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Small molecule displacement of a cryptic degron causes conditional protein degradation

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Abstract

The ability to rapidly regulate the functions of specific proteins in living cells is a valuable tool for biological research. Here we describe a novel technique by which the degradation of a specific protein is induced by a small molecule. A protein of interest is fused to a Ligand-Induced Degradation (LID) domain resulting in the expression of a stable and functional fusion protein. The LID domain is comprised of the FK506- and rapamycin-binding protein (FKBP) and a 19-amino acid degron fused to the C-terminus of FKBP. In the absence of the small molecule Shield-1, the degron binds to the FKBP protein and the fusion protein is stable. Shield-1 binds tightly to FKBP thereby displacing the degron and inducing rapid and processive degradation of the LID domain and any fused partner protein. Structure-function studies of the 19-residue peptide showed that a four-amino acid sequence within the peptide is responsible for degradation.

Introduction

The conditional control of protein levels in eukaryotic cells is a powerful tool to study complex biological systems. Protein expression can be regulated on a genetic level by small molecule-mediated transcriptional switches[1,2] however, these systems require the degradation of the regulated protein when switched to the nonpermissive state, and in some cases more rapid protein depletions may be desirable. A variety of techniques have been developed to post-translationally regulate proteins using cell-permeable small molecule ligands.[3,4] Several of these approaches involve the use of dimeric synthetic compounds that enforce the colocalization of a protein of interest (POI) with specific ubiquitin ligases that are involved in the proteasomal degradation pathway.[5-7] One strategy that involves these proteolysis-targeting chimeric molecules (known as Protacs)[8] is based on a specific Skp1-Cullin-Fbox (SCF) E3-binding peptide that can be covalently linked to a ligand that binds to a protein of interest. This bifunctional ligand enforces the colocalization of the POI with the

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E3 ligase, typically resulting in degradation of the POI. For many proteins however, ligands are not known for desired targets, which hinders the application of this method.

Another method was recently published that relies on components of a ligand-induced degradation pathway found in plants.[9] In this system, a specific F-box protein that forms a complex with a SCF E3 ubiquitin ligase promotes the complexation with a plant transcriptional repressor in the presence of the auxin, indole-3-acetic acid. Proteins of interest that are fused to the repressor and expressed in cells that also express the F-box protein can be rapidly degraded by the proteasome upon addition of the auxin.

In contrast to these systems in which administration of the ligand causes proteins to be degraded, our laboratory recently developed a technique to regulate protein stability that makes use of engineered mutants of the FK506- and rapamycin-binding protein (FKBP12) that behave as “destabilizing domains” (DDs).[10] We have also developed an orthogonal regulatory system of DDs based on *E. coli* DHFR and trimethoprim.[11] In both systems proteins that are fused to the DDs are rapidly and constitutively degraded when expressed in mammalian cells. The addition of a cell-permeable ligand called Shield-1 that binds to the DD shields them from degradation thus allowing the proteins to perform their cellular functions. Genetic fusion of the DD to the gene of interest ensures specificity, however, regulating essential genes with the DD system necessitates constant administration Shield-1 to cells or animals to maintain protein function. To complement the two existing DD systems, we set out to develop a Ligand Induced Degradation (LID) system. The goal was to engineer one or more LID domains so that proteins fused to the LID domain would be stable in the absence of ligand and rapidly degraded in the presence of a high-affinity ligand (Fig. 1).

Herein we report the discovery and characterization of an FKBP-based LID domain whose stability is regulated by Shield-1 (Supplementary Fig. 1). The LID domain rapidly destabilizes a variety of fused proteins upon addition of Shield-1, and the proteasome is responsible for degradation. Our studies suggest that the peptide appended to the C-terminus of FKBP functions as a cryptic degron that is prevented from inducing degradation when bound to the FKBP active site but revealed when displaced by Shield-1. We further demonstrate that the LID domain can be used together with our previously developed DD system, opening the possibility to simultaneously degrade one protein and stabilize a second protein by the addition of a single drug. One benefit of the LID system is that a single genetic perturbation combined with small molecule control can be used in a general fashion to regulate protein levels in cells, and that the co-expression of additional E3 ligases is not needed.

Results

Development and characteristics of the LID domain

As a starting point to identify protein domains that are stable in the absence of ligand and degraded upon addition of Shield-1, we screened FKBP proteins that possess additional residues appended to the C-terminus. FKBP is an enzyme possessing *cis*/*trans* prolyl isomerase activity, and FKBP can act on a broad spectrum of substrate polypeptides.[12] We

envisioned that a peptide of sufficient length appended to the C-terminus of FKBP might be able to bind intramolecularly to the active site. In addition, we reasoned that this peptide might encode a degradation sequence (i.e., degron) that would not be detected by cellular degradation proteins when sequestered in the active site, this rendering it a cryptic degron.

Our first attempt at engineering a LID domain involved fusing three known degron sequences (CL1,[13] CyclinB[14] and a degron sequence found in encephalomyocarditis virus[15]) to the C-terminus of FKBP12(F36V) followed by a short sequence of alternating leucine and proline residues that are preferred substrates for FKBP.[16,17] The FKBP12(F36V) mutant, hereafter referred to as FKBP, was used because this cavity-forming mutation ensures the selectivity of Shield-1 for our engineered proteins relative to endogenous FKBP12 that is present in eukaryotic cells.[18] Yellow fluorescent protein (YFP) was fused to the N-terminus of FKBP as a FACS-readable marker for protein stability. Retrovirus was used to stably transduce NIH3T3 cells with each construct, and protein stability was measured by analytical flow cytometry. In the absence of Shield-1 low levels of YFP fluorescence were observed indicating that the protein fusions are largely degraded and that the degron sequences are not rendered cryptic. In the presence of Shield-1 an increase in fluorescence was observed (Supplementary Fig. 2), which suggests that ligand binding to the FKBP domain favors protein stabilization, similar to the behavior of the existing DDs.

Known degrons fused to the C-terminus of FKBP did not prove to be cryptic in the absence of Shield-1, so we next attempted to engineer the desired behavior into the FKBP protein *de novo*. We used synthetic oligonucleotides to generate diversity in a short peptide sequence fused to the 3′-end of the YFP-FKBP gene. The library of YFP-FKBP chimeras was stably transduced into NIH3T3 cells and the transduced cells were screened by several rounds of FACS-based analysis using alternative treatments of vehicle or Shield-1 with sorting for high and low YFP levels, respectively. These screening efforts resulted in the isolation of several clones that displayed the desired ligand-dependent stability profile. Isolation of the positive cells and analysis of the clones revealed an identical construct in all cases: the YFP-FKBP fusion protein with an additional 19 amino acids (TRGVEEVAEGVVLLRRRGN) fused to the C-terminus of FKBP.

To validate and further characterize this new fusion protein, we re-cloned the YFP-FKBP gene including the additional 19-residue C-terminal extension, and transduced this construct into NIH3T3 cells. YFP is degraded in a dose dependent manner upon addition of Shield-1 (IC_{50} = 3.3 nM, Fig. 2c), and YFP fluorescence levels in Shield-treated cells dropped to 10-20% above the background autofluorescence observed in untransduced cells (Fig. 2). YFP fluorescence was reduced even further to the background level of untransduced cells when the cells were treated with the translation inhibitor cycloheximide, suggesting a time lag between the synthesis and the degradation of the LID-fusion protein (Supplementary Fig. 3). We further established that a less potent binding ligand for FKBP, Shield-2 (Supplementary Fig. 1),[19] can also destabilize the LID domain although with slightly reduced potency.

To explore the time-dependent stability of the LID domain (LID refers to the FKBP(F36V) plus the 19-residue C-terminal peptide) we monitored the degradation of YFP-LID upon

addition of Shield-1 as well as the recovery of YFP-LID upon depletion of Shield-1 from the culture media. The data revealed that YFP levels are reduced 50% in 45 minutes upon addition of Shield-1 in the absence of cycloheximide, and further that 100 minutes are required for the YFP level to double following withdrawal of Shield-1 from the media (Fig. 2d). To investigate the mechanism by which the LID domain is degraded in NIH3T3 cells we treated the cells with Shield-1 in the absence and presence of the proteasome inhibitor MG132. YFP levels measured by flow cytometry as well as Western blot reveal a significant accumulation of YFP-LID in the presence of Shield-1 and MG132 indicating the involvement of the proteasomal degradation pathway acting on the LID domain (Fig. 2a). We confirmed the role of proteasomal protein degradation by treating NIH3T3 cells with the lysosome inhibitors chloroquine and ammonium chloride that did not show the accumulation of fusion proteins (Supplementary Fig. 3). We further established that YFP-LID fusion proteins could be degraded in transient transfected HeLa and U2OS cells when treated with Shield-1, indicating the potential generality of the LID system in mammalian cells (Supplementary Fig. 4).

The binding of ligands to proteins involves a decrease in free energy and typically causes stabilization of a protein structure. Therefore, the simple binding event between FKBP and Shield-1 is unlikely to be the source of the observed cellular instability. To investigate whether the 19-residue peptide appended to the C-terminus of FKBP acts as a degron, we fused this peptide directly to the C-terminus of YFP. Fusion of this peptide to YFP causes strong degradation of the fluorescent protein, and this instability is not affected by Shield-1 (Fig. 2e). We next evaluated whether the 19-residue peptide behaves as a cryptic degron when fused to the N-terminus of FKBP. In contrast to LID, placement of the 19-residue peptide at the N-terminus of FKBP-YFP or between the FKBP and YFP domains results in Shield-1 dependent stabilization of the protein. The 19-residue sequence appears to be cryptic only when fused to the C-terminus of FKBP and only when this LID domain is fused at the C-terminus of YFP. (Supplementary Fig. 5).

The cryptic degron occupies FKBP's active site

We have established that the 19-residue peptide acts as a degron when fused directly to YFP (Fig. 2e) and also that Shield-1 has no effect on the stability of this fusion. However, when the 19-residue degron is fused to the C-terminus of FKBP to constitute the LID domain, proteins with the LID domain fused to their C-termini are stable until treatment with Shield-1 causes degradation. This behavior in which the instability imparted by the 19-residue peptide depends on FKBP suggests a mechanism by which this peptide degron is rendered cryptic by FKBP.

To further characterize the structure of the LID domain relative to that of the parent FKBP we performed NMR spectroscopy experiments using bacterially expressed and purified $^{13}C/^{15}N$ labeled FKBP and LID proteins. We were especially interested in interactions between the 19-residue degron and the parent FKBP domain. First, we measured the 2D $^{1}H/^{15}N$-HSQC correlation spectra of FKBP and LID proteins with and without Shield-1. All experiments resulted in well-dispersed correlation spectra and relevant comparisons are shown in Fig 3a-c (see Supplementary Fig. 6a-d for full-scale spectra). In

the absence of Shield-1, little overlap was observed for resonances in the spectra of FKBP compared to the LID protein suggesting an interaction between the 19-residue peptide and the core FKBP domain (Fig. 3a). When Shield-1 was added to the FKBP sample, significant chemical shift perturbations were observed between the ligand-free and Shield-bound states (Fig. 3b). Shield-1 elicited a similar dramatic effect on the LID domain (Supplementary Fig. 6d). This finding was not unexpected as the binding of Shield-1, a compound containing aromatic rings, would be expected to induce changes in the local chemical environment of its FKBP binding site. Most striking, however, was the comparison of the FKBP•Shield-1 and LID•Shield-1 spectra (Fig. 3c), which revealed a high degree of overlap. This similarity suggests that the 19-residue degron of LID is binding to the FKBP domain and once displaced by Shield-1, the chemical environment of the FKBP domain of both ligand-bound proteins becomes similar (Fig. 3c).

To verify this we identified the amino acid residues of the FKBP backbone that are most perturbed when comparing the FKBP and LID proteins. We reasoned that these perturbations would provide further information about the binding location of the 19-residue degron to the FKBP domain. For this experiment, we compared the HSQC spectrum of FKBP with that of LID (to evaluate chemical shift differences induced by the 19-residue peptide). We also compared the spectra of FKBP with and without Shield-1 to evaluate if the chemical shift perturbations induced by the C-terminal peptide are similar to the residues that are perturbed by Shield-1. In order to make the amide backbone chemical shift assignments in the HSQC-spectra, we acquired 3D-NMR spectra of the relevant samples. The sequential assignment (Supplementary Table 1) allowed us to analyze the weighted chemical shift perturbations of FKBP compared to LID as well as FKBP compared to the FKBP•Shield-1 complex that are quantitated in Fig. 3d. Similar patterns and extents of chemical shift perturbation are observed for the two comparisons, and these data were mapped onto the crystal structure of FKBP(F36V) that was co-crystallized with an analog of Shield-1 (Fig. 3e, PDB code: 1BL4). The residues that are perturbed most dramatically (colored red in Fig. 3e) are located predominantly in the ligand-binding site of FKBP. The same pattern of chemical shift perturbations is seen for both pairwise comparisons suggesting that the 19-residue peptide of the LID domain perturbs the same residues of FKBP as does Shield-1, which is suggestive of an overlapping binding site.

To probe this model further we used fluorescence polarization to monitor a competition binding experiment between a fluorescent analog of Shield-1 (fluorescein-SLF; Fl-SLF) and a synthetic sample of the 19-residue peptide. Fixed concentrations of Fl-SLF and the FKBP(F36V) protein were incubated with various concentrations of the peptide. A dose-dependent decrease in polarization signal was observed upon addition of the 19-residue peptide indicating displacement of the tracer from the protein (Supplementary Fig. 7). Fitting of the dose response curve reveals a binding affinity of 28.9 μM of the peptide for FKBP.

A tetrapeptide is responsible for protein degradation

In order to identify the specific amino acids within the 19-residue peptide that are important for binding to FKBP or serving as a degron, we substituted each of the residues within the

19-residue peptide with alanine (Fig. 4a). Mutations of the amino acids that are located at the N-terminal region of the 19-residue peptide did not significantly affect the LID behavior, whereas mutations within the C-terminal part had a more significant effect. Arginine 15 and glycine 18 appear to be especially important for degradation as substitution with alanine at these sites resulted in stable fusion proteins that were not destabilized upon addition of Shield-1. Residues that affect the ligand-dependence as well as the stability of the LID domain in the absence of Shield-1 (e.g., leucine 13 and asparagine 19) are likely to be important for binding of the 19-residue degron to the FKBP domain.

We next verified the importance of arginine 15 and glycine 18 within the peptide by preparing YFP-FKBP(F36V) constructs encoding truncated 19-residue peptides attached to the C-terminus (Fig. 4b). Deletion of the last two amino acids (glycine 18 and asparagine 19) yielded a stable fusion protein that was not destabilized upon addition of Shield-1. Further truncation did not affect protein stability. To further establish the minimal degron we made a series of truncation constructs in which the FKBP domain is deleted and the peptides are fused directly to YFP (Fig. 4c). This experiment revealed that the 19-residue peptide degron could be reduced to a tetrapeptide (RRRG) that, when fused directly to the C-terminus of YFP, lowered YFP-fluorescence to the background levels observed in untransduced NIH3T3 cells. Further truncation of the peptide resulted in high fluorescence levels confirming the importance of both arginine 15 and glycine 18 in this peptide degron. Taken together, these data support the model depicted in Fig. 1 in which the 19-residue peptide degron at the C-terminus of FKBP binds to the active site and becomes cryptic in this orientation.

LID fusion proteins are general and functional

To investigate if the LID domain can be generally used to regulate the stability of other proteins we fused the LID domain to a set of transcription factors that are involved in reprogramming differentiated cells into a pluripotent state. These reprogrammed cells are known as induced pluripotent stem cells (iPS cells).[20,21] The genes encoding each transcription factor were tagged at their 3′-ends with DNA encoding the LID domain. These constructs were separately transduced into NIH3T3 cells, and treatment of the cells with vehicle or 2 μM Shield-1 followed by immunoblotting of the lysates with appropriate antibodies showed that these transcription factors were all degraded in a Shield-1 dependent manner (Fig. 5a).

We then evaluated whether proteins that are fused to the LID domain are functional in mammalian cells by using this technology to regulate one of the transcription factors that is essential for reprogramming fibroblasts into iPS cells. Enforced expression of four transcription factors (Oct4, Sox2, Klf4 and c-Myc) has been shown to reprogram fibroblasts to iPS cells [20,22,23]. We fused the LID domain to the C-terminus of Oct4 to determine whether the Oct4-LID fusion is capable of reprogramming murine embryonic fibroblasts (MEFs) derived from transgenic mice in which GFP expression serves as a reporter for successful reprogramming.[24] Expression of GFP as well as the presence of alkaline phosphatase activity and the appearance of the cell surface marker stage-specific embryonic antigen-1 (SSEA-1) were used as three independent indicators for successful

reprogramming. Enforced expression of an unoptimized Oct4-LID fusion protein along with wild-type Sox2, Klf4, and c-Myc resulted in successful reprogramming to iPS cells, although the efficiency of this process was reduced relative to reprogramming using all four unmodified transcription factors (Fig. 5b and Fig. 5c). Cells expressing Oct4-LID were unable to undergo reprogramming to iPS cells in the presence of Shield-1, as expected.

As an additional test of the ability of the LID domain to regulate protein function, we fused LID to β-actin to determine if the actin-LID fusion protein could be efficiently integrated in microfilaments.[25] NIH3T3 fibroblasts were doubly transduced with human β-actin fused to a green fluorescent protein derived from *Aequorea coerulescens* (AcGFP) and the LID domain as well as β-actin fused to mCherry to visualize microfilaments. In the absence of Shield-1, both green and red fluorescent actin filaments are observed, indicating stable integration of the actin-GFP-LID fusion protein (Supplementary Fig. 8a and b). When cells were treated with Shield-1, GFP fluorescence was absent demonstrating that the β-actin-LID fusion protein is functional and its stability can be regulated by Shield-1.

LID system can be used simultaneously with the DD system

In some experimental designs, investigators may desire the rapid depletion of one protein and the simultaneous induction of another protein to acutely perturb their system of interest. Therefore, we set out to test whether the LID domain and our previously reported destabilizing domain (FKBP(L106P) mutant) could be used simultaneously in cells.[10] Addition of Shield-1 would be expected to induce the degradation of a protein that is fused to the LID domain and simultaneously stabilize a second protein that is fused to the DD. We doubly transduced NIH3T3 cells with YFP-LID and DD-mCherry and these cells were treated with various concentrations of Shield-1. After 24 hours, the cells were analyzed by flow cytometry and epifluorescence microscopy revealing the dose-dependent depletion of YFP-LID and simultaneous stabilization of mCherry (Fig. 6).

Discussion

The ability to rapidly and reversibly regulate protein levels using small molecules represents a valuable tool for biologists. Here we present a novel technique to regulate protein stability using the small molecule Shield-1. Our system is based on the genetic fusion of a protein of interest to a Ligand Induced Degradation (LID) domain. In contrast to our previously reported destabilizing domains, the LID domain can be classified as a novel “drug-off” system in which targeted proteins are stable in the absence of ligand and rapidly degraded upon addition of Shield-1. We have established that the 19-residue peptide that is fused to the C-terminus of FKBP is a strong but ligand-dependent degron when the LID domain is fused to the C-terminus of partner proteins. In particular, two residues (arginine 15 and glycine 18) within the 19-residue peptide are responsible for protein degradation. We further established by truncation experiments that fusion of the minimal four-residue peptide (RRRG) is sufficient to fully destabilize YFP. Additional mutagenesis experiments may reveal which residues and structural features of this degron are tolerated and important for conferring cellular instability.

We provide evidence that the instability conferred by the LID domain is general. Fusions of the LID domain to various proteins, including actin and transcription factors, providefunctional proteins in the permissive state that are degraded upon addition of Shield-1. Also, in combination with our previously described destabilizing domain (DD) a protein switch can be engineered. In this experimental manifold one protein is stable and active without ligand while a second protein is unstable, and this situation is rapidly reversed by the addition of Shield-1.

We also investigated the molecular basis by which the 19-residue degron becomes cryptic when fused to FKBP. Our studies support a model wherein the 19-residue peptide binds to the FKBP active site, likely in an intramolecular fashion though our studies cannot definitively exclude intermolecular dimers or other multiprotein assemblies. Shield-1 displaces the degron from the FKBP active site, at which time it can be recognized and targeted for degradation by one or more cellular quality control proteins. In addition, biochemical studies suggest that the 19-residue degron and Shield-1 occupy overlapping binding sites in the FKBP active site. The affinity of Shield-1 for the FKBP domain is sub-nanomolar,[18,26] which is likely strong enough to compete with a 28.9 μM intramolecular peptide ligand.

The LID domain represents a novel genetically concise strategy to conditionally degrade proteins on a post-translational level. DNA encoding the LID domain need only be fused to a gene of interest to confer ligand-dependent instability. There is no need for a known ligand for the protein of interest nor must the investigator introduce additional genetic elements (e.g., E3 ligases) in the cell. The LID domain may be especially useful to study essential genes since constant administration of a stabilizing drug is not needed. The FKBP-derived DDs have proven to be useful for conditionally regulating gene products in a variety of organisms, including *Plasmodium falciparum*, *Toxoplasma gondii*, and *Leishmania major*. Further studies on the mechanisms by which the LID domain is recognized and targeted for degradation are likely to lead to new insights into protein quality control pathways in mammalian cells.

Methods

FKBP ligands

Shield-1 was prepared as described.[18,27] Reagent requests should be directed to T.J.W.

Library preparation

We prepared synthetic oligonucleotides encoding a 10-amino acid linker ($Gly_4SerGly_4Ser$) followed by four or six random amino acids that were encoded using the NNS, NNK, and NNM mixed-base strategies (N= A,T,C,G, S=C,G and K=T,G and M = A,C). The oligonucleotides were cloned at the 3′-end of the chimeric YFP-FKBP12(F36V) gene in a pBMN vector with HcRed-tandem behind IRES (iHcRed-t). Stop codons were engineered in all three reading frames. A library of 3.5×10^5 independent clones was generated and screened in NIH-3T3 cells. The positive clone obtained from the screen [YFP-FKBP(F36V)-TRGVEEVAEGVVLLRRRGN] appears to be derived from a synthetic oligonucleotide that

experienced a dropped base during synthesis resulting in a shifted reading frame at the end of the chimeric gene.

Cloning, cell culture, transfections and transductions

Genes encoding the proteins tested as fusions to the LID domain were cloned by standard techniques in the retroviral pBMN vector encoding an iHcRed-t, iBlasticidin or iPuromycin. The ΦNX ecotropic packaging cell line was cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The NIH3T3 cell line was cultured in DMEM supplemented with 10% heat-inactivated donor bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The ΦNX cells were transfected using standard Lipofectamine 2000 protocols. Viral supernatants were harvested 48 h posttransfection and filtered through a 0.45 μm nylon syringe filter. NIH3T3 cells were incubated with the retroviral supernatants supplemented with 4 μg/mL polybrene for 4 h at 37 °C. Cells were cultured in growth media for 24 to 36 h to allow for viral integration, then assayed as described.

Flow cytometry

Transduced NIH3T3 cells were plated at 1×10^5 cells per well of a 12-well plate and treated with vehicle or Shield-1 for indicated time points prior to analysis. Cells were detached from wells using trypsin-EDTA and quenched with 1 mL of growth medium. Samples were analyzed at the Stanford Shared FACS Facility with 10,000 events typically represented. To monitor protein recovery, Shield-1 was depleted from NIH3T3 cells by changing the growth medium to DMEM supplemented with 10% heat-inactivated donor bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and 5 μM recombinant FKBP(F36V) at the indicated time points prior to analysis. The affinity of Shield-1 for LID was calculated using the nonlinear regression curve-fitting program GraphPad Prism v.4 (GraphPad Software Inc., San Diego, CA, USA).

Microscopy

NIH3T3 cells expressing fluorescent proteins were imaged with a 40x objective on a Zeiss Axioskop 2 epifluorescence microscope equipped with a QICAM FAST 1394 digital CCD camera. For nuclear stain, cells were incubated with 1 μg/mL Hoechst 33342 for 5 min before fixation.

Expression and purification of isotopically labeled protein

Genes encoding the FKBP or LID domains were cloned into a pET-15b plasmid containing a His6-tag followed by a TEV cleavage site. The vectors were transformed into *E. coli* BL21(DE3) cells and plated on LB/Amp agar plates. A single colony was picked from the plate and used to inoculate a 50 mL LB/Amp overnight culture. The overnight culture was used to inoculate a Fernbach flask containing 1.0 L M9 minimal media containing 1 g/L $^{15}NH_4Cl$ and 2 g/L U-$^{13}C_6$-glucose as the sole nitrogen and carbon sources. Cultures were incubated at 37 °C with shaking until the OD_{600} reached 0.6-0.8 (3-4 h). Protein expression was induced by the addition of 0.5 mM IPTG, and the culture was shaken overnight at 20 °C. Cells were harvested by centrifugation, resuspended in 20 mL lysis buffer (50 mM

NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) containing 1 mg/mL lysozyme and transferred to an Oak Ridge tube and incubated for 30 min on ice. A Branson sonicator with a microtip probe was used to sonicate the bacteria on ice (power = 50%, 10s on/10s off, 0.5s interval for 5 × 2 min, 2 min rest). When sonication was complete, 20% Triton X-100 was added to a final concentration of 1%, and the cells were incubated on ice for an additional 30 min. Cell lysates were clarified by centrifugation, and the supernatant was incubated on 3 mL Ni-NTA resin (QIAGEN) for affinity column purification for 1 h at 4 °C. The resin was washed and eluted according to the manufacturer's instructions.

The His_6-TEV-FKBP and His_6-TEV-LID proteins were dialyzed into TEV cleavage buffer (50 mM TRIS, 0.5 mM EDTA, 100 mM NaCl and 1 mM DTT) and 10 % (w/w) TEV protease was added. The mixture was shaken overnight at 4 °C. The mixture was added to 3 mL Ni-NTA resin and the flow-thru containing the labeled proteins were collected. For NMR experiments, the flow-thru was dialyzed in NMR buffer (50 mM NaH_2PO_4, 100mM NaCl, 0.5 mM EDTA, 1 mM TCEP, 2% glycerol, pH 6.8). Protein was concentrated, and the protein concentration was determined by A_{280} (ε = 9650 M^{-1}•cm^{-1}) and adjusted to 600 μM.

NMR Spectroscopy

$^1H/^{15}N$-NOESY-HSQC two-dimentional spectra were acquired on uniformly ^{15}N and $^{13}C/^{15}N$-labeled FKBP, FKBP•Shield-1, LID, and LID•Shield-1, and HNCACB and C(CO)NH three-dimensional spectra were acquired on uniformly $^{13}C/^{15}N$-labeled FKBP, FKBP•Shield-1, and LID samples. A 3D ^{15}N-NOESY spectrum was also recorded for the LID domain. For the measurements, a 600 μM protein solution was supplemented with 10% D_2O and spectra were acquired at 25 °C on Varian 600- and 800-MHz Inova spectrometers running VNMR v6.1c and VNMRJ 2.1B, respectively. Spectra were processed with VNMR/VNMRJ or nmrPipe[28] and analyzed utilizing Sparky (Goddard, T. D.; Kneller, D. G. SPARKY 3, UCSF.). The sequential assignments of FKBP(F36V) and LID proteins are provided in Supplementary Table 1. Chemical shift perturbations were quantitated using a weighted average chemical shift difference (δ_{avg}) that was calculated as $[(\delta^1H)^2 + (\delta^{15}N/5)^2]/2]^{1/2}$, where δ^1H and $\delta^{15}N$ are the change in ppm between the spectra. All experiments were normalized ($\delta_{avg}/\delta_{max}$) with respect to the maximum observed weighted average shift difference (δ_{max}).[29]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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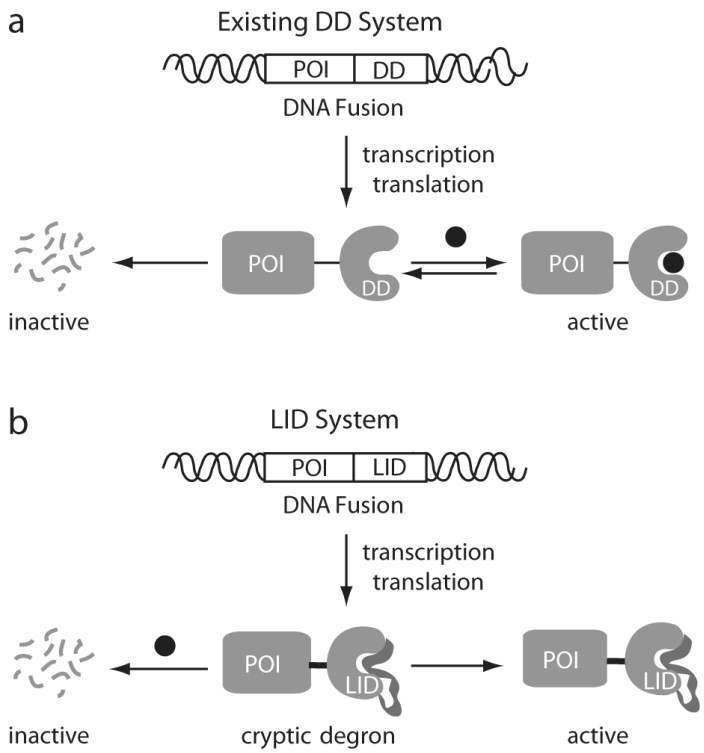

Figure 1.

Two strategies for ligand-regulated protein stability. (**a**) Schematic representation of the destabilizing domain (DD) technology. Addition of ligand stabilizes the protein-of-interest (POI). (**b**) Schematic illustration of the ligand-induced degradation (LID) technology in which addition of the ligand destabilizes rather than stabilizes its target.

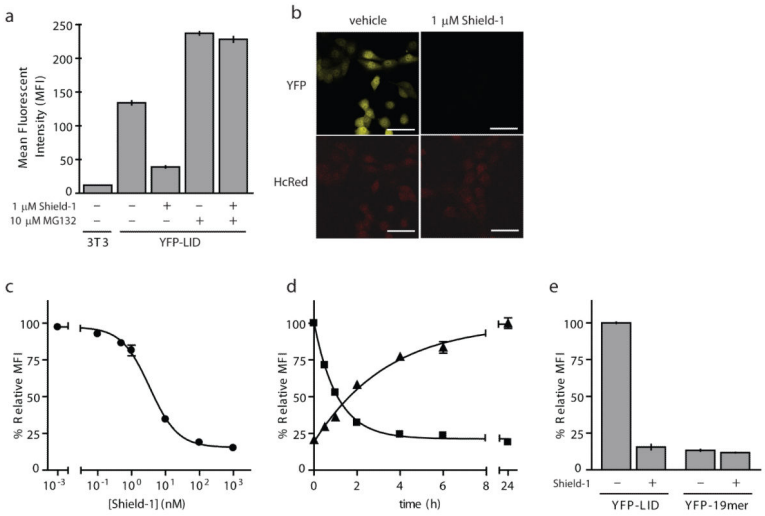

Figure 2

. Characterization of the LID domain. (**a**) NIH3T3 cells stably expressing YFP-LID were treated with either vehicle or 10 μM MG132 in the presence or absence of 1 μM Shield-1 for 6 h followed by flow cytometry analysis. Lysates from the cells described were resolved by SDS-PAGE and immunoblotted with either anti-YFP or anti-Hsp90 antibodies (Supplementary Fig. 9). (**b**) Fluorescence microscopy of cells stably expressing the YFP-LID fusion. Cells were treated with either vehicle or 1 μM Shield-1 for 24 h and analyzed using epifluorescence microscopy. HcRed serves as a marker for infection. Insert scalebars represent 10 μm. (**c**) Cells stably expressing the YFP-LID fusion were treated with various concentrations of Shield-1 (1 μM to 1 pM) and monitored by flow cytometry. (**d**) The degradation (squares) of YFP-LID was monitored at various times following addition of Shield-1. The recovery (triangles) of YFP-LID was monitored at various time points after depletion of Shield-1 from the culture media. Both experiments were analyzed by flow cytometry. The maximum observed fluorescence intensity for each construct was set to 100%. (**e**) Cells stably expressing either the YFP-LID or the YFP-19mer fusion proteins were treated with vehicle (−) or with 1 μM Shield-1 for 24 h (+) and analyzed by flow cytometry. The error bars represent the s.d. of the mean based on at least two experiments.

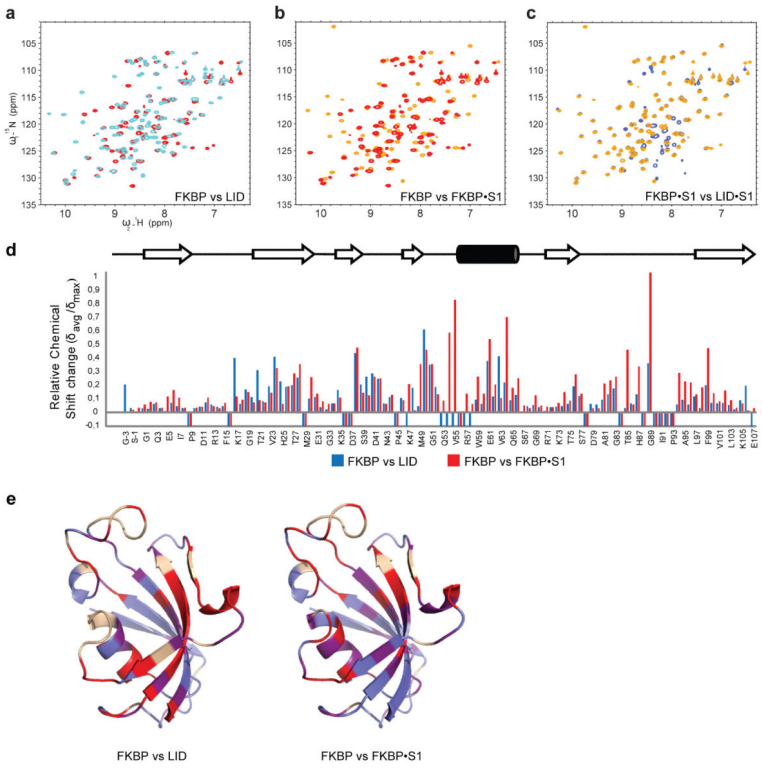

Figure 3

. NMR analysis of FKBP and the LID domain. $^{1}H/^{15}N$ HSQC spectra of (**a**) FKBP (red) compared to LID (cyan), (**b**) FKBP (red) compared to FKBP bound to Shield-1 (orange), and (**c**) FKBP bound to Shield-1 (orange) compared to LID bound to Shield-1 (blue). (**d**) Relative chemical shift perturbations of FKBP compared to LID (blue bars) as well as FKBP compared to the FKBP•Shield-1 complex (red bars). Residues that could not be assigned in the HSQC-spectra are indicated with negative bars. (**e**) Chemical shift perturbations mapped onto the structure of FKBP complexed with a Shield-1 analog (PDB:1BL4). Residues experiencing significant chemical shift perturbations ($\delta_{avg}/\delta_{max} > 0.2$) are colored red, moderate perturbations are colored purple ($0.1 < \delta_{avg}/\delta_{max} < 0.2$), and minor perturbations are shown in blue ($\delta_{avg}/\delta_{max} < 0.1$).

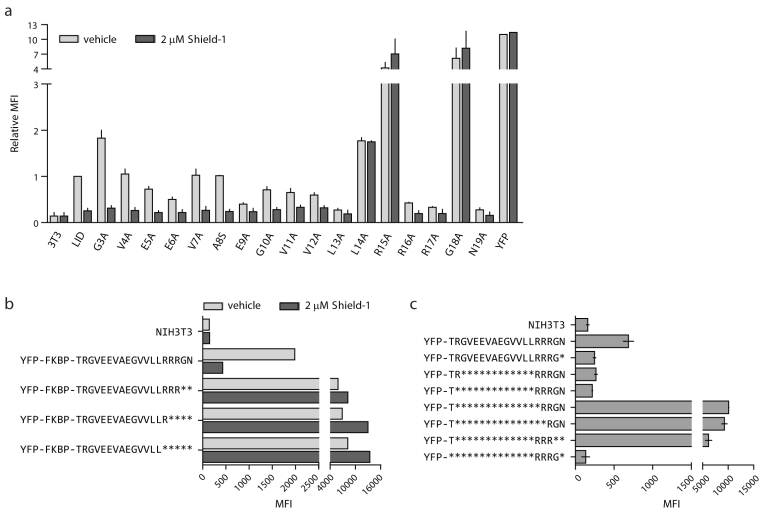

Figure 4.

Characterization of the 19-residue peptide degron. (**a**) Alanine was substituted at seventeen positions of the 19-residue peptide fused to YFP-FKBP(F36V). Cells were stably transduced with the appropriate retrovirus and after 24 hours were treated with vehicle or 2 μM Shield-1 for 24 hours and YFP levels were scored by analytical flow cytometry. (**b**) Four C-terminal truncation mutants of the YFP-LID domain were prepared, and NIH3T3 cells stably transduced with these constructs were treated with either vehicle or 2 μM Shield-1 for 24 hours and evaluated by flow cytometry. Deleted residues are indicated as asterisks. (**c**) The 19-residue peptide and deletion mutants of this degron were fused directly to YFP to evaluate the strength of these degrons. Deleted residues are indicated as asterisks. The error bars represent the s.d. of the mean based on at least two experiments.

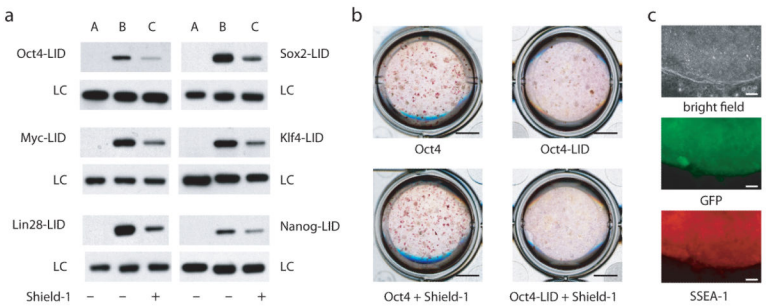

Figure 5.

LID domain regulates transcription factors. (**a**) The LID domain was fused to the C-termini of six transcription factors that were independently transduced into NIH3T3 cells. Cell populations were treated with either vehicle or 2 μM Shield-1, and cell lysates were immunoblotted with antibodies against the indicated proteins (Oct4, Klf4), anti-HA (Sox2, Myc, Lin28) or anti-Flag (Nanog). α-Tubulin serves as the loading control (LC). Lane A represents untransduced NIH3T3 cells. Lanes B and C are from cells that were treated with vehicle (−) or with 2 μM Shield-1 for 24 h (+). Full western blots are shown in Supplementary Fig. 10. (**b**) Nuclear reprogramming of MEFs derived from transgenic mice encoding an Oct4 promoter driving GFP. MEF(Oct4/GFP) cells (ref. 24) were transduced with *Sox2*, *Myc*, *Klf4*, and *Oct4* (left) or with *Sox2*, *Myc*, *Klf4*, and *Oct4-LID* (right). Cells were treated with vehicle or with 2 μM Shield-1 on day 3 post-infection. Alkaline phosphatase (AP) staining was performed on day 14 post-infection. Insert scalebars represent 5 mm. (**c**) GFP positive cells were transferred on day 19 to a new feeder layer. APC conjugated *SSEA-1 (Stage-Specific Embryonic Antigen-1)* antibody staining was performed on day 24 post-infection. Insert scalebars represent 50 μm.

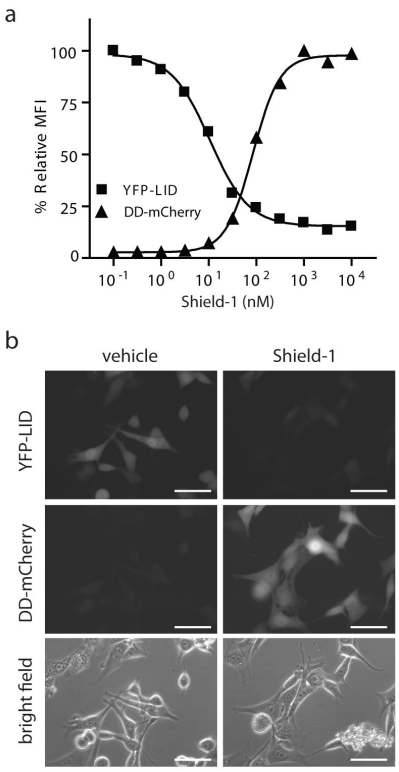

Figure 6.

Shield-1 can simultaneously stabilize and destabilize specific targets. NIH3T3 cells stably expressing both YFP-LID and DD-mCherry were treated with various concentrations of Shield-1 for 24 h, and the fluorescent signals in the appropriate channels were monitored by flow cytometry (**a**) and fluorescence microscopy (**b**). Insert scalebars represent 10 μm.