Protein Biology

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Precise Control of Protein Concentration in Living Cells**

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Despite the importance of cellular protein concentration in many areas of biology,[1–3] relatively few technologies have been developed for the precise manipulation of protein concentration. One of the most successful strategies is based on the degradation of destabilization domains (DDs) by the ubiquitin proteasome system. One such method was developed by Crabtree and co-workers and relied on a triple mutant of the protein FRB, termed FRB*.[4–6] Genetic fusion of a protein of interest to FRB* resulted in degradation of the chimera; however, FRB* can be stoichiometrically stabilized or "shielded" by the addition of a rapamycin analogue, MaRap, in a ternary complex with the endogenous protein FKBP.[7] The Wandless research group subsequently improved upon this result through the discovery of an FKBP-based DD (FKBP*, FKBP L106P).[8–11] Fusion proteins to this DD can be shielded by a synthetic small molecule Shld1. These technologies are powerful, but they result in a fusion of FRB* or FKBP* to the protein of interest, which may affect its endogenous biological activity.

We previously reported a complementary strategy, termed split ubiquitin for the rescue of protein function (SURF), in which rapamycin was used to both shield proteins from degradation and affect their release from the DD, an approach that resulted in native protein.[12] This technology relied upon the small-molecule-induced complementation of split ubiquitin[13,14] and was used to control several different proteins. Despite our successful application of SURF, the system is refractory to the facile generation of stably transformed cell lines, which are a better model system for the precise analysis of protein concentration. Specifically, the repetitive nature of the DNA encoding for our DD (three copies of FRB) precludes the use of retroviral technologies for the production of stably transformed cell lines, and SURF is expressed from two separate, large plasmids.

Herein we report a greatly improved system, termed traceless shielding (TShld), which overcomes these limitations. TShld retains the features of SURF and enables the rapid generation of stably transformed cell lines. We were able to precisely control the concentration of green fluorescent protein (GFP) over a 46-fold range. The system is therefore complementary to transcription-based systems, such as tetracycline-inducible promoters. Furthermore, cells transformed with TShld displayed resistance to the inhibition of cellular signaling by rapamycin. This characteristic of the system is important, as rapamycin is more readily available than synthetic small molecules, such as MaRap and Shld1.

SURF required two constructs that were transiently transfected into mammalian cells (Figure 1 A). The first construct, **1**, consisted of maltose-binding protein (MBP) fused to a mutant (I13A) of the N-terminal fragment of ubiquitin (UbN, residues 1–37), followed by FKBP. The I13A mutation prevented constitutive ubiquitin complementation. The second construct, **2**, consisted of the DD (three copies of the FRB mutant T2098L) followed by the C-terminal fragment of ubiquitin (UbC, residues 35–76) and the protein under investigation (Pro). When mammalian cells transiently cotransfected with these two constructs were treated with rapamycin, a ternary complex between FRB and FKBP resulted. This complex would both shield FRB and enable the complementation of split ubiquitin and subsequent release of Pro by endogenous ubiquitin hydrolases.

To improve SURF and create TShld, we first cloned constructs **3** and **4** (Figure 1 a). Construct **3** consisted of FRB followed by UbN. In construct **4**, we replaced our FRB-based DD with FKBP*, followed by UbC and Pro (see the Supporting Information for full construct details). The addition of rapamycin should initially shield FKBP* and then recruit FRB to result in ubiquitin complementation and the release of Pro. To characterize TShld, we used enhanced GFP as Pro in construct **4**. NIH 3T3 cells were then transiently cotransfected with **3** and GFP-encoding **4**, and treated with rapamycin (100 nM) or a dimethyl sulfoxide (DMSO) vehicle for 48 hours. The shielding and release of GFP were then analyzed by western blotting and flow cytometry (Figure 1 b). The addition of rapamycin resulted in modest (ca. threefold) shielding and release of GFP; however, significant background ubiquitin complementation and GFP release in the DMSO control were also observed.

Despite this inefficient shielding, we chose to move forward and generate a single construct that would encode both proteins of the TShld system. We took advantage of a "self-cleaving" 2A peptide from the *Thosea asigna* insect virus (T2A: EGRGSLLTCGDVEENPGP) that induces cotranslational cleavage between the underlined glycine and

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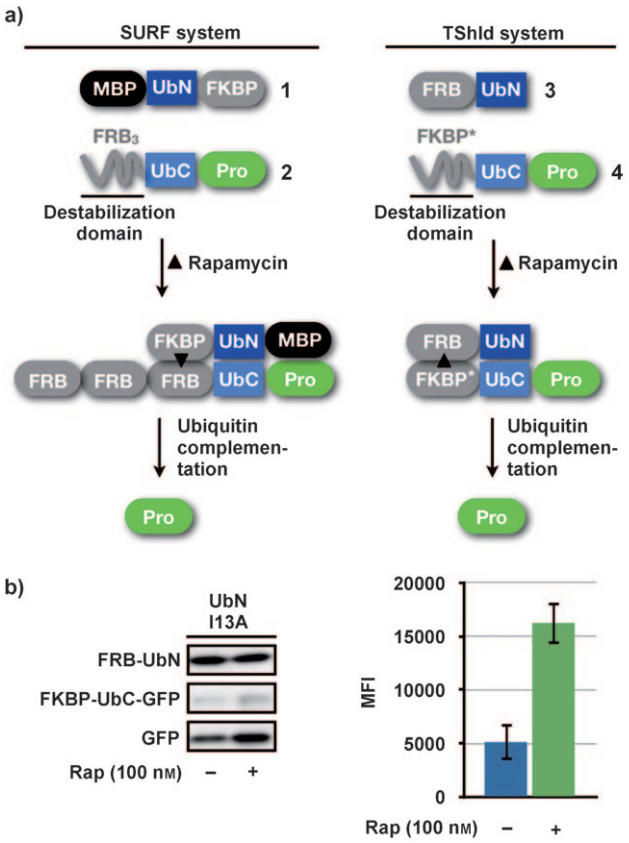

Figure 1. Creation and analysis of TShld. a) Comparison of SURF with TShld. b) NIH 3T3 cells were transiently transfected with TShld-GFP plasmids and treated with rapamycin (100 nM) for 24 h before analysis by western blotting (FRB-UbN: anti-FLAG, FKBP-UbC-GFP and GFP: anti-HA). c) Cells were transfected and treated as in (b) and analyzed for GFP fluorescence by flow cytometry. Error bars show the standard deviation from three experiments. MFI = mean fluorescence intensity.

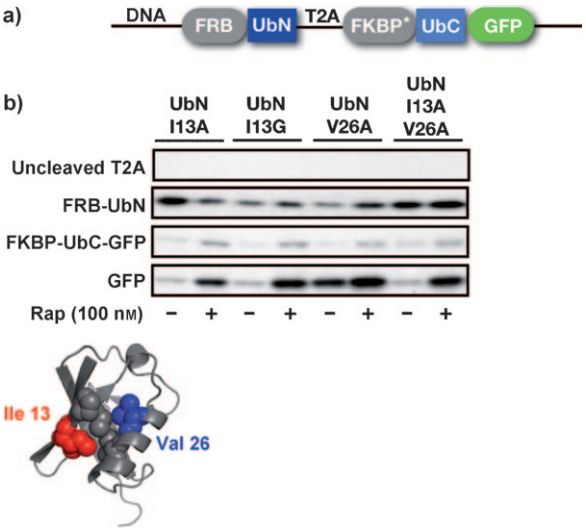

Figure 2. Analysis of TShld expressed from one plasmid. a) Architecture of TShld-GFP. b) NIH 3T3 cells were transiently transfected with TShld-GFP plasmids containing the indicated mutations in UbN and treated with rapamycin (100 nM) for 48 h before analysis by western blotting.

proline residues.[15,16] This peptide was then cloned between FRB–UbN and FKBP*–UbC–GFP to generate a single construct, termed TShld-GFP (Figure 2a). Because of the high level of shielding and release of GFP without rapamycin treatment in our previous experiment, we also generated three additional mutants in UbN in an attempt to lower this background (Figure 2b). The first mutant generated was I13G, which should display greater destabilization of the ubiquitin fold than that observed with I13A. The second mutant was V26A, as this residue has been shown to be important for the stability of ubiquitin in vitro. Finally, a double mutant of both important residues (I13A and V26A) was created. NIH 3T3 cells were transiently transfected with the four different TShld-GFP constructs (I13A, I13G, V26A, and I13AV26A) and treated with rapamycin (100 nM) or DMSO for 48 hours. Western blotting analysis was then performed to visualize the shielding and release of GFP (Figure 2b). It was clear that the T2A peptide had been cleaved efficiently, as no full-length protein was observed. Surprisingly, we observed significantly less background release of GFP with the TShld-GFP I13A mutant than in the I13A cotransfection experiment. We do not have a definitive explanation for this result but reason that the T2A-containing TShld plasmid may yield lower protein levels, so that the use of this plasmid results in less background ubiquitin complementation. I13A, I13G, and I13AV26A all showed efficient shielding and release of GFP upon treatment with rapamycin; only V26A displayed significant background. A comparison of I13A and I13G by flow cytometry (see Figure 1 in the Supporting Information) revealed the superior induction of I13G, which was therefore used in future experiments.

We next created two TShld-GFP constructs for stable expression in mammalian cells. One, **5**, utilizes the murine stem cell virus (MSCV) promoter, which gives low levels of expression, and the other, **6**, uses the cytomegalovirus (CMV) promoter, which produces higher protein levels. We reasoned that, in combination, these two promoters would increase the dynamic range of shielding. The TShld-GFP constructs were stably integrated into NIH 3T3 cells through the use of an amphotropic retroviral expression system, and the cells were treated with rapamycin (100 nM) or DMSO for 48 hours. Analysis by western blotting showed robust shielding of GFP regardless of the promoter (Figure 3a) and a qualitative dose dependence on the rapamycin concentration (Figure 3b).

We next used flow cytometry to quantitatively analyze GFP expression (Figure 4a; see also Figure 2 in the Supporting Information). TShld-GFP constructs **5** and **6** showed precise control of protein concentration over rapamycin concentrations from 0.01 to 1000 nM. Separately, **5** and **6** showed good control over non-overlapping ranges of protein concentration. For this reason, the use of both promoters together enables accurate analysis of protein levels with excellent coverage over a 46-fold dynamic range. To determine the kinetics of GFP shielding, we treated transformed NIH 3T3 cells with rapamycin (500 nM) and performed flow cytometry at different time points (Figure 4b). GFP fluorescence was observable at 2 hours and began to plateau between 24 and 48 hours. Understandably, much like those

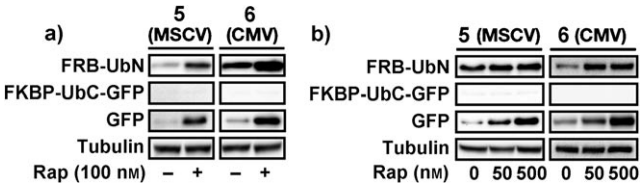

Figure 3. Analysis of TShld-GFP in stably transformed cells. a) NIH 3T3 cells infected with TShld-GFP expressed from an MSCV (in the case of **5**) or CMV promoter (in the case of **6**) were treated with rapamycin (100 nM) or DMSO for 48 h and analyzed by western blotting. b) NIH 3T3 cells containing **5** or **6** were treated with rapamycin at the indicated concentrations or DMSO for 48 h and then analyzed by western blotting.

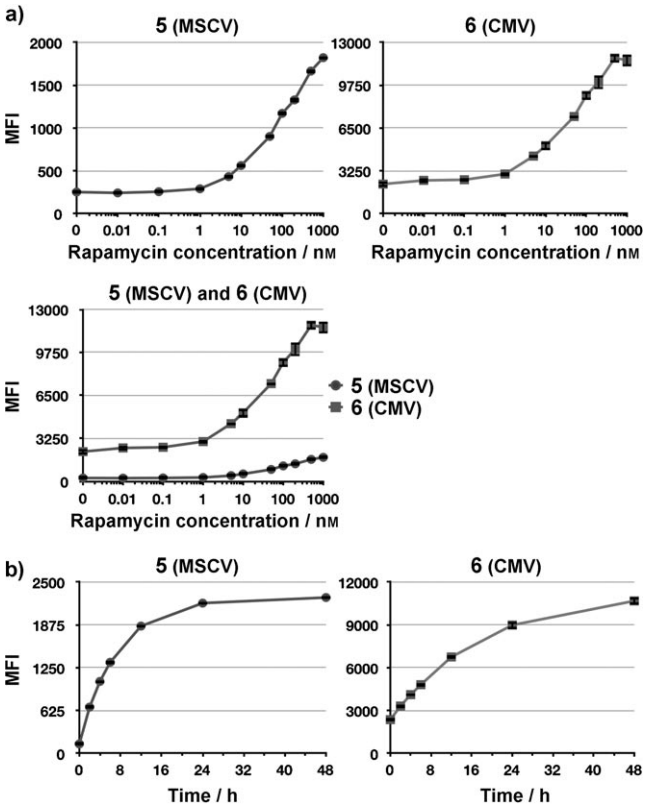

Figure 4. Quantitative analysis of TShld-GFP by flow cytometry. a) NIH 3T3 cells infected with TShld-GFP containing **5** or **6** were treated with rapamycin at the indicated concentrations or DMSO for 48 h, and GFP fluorescence was quantified by flow cytometry. b) NIH 3T3 cells containing **5** or **6** were treated with rapamycin (500 nM) for the indicated amount of time, and GFP fluorescence was quantified by flow cytometry. All experiments were performed in triplicate; error bars show the standard deviation.

of other shielding technologies,[4,9,11] these kinetics seem to be limited by the rate of protein synthesis.

We next wanted to examine whether TShld displayed advantages over transcription-based systems, such as the commercially available tetracycline-inducible expression system Tet-On. Towards this goal, we treated NIH 3T3 cells stably transduced with tetracycline-inducible GFP with doxycycline at different concentrations. For comparison, we treated NIH 3T3 cells containing **5** or **6** with rapamycin and analyzed GFP levels in all three cell types by flow cytometry (Figure 5). The results showed that TShld can access proteins in a lower concentration range than is possible with Tet-On. Furthermore, after normalization (see the Supporting Information) of the mean fluorescence intensity (Figure 5), TShld displays a superior (more linear) dose response to the small molecule. Furthermore, the background level of untreated **5** is very low: it represents only a 150% increase in fluorescence over that of noninfected cells, whereas a corresponding 850% increase is observed for untreated Tet-On.

One concern we had regarding the use of rapamycin was its ability to downregulate cell proliferation through inhibition of the mTOR/raptor kinase.[17,18] We reasoned that our cell lines expressing TShld might resist treatment by sequestering rapamycin away from mTOR in FKBP*/FRB ternary complexes. To test this possibility, we treated NIH 3T3 cells that had been virally infected with an empty vector (mock), **5**, or **6** with rapamycin at different concentrations for 48 hours. Proliferative signaling through mTOR was then induced by treatment with 20% fetal calf serum for 30 minutes, and mTOR activity was analyzed by western blotting against phosphorylation of a downstream target, S6 kinase (S6K threonine 389, Figure 6).[17] Rapamycin inhibited mTOR in mock-infected cells at all concentrations; however, phosphorylation of S6K was detectable in cells infected with **5** or **6** even at high rapamycin concentrations (500 nM), which suggests that TShld does confer some protection against rapamycin.

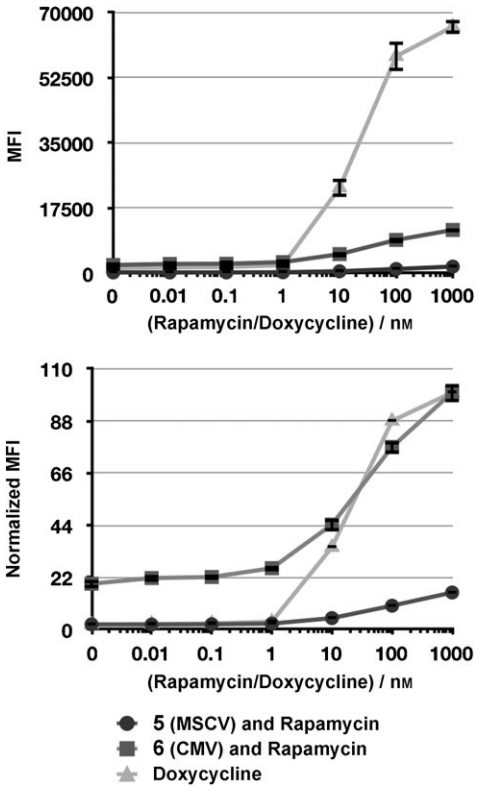

Figure 5. Comparison of TShld with Tet-On. NIH 3T3 cells infected with GFP by using the Tet-On system or TShld-GFP containing **5** or **6** were treated with doxycycline or rapamycin at the indicated concentrations or DMSO for 48 h, and GFP fluorescence was quantified by flow cytometry. All experiments were performed in triplicate; error bars show the standard deviation.

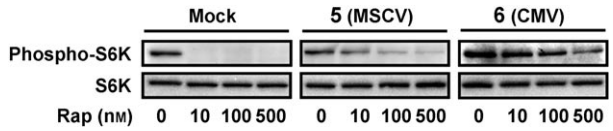

Figure 6. Analysis of mTOR inhibition in TShld-expressing cells. NIH 3T3 cells infected with an empty plasmid (mock), **5**, or **6** were treated with rapamycin at the indicated concentrations for 48 h and then with 20% fetal calf serum for 30 min. mTOR activity was then visualized by western blotting for the phosphorylation of threonine 389 on S6 kinase.

Finally, to explore the generality of TShld, we performed two experiments. First, we generated RPE and HEK 293 cells stably transformed with **5** by using the amphotropic retroviral expression system described above. Treatment of these cells with rapamycin (100 nM) for 48 hours and analysis by western blotting showed good shielding and release of GFP (see Figure 3 in the Supporting Information). Second, we generated TShld constructs for caspase 3 (TShld-Caspase3) and α-synuclein (TShld-αSyn), both under the control of the CMV promoter. NIH 3T3 cells expressing the above constructs were treated with rapamycin (500 nM) for 48 hours and analyzed by western blotting (Figure 7). In both cases, we

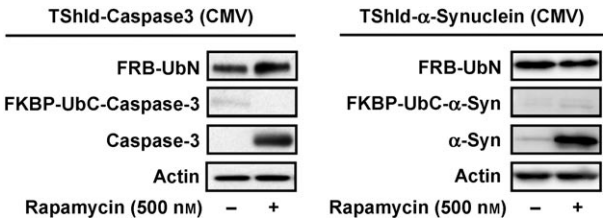

Figure 7. Analysis of TShld-Caspase3 and TShld-αSyn. NIH 3T3 cells expressing TShld-Caspase3 or TShld-αSyn from the CMV promoter were treated with rapamycin (500 nM) or DMSO, followed by visualization by western blotting.

observed excellent shielding and release that was qualitatively superior to that observed for TShld-GFP.

In summary, we have developed and characterized a new technology, termed traceless shielding (TShld), for controlling protein concentration in living cells. TShld represents a large improvement over our previously reported system,[12] as it is expressed from a single plasmid and enables the rapid generation of stably transformed cell lines. When GFP and two different promoters, MSCV and CMV, were used, TShld was highly responsive to the concentration of rapamycin over an approximately 50-fold range of protein abundance, and the kinetics of shielding were linked to protein synthesis. Moreover, TShld shows improved dose dependence and can access different protein concentrations when compared to transcription-based systems, such as Tet-On; thus, these technologies are complementary. Some researchers may be hesitant to use rapamycin because it inhibits the mTOR kinase.[17,18] We were able to show that cells expressing TShld were resistant to the effects of rapamycin by examining the phosphorylation status of a downstream target of mTOR, S6 kinase.[17] This resistance should facilitate the use of all shielding technologies, as FK506 and rapamycin are inexpensive and more readily available than nontoxic analogues, such as MaRap[4] and Shld1.[11] Therefore, TShld represents a straightforward alternative to existing shielding technologies that require permanent fusion of the protein under investigation to the DD. We feel that the feature of tracelessness will be particularly attractive for the study of proteins that must participate in protein–protein interactions or multimeric complexes, such as caspase 3 and oligomeric α-synuclein. In ongoing studies, we are investigating how the concentration of these proteins affects cellular phenotypes. Furthermore, we are confident that the concentration of a variety of other proteins can be controlled in cells and in vivo on the basis of results described previously by us and others.[5,10,12]

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