (FBXO7), a member of the F-box protein family, which confers substrate

specificity to SCF-Ub ligase complexes. We show that FBXO7 is a negative regulator of NF- κ B signaling, modulating ubiquitination levels of several components of the TNF-RSC, and ultimately lowering NF- κ B signaling activity.

Results

A siRNA screen searching for novel regulators of NF- κ B signaling

To identify modulators of regulatory ubiquitination in NF- κ B signaling we performed an imaging-based screen based on sub-cellular redistribution of NF- κ B p65, using a commercially available siRNA library targeting DUBs and other proteins involved in ubiquitination. Cells were transfected with the siRNA library, left for 72 hours, stimulated with TNF α for 20 minutes or left untreated prior to fixation (Fig 1A). Immunofluorescence labeling was used to stain endogenous p65, and DNA was stained with DAPI to define nuclei. Images were acquired using an automated microscope, analyzed with CellProfiler software (Carpenter et al, 2006), and the nuclear - cytoplasmic (Nuc/Cyt) ratio of the p65 signal was calculated (Fig 1B).

The siRNA pools that significantly altered Nuc/Cyt ratio of p65 were selected for further validation. Of the selected 15, including CYLD and A20, 6 showed reproducible phenotypes, and 3 were validated with 2 unique siRNAs, decreasing the chance of off-target effects. Inhibition of these three proteins lead to increased nuclear accumulation of p65 upon TNF α stimulation (Fig 1C,D). These novel modulators of NF- κ B signaling include the DUBs OTU domain-containing aldehyde-binding protein 2 (OTUB2), and signal transducing adaptor molecule binding protein (STAMBP) (Nijman et al, 2005). The remaining hit FBXO7 belongs to the F-box protein family, which in the context of Skp, Cullin, F-box (SCF) Ub ligase complexes, confer substrate specificity (Winston et al, 1999). Interestingly, FBXO7 can both stabilize and destabilize its interactors (Hsu et al, 2004; Laman et al, 2005), and certain recessive mutants of FBXO7 have been linked

to early-onset Parkinson-pyramidal syndromes (Di Fonzo et al, 2009; Shojaee et al, 2008). Furthermore a study suggested that cIAP1, a well-established positive regulator of NF- κ B signaling, is ubiquitinated by SCF^{FBXO7} (Chang et al, 2006). However, the effect of this modification on NF- κ B signaling was not examined. We therefore set out to investigate how FBXO7 might influence the NF- κ B pathway.

F-box only protein 7 is a negative regulator of NF- κ B signaling

Having established that FBXO7 inhibition leads to increased accumulation of nuclear p65, we examined whether NF- κ B target gene activation is affected. We measured activation of the NF- κ B target genes IL8 and A20 by Q-RT PCR in cells transfected with siRNAs targeting TNF-R1, CYLD, or FBXO7. As expected, knockdown of TNF-R1 impairs activation of IL8 and A20, while knockdown of the negative regulator of NF- κ B signaling CYLD results in transcriptional hyper-activation (Fig 2A). Similarly, FBXO7 knockdown also results in hyper-activation of IL8 and A20 after TNF α treatment. Importantly, in an independent cell line, immortalized BJ primary fibroblasts, knockdown of FBXO7 shows similar effects (Fig 2B).

Next, we tested whether over-expression of FBXO7 suppresses NF- κ B signaling. A NF- κ B luciferase reporter was co-transfected with plasmids encoding either wild type (WT), or a F-box deletion mutant (Δ F) FBXO7 that no longer forms a SCF complex. We found that over-expression of WT but not Δ F FBXO7 suppresses the activity of the NF- κ B luciferase reporter upon stimulation with TNF α (Fig 2C). We conclude that the regulation of NF- κ B signaling by FBXO7 depends on SCF^{FBXO7} complex formation, and that FBXO7 negatively regulates NF- κ B signaling.

FBXO7 acts upstream of I κ B α degradation

Next, we investigated whether FBXO7 influences I κ B α protein degradation upon TNF α stimulation. Cells were transfected with siRNAs targeting CYLD or FBXO7. TNF α time-course experiments were performed and I κ B α phosphorylation and degradation was visualized by Western blots. As expected, knockdown of CYLD causes increased I κ B α protein degradation, even without TNF α stimulation (Fig 3A). Knockdown of FBXO7 results in a similar phenotype but degradation is primarily affected upon TNF α treatment, in agreement with the nuclear translocation assays (Figure 1C). Conversely, over-expression of WT but not the Δ F FBXO7 results in I κ B α stabilization, explaining the diminished signaling seen upon over-expression in reporter experiments (Fig 3B & 2D). These observations point to the TNF-RSC as the site of action for FBXO7, supported by the previously reported FBXO7 - cIAP1 interaction (Chang et al, 2006).

FBXO7 promotes ubiquitination of bound cIAP1

To gain further insight into possible sites of action of FBXO7 in the context of the TNF-RSC, we examined if FBXO7 interacts with TNF-RSC components. First, in co-transfection experiments with tagged versions of either WT or Δ F FBXO7 and cIAP1, we confirmed that cIAP1 binds to WT, but also Δ F FBXO7 (Fig 4A). These interactions were not influenced by TNF α stimulation (data not shown). Next, we investigated whether cIAP1 is ubiquitinated by SCF^{FBXO7}. We observed no change in the ubiquitination levels of total cellular cIAP1 in the presence of exogenous FBXO7 (data not shown). However, when scaling up FBXO7-cIAP1 co-immunoprecipitation

experiments (IP), we found that cIAP1 specifically bound to WT but not Δ F FBXO7, is highly ubiquitinated (Fig 4B).

FBXO7 binds to, and ubiquitinates TRAF2

TRAF2 and cIAP1/2 are constitutively bound to each other and co-recruited to the TNF-RSC upon TNF α stimulation (Chan, 2007). We therefore asked whether FBXO7 might also interact with TRAF2 and be a constitutive member of the complex. We immunoprecipitated Flag-TRAF2 from cells that had been co-transfected with GFP-FBXO7 WT or Δ F constructs. We found that FBXO7 binds to TRAF2 (Fig 5A), independently of TNF α (data not shown). We next examined whether FBXO7 also affects TRAF2 ubiquitination. Cells were transfected with constructs expressing Flag-TRAF2, HA-Ub and GFP-FBXO7 WT or Δ F. To avoid possible ubiquitination contamination from other proteins we lysed cells in denaturing RIPA buffer. We then performed Flag-IPs and immuno-blots for HA and found that there is a significant increase in TRAF2 ubiquitination upon over-expression of WT FBXO7 (Fig 5B). In summary FBXO7 binds to cIAP1 and TRAF2, and promotes ubiquitination of both proteins, the consequence being decreased NF- κ B signaling.

FBXO7 lowers RIP1 ubiquitination

Since TRAF2 and cIAP1 are both important for ubiquitination of RIP1, we investigated whether FBXO7-induced changes in TRAF2 and cIAP1 ubiquitination alter RIP1 ubiquitination. In co-IP experiments we tried to determine whether FBXO7 and RIP1 interact but we were unable to detect this interaction (data not shown). We then measured

RIP1 ubiquitination levels in cells transfected with Flag-RIP1, HA-Ub and GFP-FBXO7 WT or ΔF . We found that over-expression of WT FBXO7 diminishes RIP1 ubiquitination compared to the inactive ΔF FBXO7 (Fig 5C). We hypothesize that this decrease reduces recruitment and activation of kinase complexes, preventing I κ B α degradation, and as a consequence lowers NF- κ B signaling.

Discussion

We screened a siRNA library for modulators of regulatory ubiquitination in NF- κ B signaling. Employing cellular redistribution of the NF- κ B subunit p65 as a read-out, we identified and verified three new regulators. We focused our attention on F-Box only protein 7 (FBXO7), which has different known functions. In the context of SCF^{FBXO7} proteasomal degradation of Hepatoma Up-Regulated Protein is promoted (Hsu et al, 2004). Other functions such as stabilization of Cdk6/CyclinD complexes, and pro-B cell differentiation have recently been linked to FBXO7 nuclear localization (Nelson & Laman, 2011; Meziane et al, 2011). Contrarily, our data show that in NF- κ B signaling, FBXO7 exerts its function in the cytoplasm.

RIP1 is a key protein within the NF- κ B signaling pathway with K63 Ub chains serving as recruitment platforms for the two kinase-complexes TAK/TAB and IKK (Kanayama et al, 2004; Wu et al, 2006). This K63-linked Ub conjugation depends on recruitment of TRAF2-cIAP1/2 complexes to the TNF-RSC with TRAF2 serving as a scaffold for cIAP1/2 (Mahoney et al, 2008). We showed that FBXO7 binds to both cIAP1 and TRAF2 independently of TNF α , suggesting constitutive binding and co-recruitment to the TNF-RSC upon stimulation. We found that FBXO7 promotes ubiquitination of cIAP1 and TRAF2, leading to a decrease in RIP1 ubiquitination and lowered NF- κ B signaling activity. Different scenarios can be envisioned to account for this decrease. SCF^{FBXO7} could target both cIAP1 and TRAF2 for proteasomal degradation thus preventing ubiquitination of RIP1 and signaling activation. However, in our experiments we did not see alterations in neither cIAP1 nor TRAF2 protein levels as a consequence of FBXO7 knock down or over-expression arguing against this idea (data not shown). We

note that this could also be due to rapid de novo protein synthesis. Within the context of NF- κ B signaling, in addition to RIP1, cIAP1/2 can promote ubiquitination of NEMO, TRAF2 as well as auto-ubiquitination. However, at present it remains unclear how cIAP1/2 substrate selectivity is determined. We observed that cIAP1 specifically bound by FBXO7 is ubiquitinated. Perhaps this modification could switch cIAP1 substrate selectivity away from RIP1, thus decreasing kinase complex recruitment. One could even speculate that the Ub ligase activity of cIAP1 could be turned to TRAF2, possibly targeting this protein for proteasomal degradation.

Some evidence exists that NF- κ B induced inflammation may be involved in development of Parkinson's disease (reviewed in (Flood et al, 2011)). As mentioned earlier, certain recessive mutants of FBXO7 have been linked to early-onset Parkinson-pyramidal syndromes. We investigated whether such mutations might influence NF- κ B signaling and thereby promote disease phenotypes using two FBXO7 patient mutants (R378G and R498X). However, neither binding to, nor ubiquitination of cIAP1 or TRAF2 was affected significantly by these mutations, and in NF- κ B luciferase experiments patient mutants behaved like WT FBXO7 (data not shown). It remains possible that the investigated patient mutations give highly subtle phenotypes, or that defects are only noticeable in neuronal cell types. Alternatively, development of Parkinson-pyramidal syndrome may be a consequence of yet to be determined functions of FBXO7.

In conclusion, this study strongly implicates a novel inhibitory role of FBXO7 at the TNF-RSC in modulating regulatory ubiquitination events in NF- κ B signaling. Future

experiments will elucidate the precise mode of action further, expanding our knowledge of this complex regulated signaling pathway.

Methods

The screen, siRNA sequences, Q-RT PCR primers, cell culture, and immunoblotting conditions used in this study are described in the supplementary information online.

siRNA transfections and qRT-PCR

U2OS cells and immortalized BJ primary human fibroblasts (BJET) were transfected with Dharmafect 1 or 3 (Thermo Scientific) transfection agents. Cells were refreshed 24 hours after transfection, incubated for 48 hours, and treated with 10 ng/mL TNF α (Sigma) for 45min. RNA was isolated with Trizol (Invitrogen) or RNAeasy mini kit (Qiagen). cDNA was generated with SuperScript II kit (Invitrogen). FastStart MasterPLUS SYBR Green kit (Roche), and appropriate primers (supplementary data) were used to measure relative mRNA levels. The mean of the technical replicates is either shown directly, applicable to the representative figures, or used to summarize three independent experiments.

Plasmids and Antibodies

The Luciferase plasmid NF- κ B-Luc is from Clontech, SV40-Renilla from Promega. pEGFP-FBXO7 and pVlag- FBXO7 were cloned by PCR amplification of IMAGE clone 3611049. Sal I site containing PCR primers were used to make the Δ F mutant. pEGFP-cIAP1 was cloned by PCR amplification of IMAGE clone 3908352.

Antibodies: anti-GFP (FL) and (B-2), anti-p65 (C-20), anti-CDK4 (C-22), anti-Ub (P4D1) from Santa Cruz (S.C), anti-flag (M2) from Sigma, anti-pIkB α (5A5) and anti-IkB α (L35A5) from Cell Signaling Technology, anti-cIAP1 (AF8181) from R&D

Systems, and anti-HA (12CA5) from a hybridoma culture supernatant from our lab. FBXO7 rabbit antibody (Laman et al, 2005). Normal mouse IgG from S.C was non-immune control in IP experiments.

References

- Baldwin AS, Jr. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* **14**: 649-83
- Brummelkamp TR, Nijman SM, Dirac AM, Bernards R (2003) Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. *Nature* **424**(6950): 797-801.
- Carpenter AE, et al. (2006) CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol* **7**(10): R100
- Chan FK (2007) Three is better than one: pre-ligand receptor assembly in the regulation of TNF receptor signaling. *Cytokine* **37**(2): 101-7
- Chang YF, Cheng CM, Chang LK, Jong YJ, Yuo CY (2006) The F-box protein Fbxo7 interacts with human inhibitor of apoptosis protein cIAP1 and promotes cIAP1 ubiquitination. *Biochem Biophys Res Commun* **342**(4): 1022-6
- Chen G, Goeddel DV (2002) TNF-R1 signaling: a beautiful pathway. *Science* **296**(5573): 1634-5
- Di Fonzo A, et al.(2009) FBXO7 mutations cause autosomal recessive, early-onset parkinsonian-pyramidal syndrome. *Neurology* **72**(3): 240-5
- Flood PM, Qian L, Peterson LJ, Zhang F, Shi JS, Gao HM, Hong JS Transcriptional Factor NF-kappaB as a Target for Therapy in Parkinson's Disease. *Parkinsons Dis* **2011**: 216298
- Haas TL, et al.(2009) Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. *Mol Cell* **36**(5): 831-44
- Harhaj EW, Dixit VM (2011) Deubiquitinases in the regulation of NF-kappaB signaling. *Cell Res* **21**(1): 22-39
- Hsu JM, Lee YC, Yu CT, Huang CY (2004) Fbx7 functions in the SCF complex regulating Cdk1-cyclin B-phosphorylated hepatoma up-regulated protein (HURP) proteolysis by a proline-rich region. *J Biol Chem* **279**(31): 32592-602
- Kanayama A, Seth RB, Sun L, Ea CK, Hong M, Shaito A, Chiu YH, Deng L, Chen ZJ (2004) TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. *Mol Cell* **15**(4): 535-48

- Laman H, Funes JM, Ye H, Henderson S, Galinanes-Garcia L, Hara E, Knowles P, McDonald N, Boshoff C (2005) Transforming activity of Fbxo7 is mediated specifically through regulation of cyclin D/cdk6. *Embo J* **24**(17): 3104-16
- Li S, Wang L, Dorf ME (2009) PKC phosphorylation of TRAF2 mediates IKKalpha/beta recruitment and K63-linked polyubiquitination. *Mol Cell* **33**(1): 30-42
- Mahoney DJ, et al.(2008) Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation. *Proc Natl Acad Sci U S A* **105**(33): 11778-83
- Meziane el K, Randle SJ, Nelson DE, Lomonosov M, Laman H (2011) Knockdown of Fbxo7 reveals its regulatory role in proliferation and differentiation of haematopoietic precursor cells. *J Cell Sci* **124**(Pt 13): 2175-86
- Nelson DE, Laman H (2011) A Competitive Binding Mechanism between Skp1 and Exportin 1 (CRM1) Controls the Localization of a Subset of F-box Proteins. *J Biol Chem* **286**(22): 19804-15
- Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK, Bernards R (2005) A genomic and functional inventory of deubiquitinating enzymes. *Cell* **123**(5): 773-86
- Samuel T, Welsh K, Lober T, Togo SH, Zapata JM, Reed JC (2006) Distinct BIR domains of cIAP1 mediate binding to and ubiquitination of tumor necrosis factor receptor-associated factor 2 and second mitochondrial activator of caspases. *J Biol Chem* **281**(2): 1080-90
- Schweitzer K, Bozko PM, Dubiel W, Naumann M (2007) CSN controls NF-kappaB by deubiquitylation of IkappaBalpha. *Embo J*
- Shojaee S, Sina F, Banihosseini SS, Kazemi MH, Kalhor R, Shahidi GA, Fakhrai-Rad H, Ronaghi M, Elahi E (2008) Genome-wide linkage analysis of a Parkinsonian-pyramidal syndrome pedigree by 500 K SNP arrays. *Am J Hum Genet* **82**(6): 1375-84
- Tang ED, Wang CY, Xiong Y, Guan KL (2003) A role for NF-kappaB essential modifier/IkappaB kinase-gamma (NEMO/IKKgamma) ubiquitination in the activation of the IkappaB kinase complex by tumor necrosis factor-alpha. *J Biol Chem* **278**(39): 37297-305
- Trompouli E, Hatzivassiliou E, T T, Farmer H, Ashworth A, Mosialos G (2003) The familial cyclindromatosis gene product is a deubiquitinating enzyme that negatively regulates NF-kB activation by members of the TNF receptor family. *Nature*(This issue)
- Varfolomeev E, et al. (2007) IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell* **131**(4): 669-81

Vince JE, et al. (2009) TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (tnf) to efficiently activate nf- κ b and to prevent tnf-induced apoptosis. *J Biol Chem* **284**(51): 35906-15

Wertz IE, et al. (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF- κ B signalling. *Nature* **430**(7000): 694-9

Winston JT, Koepp DM, Zhu C, Elledge SJ, Harper JW (1999) A family of mammalian F-box proteins. *Curr Biol* **9**(20): 1180-2

Wu CJ, Conze DB, Li T, Srinivasula SM, Ashwell JD (2006) Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF- κ B activation [corrected]. *Nat Cell Biol* **8**(4): 398-406

Yaron A, Hatzubai A, Davis M, Lavon I, Amit S, Manning AM, Andersen JS, Mann M, Mercurio F, Ben-Neriah Y (1998) Identification of the receptor component of the I κ B α -ubiquitin ligase. *Nature* **396**(6711): 590-4

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Author Contributions Statement

HJK designed, performed and analyzed experiments. DE did screen set-up. HL provided antibody and manuscript input. RB and RB supervised experiments and manuscript writing. AMD designed, performed and analyzed experiments, supervised project and wrote manuscript

Figures

Fig 1

Screen for modulators of NF- κ B signaling. (A) U2OS cells were transfected with the siRNA library, stimulated with TNF α or left untreated, and analyzed for cellular redistribution of NF- κ B p65. Representative photos of unstimulated and TNF α stimulated cells showing endogenous p65 staining. (B) Control normalized ratios of Nuc/Cyt ratio of p65 intensity for the siRNA pools included in the screen. Error bars shown in red. (C) Summary of three independent experiments for redistribution of p65 upon knockdown of identified modulators or controls. A non-targeting (scrambled) siRNA smartpool was the negative control. (D) Summary of three independent experiments showing the knockdown efficiency determined by Q-RT PCR for mRNA levels of the identified genes. (C and D) $*P < 0.05$, values are mean \pm standard deviation. P -value computed from unpaired two-tailed t -test.

Fig 2

FBXO7 regulates NF- κ B signaling. (A & B) Q-RT PCR for NF- κ B target genes, A20 and IL8, activation in (A) U2OS and (B) immortalized primary human fibroblasts (BJET). Scrambled negative control was used for normalization. Values are mean \pm standard deviation of replicate measurements from a representative experiment. (C) NF- κ B luciferase reporter assay in U2OS cells expressing either empty vector, Flag-tagged wild type FBXO7 (WT), or F-box deletion mutant FBXO7 (Δ F). Values represent ratio of Luciferase over Renilla control activity \pm TNF α . $*P < 0.05$, values are mean \pm

standard deviation of four independent experiments. *P*-value computed from unpaired two-tailed *t*-test.

Fig 3

FBXO7 regulates NF- κ B signaling upstream of I κ B α degradation. (A) Western blot analysis of U2OS cells transfected with scrambled control, CYLD, or FBXO7 siRNAs, stimulated with TNF α for 5, 10, 15, 20 mins, or left untreated. L.E. and S.E.: Long and short exposure. (B) Western blot analysis of U2OS cells expressing empty vector (EV), Flag-FBXO7 WT, or Δ F.

Fig 4

FBXO7 interacts with and mediates ubiquitination of cIAP1. (A) Western blot analysis of a co-immunoprecipitation (IP) experiment in U2OS cells expressing GFP-cIAP1 and Flag-FBXO7 WT or Δ F. (B) Western blot analysis of *in vivo* ubiquitinated GFP-cIAP1 co-IPed with Flag-FBXO7 WT or Δ F.

Fig 5

FBXO7 interacts with TRAF2 and alters ubiquitination of TRAF2 and RIP1. (A) Western blot analysis of a co-IP experiment in U2OS cells expressing Flag-TRAF2 and GFP-tagged WT or Δ F FBXO7. (B & C) Western blot analysis of *in vivo* ubiquitination assays in HEK293 cells expressing HA-Ub, Flag-TRAF2 or Flag-RIPK1, GFP only, GFP-FBXO7 WT or Δ F.

Figure 1. Identification of F-box only protein 7 as a negative regulator of NF- κ B signalling.

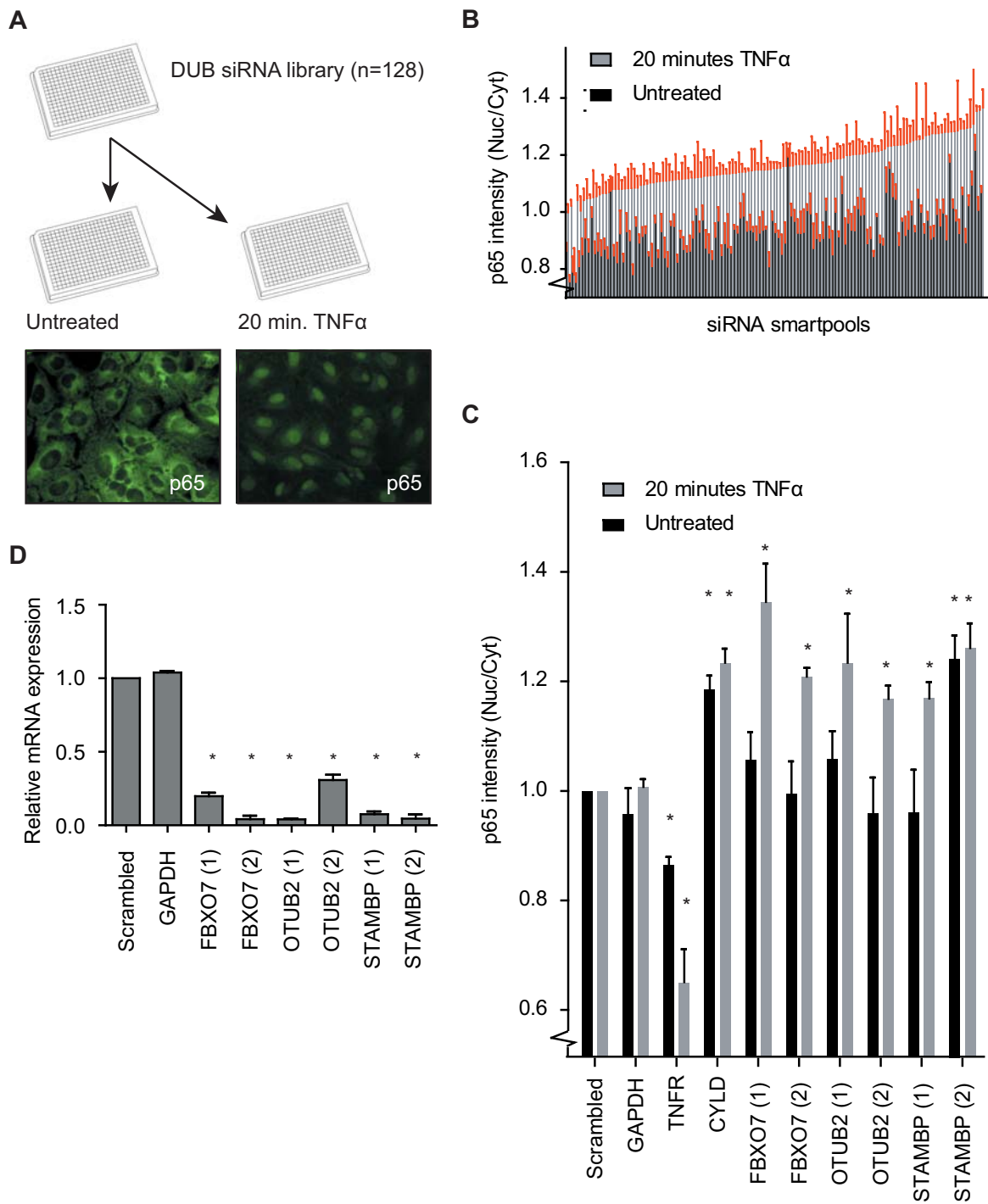


Figure 2. Identification of F-box only protein 7 as a negative regulator of NF- κ B signalling.

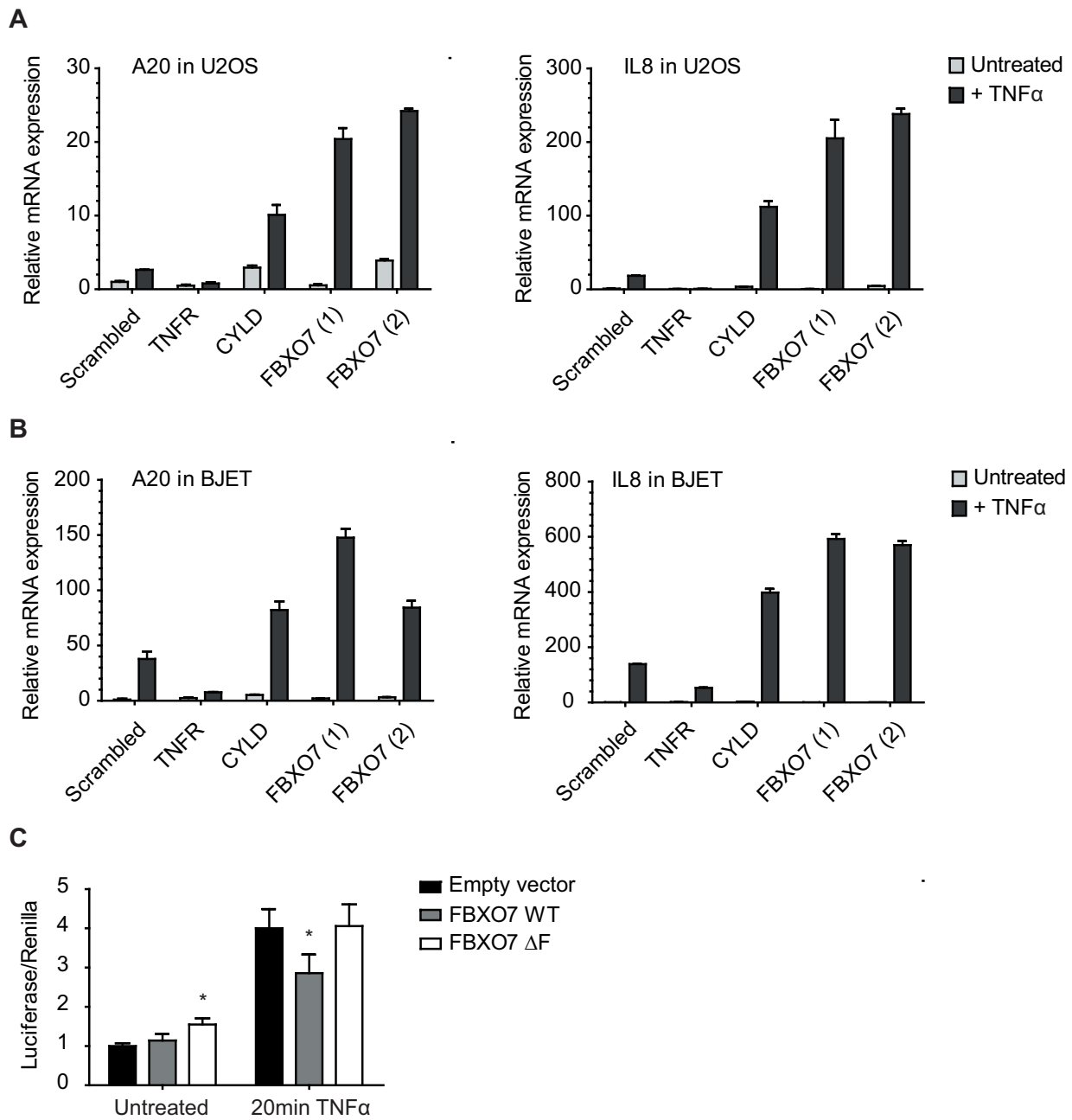
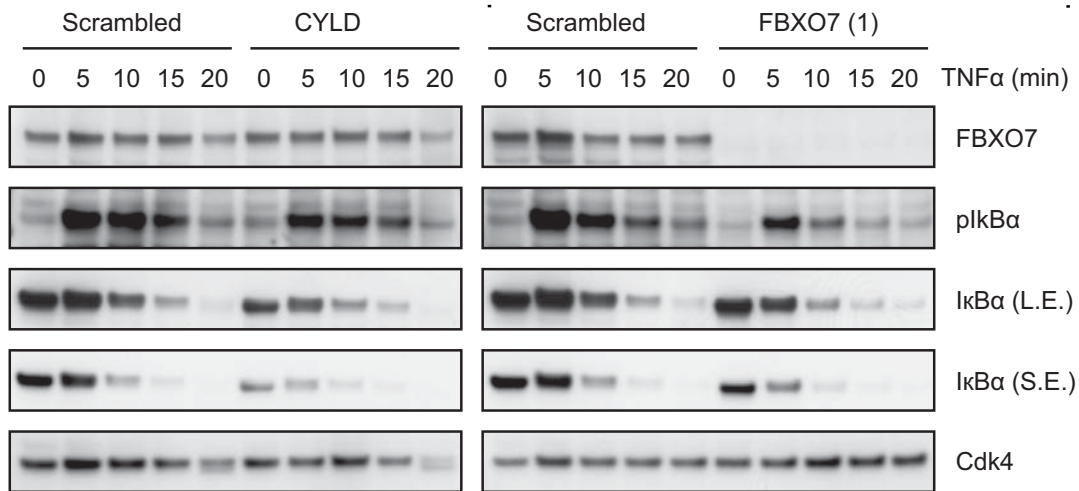


Figure 3. Identification of F-box only protein 7 as a negative regulator of NF- κ B signalling.

A



B

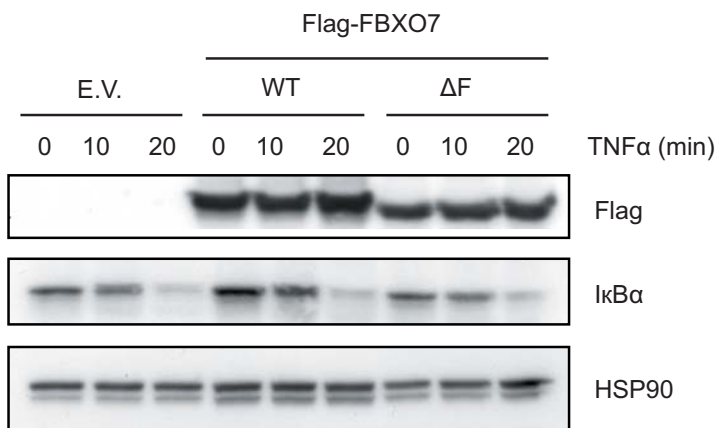


Figure 4. FBXO7 interacts with cIAP1 resulting in cIAP1 ubiquitination

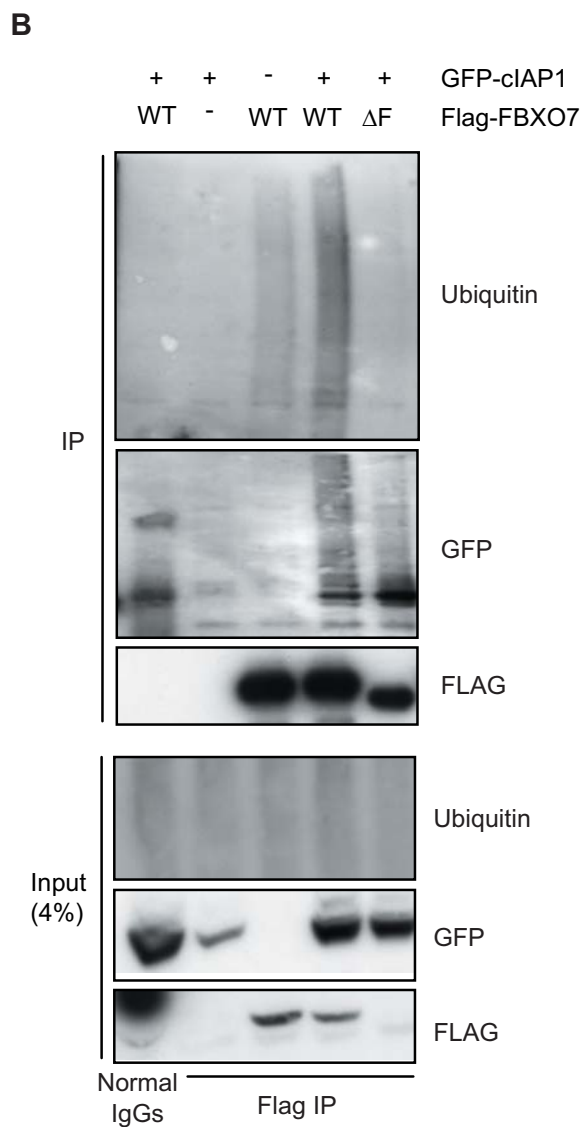
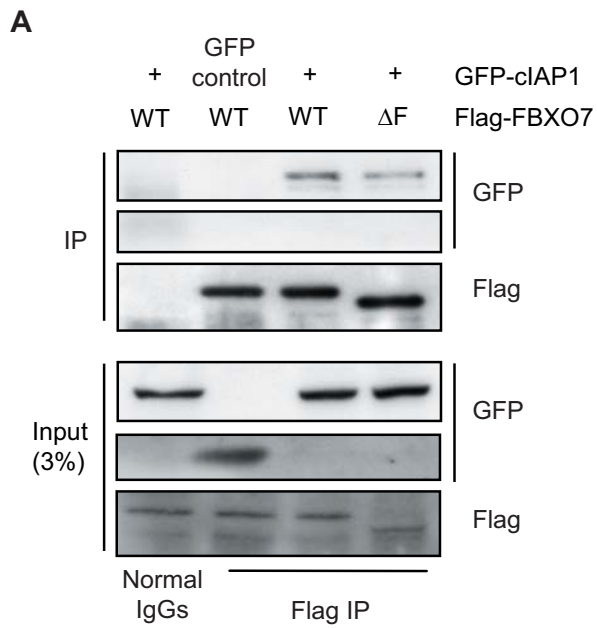


Figure 5. Identification of F-box only protein 7 as a negative regulator of NF- κ B signalling.

