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Ubiquitin tagged dominant negative induces degradation of B-ZIP proteins

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ABSTRACT

B-ZIP transcription factors heterodimerize with dominant negative designs, termed A-ZIPs, in a dimerization specific manner and inhibit its ability to bind DNA. Different A-ZIPs produce unique phenotypes *in vivo* suggesting that they have distinct B-ZIP heterodimerization partners. However, the identification of the *in vivo* heterodimerization partners of different A-ZIPs remains problematic. To identify the *in vivo* heterodimerization partners, a chimeric protein containing two ubiquitin motifs at the N-terminal of the A-ZIP domain was designed. The presence of ubiquitin reduced the concentration of specific co-transfected B-ZIP proteins. The ubiquitin enhanced degradation of the B-ZIP heterodimeric partner is inhibited by the proteasome inhibitor MG-132. These ubiquitin tagged A-ZIP dominant negatives may be more active *in vivo* because their endogenous heterodimerization partners are degraded more efficiently. This may be a general strategy to identify protein interaction partners.

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1. Introduction

The 55 human B-ZIP proteins function as homodimers or heterodimers to bind DNA in a sequence-specific manner and regulate genes involved in cell growth and differentiation [1,2]. To understand the function of structurally related B-ZIP proteins, we have designed dominant negatives, termed A-ZIPs, which contain only the B-ZIP leucine zipper domain and an appended amphipathic α-helical sequence that replaces the B-ZIP basic region [3–7]. The A-ZIP proteins heterodimerize with B-ZIP proteins via their leucine zippers and the acidic extension forms a coiled-coil structure with the B-ZIP basic region, essentially zippers the leucine zipper into the basic region [2]. In our previous studies, we have shown that the inhibition of B-ZIP DNA binding results in physiological effects in transgenic mouse models [8–11].

Expression of the A-ZIP protein A-C/EBP in the epidermis of adult mice prevents skin papilloma formation. If A-C/EBP is expressed after papilloma formation, the papillomas regress [8]. In contrast, A-Fos expression in mammalian cells reduces Ha-*ras*-mediated cellular transformation [4] whereas A-Fos expression in mouse epidermis converts papillomas to benign sebaceous adenomas and prevents conversion into carcinomas [12]. A-CREB expression prevents pappilloma formation but do not cause regression [9]. Taken together, these results suggest that the effective inhibition of B-ZIP DNA binding could be a clinically relevant molecular target.

We observe that the expression of the A-ZIP sometimes causes its *in vivo* B-ZIP target protein expression to be reduced [8] suggesting that the inhibition of B-ZIP DNA binding accelerates degradation. To enhance B-ZIP degradation, we appended two copies of ubiquitin to the A-ZIP. Ubiquitin is a 76-amino acid peptide that is covalently attached to proteins targeting them to the proteasome complex for degradation [13]. By tagging the A-ZIPs with ubiquitin, the B-ZIP|A-ZIP heterodimer may enter the ubiquitin proteasome pathway, leading to the degradation of the heterodimer [14,15]. The induced rapid turnover of pathological B-ZIP proteins may transform ubiquitin–A-ZIPs into more potent dominant negatives.

2. Materials and methods

2.1. B-ZIP, A-ZIP and ubiquitin–A-ZIP Plasmids

The B-ZIP domains of 10 different B-ZIP proteins (ATF2, C/EBPα, C/EBPβ, CREB, cFos, FosB, cJun, JunB, JunD, VBP) containing a green fluorescent protein (GFP) tag were previously designed [16]. Additionally, the A-ZIP domain plasmids were constructed as described previously [17]. Three dominant negative designs (A-FOS, A-C/EBPα, A-CREB) containing plasmids were digested with HindIII and BamHI restriction enzymes. Ubiquitin protein domain was PCR amplified using primers containing HindIII or BamHI restriction enzyme sites and digested with HindIII and BamHI. Gel purified ubiquitin fragments were subsequently ligated into the HindIII-BamHI open site of pT5 plasmid. The resulting plasmid was DNA sequenced to insure both the ubiquitin and A-ZIP design

Abbreviations: B-ZIP domain, basic leucine zipper domain; CREB, CRE-binding protein; VBP, vitellogenin gene-binding protein; C/EBP, CAAT/enhancer binding protein; AP1, activator protein 1.

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was present. After miniprep isolation and small-scale simplification, the ubiquitin–A-ZIP fragment was removed by its HindIII-NdeI sites and inserted into the complementary site on the CMV-500 vector; allowing for efficient cell transfections. The final construct was verified by digestion analysis with the HindIII and NdeI restriction enzymes. The protein sequence of ubiquitin–A-FOS is

*MQIFVKTLTGKTITLEVESSDTIDNVKSKIQDKEGIPPDQQRLIFAGKQLED GRTLSDYNIQKESTLHLVLRLRGG*MQIFVKTLTGKTITLEVESSDTIDNVKSK IQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLTGG**PVAT RDPDLEQRAEELARENEELEKEAEELEQELAELEAETDQLEDEKSALQ-TEIANLLKEKEKL**, (ubiquitin tags in italics and underlined, A-FOS in bold).

2.2. Cell culture and transfections

NIH3T3 (ATCC) cells were maintained in Dulbecco's Modified Eagle Medium with high glucose (Invitrogen), 10% bovine calf serum (Hyclone), and 1% Antibiotic/Antimycotic (Invitrogen) in a 5% CO_2 incubator at 37 °C. The cells were transfected with either GFP-tagged B-ZIP alone or co-transfected with A-ZIP or ubiquitin–A-ZIP. Transfections were carried out following standard protocol (Invitrogen); cells were plated at a density of 6×10^4 on 12-well cell culture clusters (Corning Incorporated) and were transiently transfected with a standardized total DNA plasmid amount (1.3 μg) and lipofectamine (4.0 μl per well) in serum-reduced OPTI-MEM media (Gibco-Invitrogen). After 4 h of transfection, OPTI-MEM media were replaced with serum rich media and cultured for 24 h. Cultures were checked for GFP expression with fluorescence microscopy and harvested 24 h post-transfection using RIPA buffer with a Protease Inhibitor Cocktail Tablet (complete-Mini EDTA-free, Roche). Subsequent BSA protein concentration assays were carried out using standard procedures (Thermo Scientific).

For proteasome inhibition assays, MG-132 (Sigma–Aldrich) was added to the cells at 0, 2.5, 5, and 10 μM concentrations [18]. The compound was suspended in DMSO and added to the cells 4 h post-transfection in fresh media. Controls were kept in DMSO without inhibitor.

2.3. Western blot analysis

Pre-determined amounts of protein were denatured in 1× NuPAGE loading buffer (Invitrogen), 50 mM DTT, and RIPA buffer-Protease inhibitor solution at 70 °C for 10 min. Samples were loaded onto NuPAGE 4–12% Bis–Tris Gel (Invitrogen) and run for SDS electrophoresis (150 V, 90 min). Proteins were then transferred onto a polyvinylidene difluoride membrane (PVDF, Invitrogen) at 30 V for 90 min. The membrane was incubated for 1 h at room temperature in PBS-T (pH7.4, 1× PBS, 0.1% Tween20) containing 5% non-fat dry milk (American Bioanalytical), and then incubated overnight at 4 °C with primary antibodies in milk solution. Antibodies were used for GFP (1:1000 dilution, sc-9996) and the Beta-Actin control (1:2000 dilution, sc-47778). Following the incubations, membranes were rinsed 3 × 10 min with PBS-T. Anti-Mouse IgG secondary antibody (1:5000 dilution, Amersham Biosciences) was suspended in the milk solution and subsequently added to the membrane. The incubation period was kept rocking at RT for 1 h. Membranes were then washed another 3 × 10 min. Chemofluorescence was detected using the ECL Plus western blotting detection kit (Thermo Scientific).

2.4. Reverse transcriptase PCR (RT PCR)

To insure the effects of the dominant negative was not affecting the transcriptional level, the RNA was analyzed for a two-step RT-PCR. RNA was isolated using TRIzol (Invitrogen) using standard protoco. The isolated product was treated with RNase-Free DNase I to digest the DNA impurities and subsequently inactivated by incubating at 65 °C for 5 min. cDNAs were generated using cDNA preparation Kit (Promega) as suggested by the manufacturers protocol. PCR amplifications were performed with the 1 μl of cDNA template of the cJun|FOS system, 4 μl of PCR ReadyMix (Sigma–Aldrich), 3 μl of PCR quality water and 1 μl each of forward and reverse primers. GFP primers, A-FOS primers, and GAPDH primers used are listed below.

GFP Primers:	Forward 5' GCACGACTTCTTCAAGTCCGCCATGCC 3'
	Reverse 5' GCGGATCTTGAAGTTCACCTTGATGCC 3'
A-FOS Primers:	Forward 5' CCGAAGAGCTGGAGCAGGAA 3'
	Reverse 5' TAATCAGGGATCTTGCAGGC 3'
GAPDH Primers:	Forward 5' ATGTTCCAGTATGACTCCACTCACG 3'
	Reverse 5' GAAGACACCAGTAGACTCCACGACA 3'

The reaction conditions were: 1 hold for 9 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 62 °C, and 30 s at 72°C; 1 hold for 7 min at 72 °C. The PCR product was visualized on a 2% agarose gel.

3. Results

3.1. Expression of ubiquitin tagged A-ZIPs

We evaluated if the addition of ubiquitin to the A-ZIP dominant negatives would cause their B-ZIP dimerization partner to be degraded more efficiently. We generated plasmids encoding chimeric proteins termed ubiquitin–A-ZIPs which contained two copies of the 76-a.a. yeast ubiquitin appended to the N-terminus of A-ZIPs (Fig. 1). Three plasmids containing ubiquitin tagged A-ZIP domi-

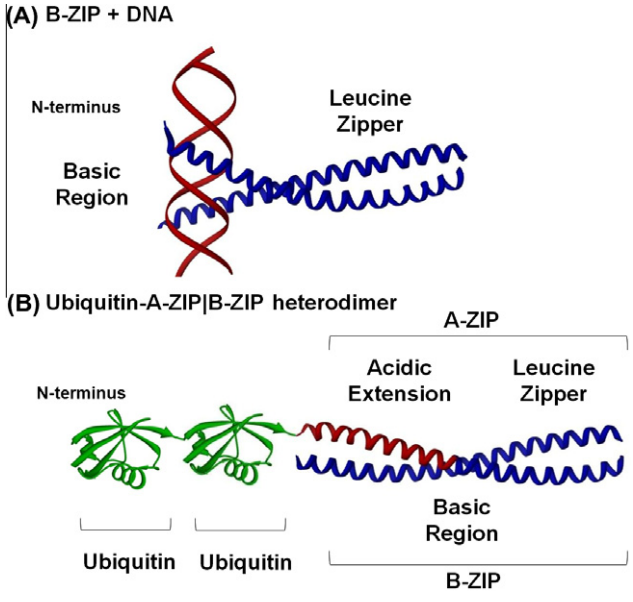

Fig. 1. Schematic representation of A-ZIP dominant negative and ubiquitin–A-ZIP, (A) Structure of B-ZIP domain (Fos|Jun heterodimer (1FOS)) in blue bound to DNA in red. (B) Coiled-coil heterodimer (359 G) between a B-ZIP monomer (blue) and A-ZIP where the acidic extension is in red with two ubiquitin domains (in green) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

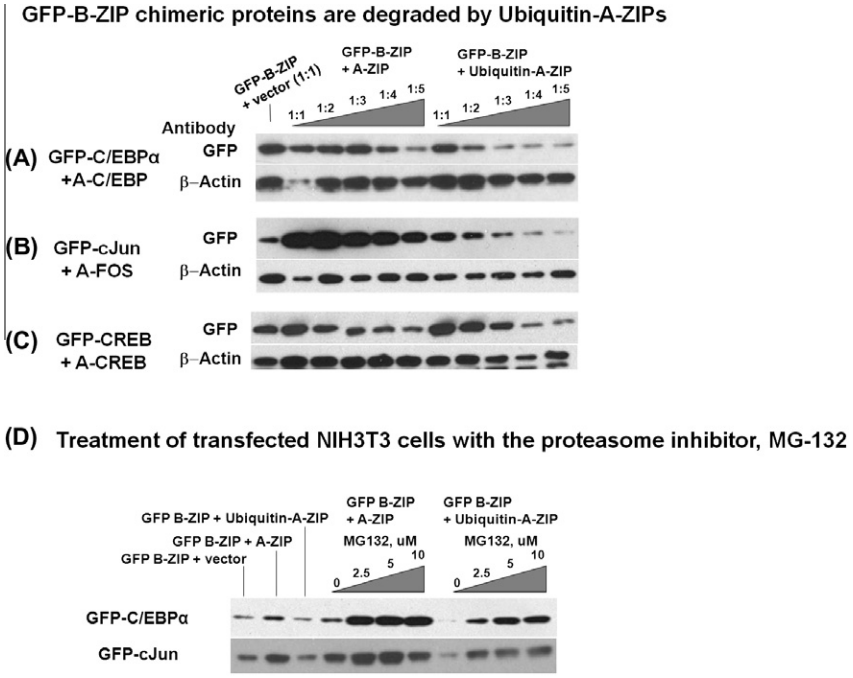

Fig. 2. GFP–B-ZIP chimeric proteins are degraded by ubiquitin–A-ZIPs. (A) GFP–C/EBPα plasmid co-transfected with increasing equivalent concentrations of A–C/EBPα or ubiquitin-A-C/EBPα plasmid in NIH3T3 cells. (B) GFP–cJun + A-FOS and GFP–cJun + ubiquitin–A-FOS (C) GFP–CREB + A-CREB and GFP–CREB + ubiquitin–A-CREB (D) GFP–ATF2 + A-ATF2 and GFP–ATF2 + ubiquitin–A-ATF2. The empty vector was added to all transfections to make DNA concentrations constant. In the western blot, antibodies were used either to GFP or to β-Actin. (D) MG-132 inhibits ubiquitin–A-ZIP degradation of B-ZIPs: in the top panel, GFP–C/EBPα and A–C/EBPα or GFP–C/EBPα and ubiquitin–A-C/EBPα were co-transfected with increasing concentrations (0, 2.5, 5, 10 μM) of the proteasome inhibitor MG-132. Cells were harvested and western blots probed with antibody to GFP. In the bottom panel, co-transfected GFP–cJun and A-FOS or GFP–cJun and ubiquitin–A-FOS NIH3T3 cells were treated with MG-132. GFP antibodies were used for measuring the protein concentration of GFP–C/EBPα or GFP–cJun.

nant negatives were examined ubiquitin–A-C/EBP that heterodiomerizies with C/EBP family members [2], ubiquitin–A-CREB that heterodimerizes with CREB family members [5], and ubiquitin–A-FOS that heterodimers with JUN family members [4].

Initially, we examined how different concentrations of A-ZIP and ubiquitin–A-ZIP would affect co-transfected GFP–B-ZIP protein concentrations. In the co-transfection experiments, the GFP–B-ZIP protein levels were monitored using a GFP antibody (Fig. 2A–C).

Ubiquitin–A-C/EBP is more potent than A-C/EBP at diminishing GFP–C/EBPα protein concentrations (Fig. 2A). A GFP–C/EBPα to ubiquitin–A-C/EBP plasmid ratio of 1:3 produced lower GFP–C/EBPα protein concentrations than a GFP–C/EBPα to A-C/EBP plasmid ratio of 1:3. When the ratio was 1:5, both dominant negatives produced low GFP–C/EBPα concentrations. Similarly, GFP–cJun proteins concentrations were lower when ubiquitin–A-FOS is used. Ubiquitin–A-CREB also showed some activity.

3.2. Addition of MG-132 proteasome inhibitor

We hypothesize that the reduction in B-ZIP protein concentration with the addition of ubiquitin to the A-ZIP is mediated through the proteasomal degradation pathway. To test this hypothesis, we inhibited the proteasome using the protesome inhibitor MG-132 [18] and measured GFP–B-ZIP protein concentration. This inhibition prevented both ubiquitin–A-C/EBP and ubiquitin–A-FOS to decrease their specific B-ZIP protein concentrations (Fig. 2D). The proteasomal inhibitor increased both GFP–C/EBPα and GFP–cJun protein levels even when no dominant negative is present. These data suggest that the ubiquitin is targeting the ubiquitin–A-ZIP|B-ZIP heterodimer into the proteasome degradation pathway.

3.3. GFP–C/EBP localization

GFP–C/EBPα cellular localization using fluorescence microscopy was determined when co-transfected in the 3T3 cell line either with A-C/EBP or ubiquitin–A-C/EBP. When GFP–C/EBPα was transfected alone it primarily localized in the nucleus and co-transfection with A-C/EBP changes the localization to the cytoplas which is consistent with our previous observations (Fig. S1) [16]. However, co-transfection with the ubiquitin tagged A-C/EBP drastically reduced the cytoplasmic localization of GFP–B-C/EBPα thus suggesting their degradation. Addition of the protesome inhibitor, MG-132, again increases the cytoplasmic localization and supports our hypothesis that degradation of the A-ZIP|B-ZIP complex is mediated through the proteosomal degradation pathway.

3.4. RT-PCR of MG-132 treated GFP–cJun|A-FOS

To evaluate if the dominant negatives are acting at a transcriptional level, the mRNA concentrations of the exogenous B-ZIPs were analyzed (Fig. 3). A-FOS primers were designed to cover the leucine zipper as well as part of the acidic extension, so that it would not amplify the endogenous cFOS. The A-FOS primers on the cDNA amplify all the appropriate experimental samples along with control plasmids (Fig. 3B). GFP and GAPDH primers were used as positive controls (Fig. 3C, D). All samples have expected band except for the stock plasmids, which were not transfected into the cells. Fig. 3E confirms that there was no DNA contamination present during the isolation of mRNA samples, only the A-FOS and ubiquitin–A-FOS plasmids produced the proper PCR products. These results confirm that the A-FOS and cJun expression seen on the western blot occurred is not at the transcriptional level.

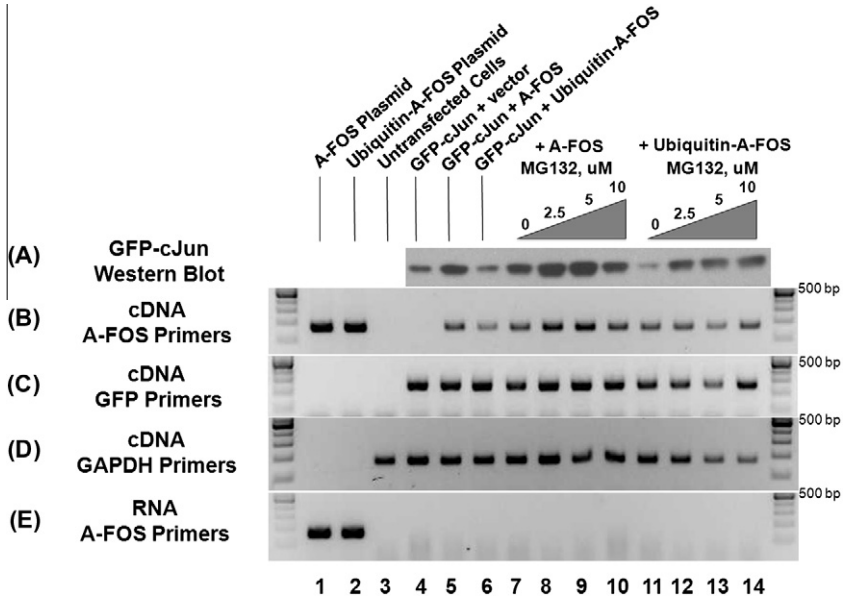

Fig. 3. Protein and mRNA analysis of GFP–cJun cotransfected with A-FOS or ubiquitin–A-FOS. (A) GFP–cJun was co-transfected with A-FOS or ubiquitin–A-FOS and treated with increasing MG132 concentrations (0, 2.5, 5, 10 μM) and visualized with a GFP antibody. (B) PCR product using A-FOS primers with plasmids (lanes 1–2) or cDNA (lanes 3–14) used as template. (C) PCR product using GFP primers and plasmids (lanes 1–2) or cDNA (lanes 3–14) used as template. (D) PCR product using GAPDH primers with plasmids (lanes 1–2) or cDNA (lanes 3–14) used as template. (E) PCR product using A-FOS primers with plasmids (lanes 1–2) or RNA (lanes 3–14) used as template. All PCR products produced the expected band size (A-FOS primers, 160-bp; GFP primers, 280-bp; GAPDH, 190-bp) when visualized on 2% agarose gel.

3.5. Ubiquitin–A-ZIP specificity to B-ZIPs

To evaluate the specificity of the ubiquitin tagged dominant negatives, three different ubiquitin tagged A-ZIPs were co-transfected with one of eleven GFP–B-ZIP encoding plasmids (Fig. 4). The most drastic effect came with ubiquitin A-FOS specifically inhibiting protein concentrations of the three Jun family members examined (GFP–cJun, GFP–JunB, and GFP–JunD). Ubiquitin–A-C/EBP produced a specific drop in GFP–C/EBPα protein. These results demonstrate the selective action of the ubiquitin–A-ZIP dominant negative constructs.

4. Discussion

We have previously reported the inhibition of DNA binding of B-ZIP transcription factors using dominant negative proteins that interact with the DNA binding region of the B-ZIP domain [2–5].

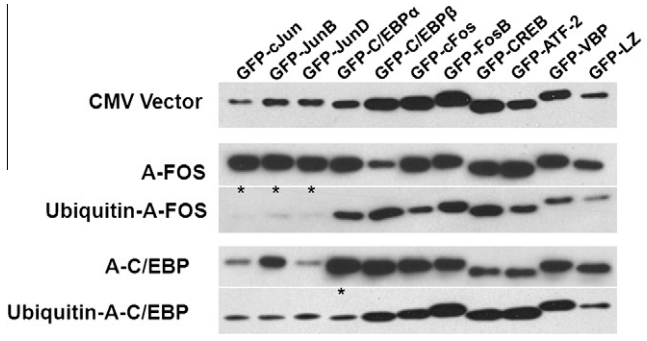

Fig. 4. GFP–B-ZIP proteins co-transfected with ubiquitin–A-ZIP proteins. A library of 11 GFP–B-ZIP constructs (cJun, JunB, JunD, C/EBPα, C/EBPβ, cFos, FosB, CREB, ATF-2, VBP, and LZ) were co-transfected with 2 A-ZIP constructs (A-FOS or A-C/EBP) with or without ubiquitin. GFP–B-ZIP protein concentration was monitored using a GFP antibody. The plasmid ratio between the GFP–B-ZIPs and A-ZIP or ubiquitin–A-ZIPs is 1:3.

These A-ZIP dominant negative produce distinct phenotypes in transgenic mice and sometimes cause their target B-ZIP proteins to disappear [8,9]. Two possible mechanisms are the A-ZIP|B-ZIP heterodimer cannot be retained in the nucleus because it does not bind to DNA, is shuttled to the cytoplasm where it is degraded or the A-ZIP|B-ZIP heterodimer forms in the cytoplasm and cannot be imported into the nucleus because the nuclear localization signal, which is the basic region critical for sequence specific DNA binding [19], is sequestered in the coiled coil extension of the leucine zipper. Independent of the exact mechanism that cause the B-ZIP proteins to disappear when the A-ZIP is expressed [16], we added two copies of ubiquitin to enhance this process, exploring if ubiquitin could act in trans to degrade the B-ZIP protein in the ubiquitin–A-ZIP|B-ZIP heterodimer. This could both help identify endogenous B-ZIP targets and also increase the potency of the dominant negative. The approach is conceptually similar to siRNA technologies. The siRNA targets specific mRNAs for degradation, which can be monitored experimentally. Obtaining conceptually similar data for the A-ZIPs degradation of the B-ZIP heterodimeric partners would strengthen their utility in various experimental paradigms.

The transient transfections revealed several interesting phenomena. Expression of the A-FOS dominant negative induced co-transfected GFP–cJun protein concentrations by a mechanism that does not involve increased GFP–cJun mRNA. Neither GFP–C/EBPα nor GFP–CREB have this property when their A-ZIPs are expressed suggesting that it could be possible to map onto the A-FOS construct to explore which property causes GFP–cJun to increase in protein concentration.

GFP–B-ZIP protein concentration increased in the presence of the proteasome inhibitor MG-132 indicating that the proteasome is critical for regulating GFP–cJun protein. Both A-FOS and particularly ubiquitin–A-FOS can accelerate GFP–cJun degradation, again indicating that one can map onto A-FOS properties that are critical for the accelerated degradation. This may be a general strategy to identify complexes *in vivo*. A component of a complex can be tagged with ubiquitin and the concentration of other proteins could be monitored to reveal which proteins are in the complex.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.007.

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