Quick & Reversible Control of Your Protein of Interest

Manipulate protein quantity in cells and organisms with fast kinetics, using the ProteoTuner™ Systems

• Quickly stabilize or destabilize your protein of interest
• Gain firsthand information about protein function
• Tunable & repeatable for precise control of protein levels

Analyzing protein function is a key focus in discovery-based cell biology research. Clontech’s revolutionary new ProteoTuner Systems, based on a technology developed by Dr. Thomas Wandless and colleagues (1), allow you to directly investigate the function of a specific protein of interest—by manipulating the level of the protein itself. This technology has already been successfully used in a variety of applications and organisms, resulting in several publications in outstanding peer-reviewed journals (1–7).

The ProteoTuner Systems are based on a 12 kDa mutant of the FKBP protein (the destabilization domain, or DD) that can be expressed as a tag fused to your protein of interest. The DD fusion protein is reversibly protected from proteasomal degradation in the presence of the small (750 Da) membrane-permeable ligand, Shield1 (Figure 1).

Fast, Focused Results

Quickly changing the amount of your protein of interest within a cell enables you to gain valuable information about its function. Unlike other systems which regulate the amount of a protein indirectly (at the transcriptional level), this system targets the protein of interest itself, guaranteeing a much quicker response than other methods. It has been shown that a DD fusion protein can accumulate to detectable levels in just 15–30 minutes after adding the stabilizing ligand Shield1 (1).

Adjustable Protein Stabilization

In the presence of Shield1, the DD-tagged protein of interest is stabilized and accumulates inside the cell. Conversely, in its absence, the DD-tagged protein is degraded very rapidly by proteasomes. Thus, it is possible to “tune” the amount of stabilized, DD-tagged protein present in the cell by titrating the amount of Shield1 in the culture medium (Figures 2–3). The degree of stabilization increases as the Shield1 concentration increases within the range of ~50–1,000 nM.

This was demonstrated by cloning DsRed-Express into the pRetroX-PTuner Vector (sold as part of Cat. No. 632171) in-frame with the 5 sequence encoding the DD. Infected cells were treated with different concentrations of Shield1, and the fluorescence intensity of treated cells was analyzed using flow cytometry. Higher fluorescence intensities were observed in samples treated with higher concentrations of Shield1, due to greater amounts of stabilized DD-DsRed-Express protein present in the cells (Figure 2).

These results were confirmed by Western blot, using the Living Colors® DsRed Polyclonal Antibody (Cat. No. 632496). DD-DsRed-Express can be stabilized to levels comparable with levels of the untagged protein in cells stably expressing untagged DsRed-Express from the CMV promoter (Figure 3). At lower concentrations of Shield1, there is a direct relationship between the concentration of Shield1 and the amount of destabilized DsRed-Express protein detected by flow cytometry or in the cell lysate (Figures 2 and 3, respectively).
Quick & Reversible Control of Your Protein…continued

Reversible & Repeatable

The ProteoTuner method is not restricted to protein stabilization—it can also be used to destabilize your protein of interest by withdrawing Shield1 from cell cultures that previously contained Shield1. This makes it possible to repeatedly stabilize and destabilize your protein using the same set of cells (1; see pages 3–4).

The ability to quickly and directly control protein levels with the ProteoTuner Systems provides you with a novel tool for studying transient effects that might otherwise be masked, by directly and specifically “tuning” the level of a protein of interest in the cell. Four systems are available: with either plasmid or retroviral vectors, and with or without a Living Colors Fluorescent Protein marker for transfection efficiency (Table 1). Shield1 is available as part of each system, as well as separately.

Figure 3. DD-DsRed-Express fluorescence is directly related to the concentration of the stabilizing ligand Shield1. In order to visualize the amount of DD-DsRed-Express expressed in a cell, cells were infected with pRetroX-PTuner DsRed-Express IRES ZsGreen1 and treated with different concentrations of Shield1. The amount of DD-tagged DsRed-Express stabilized by different concentrations of Shield1 was detected via Western blot using the Living Colors DsRed Polyclonal Antibody. Lane 1: molecular weight marker. Lane 2: 1X loading buffer. Lane 3: untreated HeLa cells (no virus, no Shield1). Lane 4: HeLa cells infected with the DD-DsRed Express construct; no Shield1. Lanes 5–8: HeLa cells infected with the DD-DsRed-Express construct and treated with 50, 250, 500, and 1,000 nM Shield1 respectively. Lane 9: 1X loading buffer. Lane 10: HEK 293 DsRed-Express stable cell line.

Table I: Four Single-Vector ProteoTuner Systems Now Available

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<td><strong>System</strong></td>
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<td>ProteoTuner System</td>
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<td>Retro-X™ ProteoTuner System</td>
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<td>Retro-X ProteoTuner IRES System</td>
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¹ Bacterial/eukaryotic

ProteoTuner™ System Components

- pPTuner Vector
- Shield1

ProteoTuner™ IRES2 System Components

- pPTuner IRES2 Vector
- Shield1

Retro-X™ ProteoTuner™ System Components

- pRetroX-PTuner Vector
- Shield1

Retro-X™ ProteoTuner™ IRES System Components

- pRetroX-PTuner IRES Vector
- Shield1

Related Products

- Tet-On™ Advanced Inducible Gene Expression System (Cat. No. 630930)
- Tet-Oct™ Advanced Inducible Gene Expression System (Cat. No. 630934)
- Knockout™ Single Vector Inducible RNAi System (Cat. No. 630933)

Notice to Purchaser

Please see the CMV Sequence, Living Colors® Fluorescent Protein Products, and ProteoTuner™ Protein Stabilization/Destabilization Products licensing statements on page 29.

References

A Rapid, Reversible, & Tunable Method to Regulate Protein Function in Living Cells

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Rapid and reversible methods for perturbing the function of specific proteins are desirable tools for probing complex biological systems. We have developed a general technique to regulate the stability of specific proteins in mammalian cells in a reversible manner using Shield1, a membrane-permeable ligand. We engineered mutants of the human FKBP12 protein that are rapidly and constitutively degraded when expressed in mammalian cells, and this instability is conferred to other proteins fused to these destabilizing domains. When Shield1 is added, it binds to the destabilizing domain and shields it from degradation, allowing fused proteins to accumulate and perform their cellular function.

Library Construction & Identification of Ligand-Responsive Destabilizing Domains

To identify mutants that display ligand-dependent stabilization, we implemented a cell-based screen in which the fluorescence of yellow fluorescent protein (YFP) served as an indicator of FKBP12 fusion protein stability. A library of over 20,000 sequences based on the FKBP12 F36V gene sequence (hereafter referred to as FKBP) was generated using error-prone PCR and cloned in-frame onto the N-terminus of YFP. The expressed proteins were then assayed for ligand-dependent stabilization.

Destabilizing Domain Characterization

Five FKBP mutants (F15S, V24A, H25R, E60G, and L106P) were chosen from the library for further analysis. To measure intracellular protein levels, cells stably expressing each destabilizing domain fused to YFP were either mock-treated or treated with Shield1. Western blot analysis was used to detect FKBP12-YFP in the lysate from four of the mutants (Figure 1).

No FKBP12 protein was detected in lysates of cells not treated with Shield1, whereas Shield1-treated cells showed strong expression of the expected fusion proteins (Figure 1).

We then tested each mutant’s response to the addition or removal of Shield1. In its absence, all five mutants showed decreased fluorescence levels and varying degrees of destabilization with respect to a positive control. When Shield1 was added, all five mutants showed increased fluorescence, with observed stabilization efficiencies varying by an order of magnitude (Figure 2, Panel A). Upon withdrawal of Shield1, we observed distinct differences in fluorescence decay profiles among the destabilizing domains (Figure 2, Panel B). This study revealed a correlation between the rate of degradation and the degree of destabilization conferred by each mutation, and based on the results, mutant L106P (hereafter referred to as the destabilization domain or DD) was chosen for further study.

Editor’s Note: This technology is now available as Clontech’s ProteoTuner™ Systems (Cat. Nos. 632167, 632168, 632171, & 632172) and Shield1 (Cat. Nos. 631037 & 631038; see pages 1–2 for more information).
Rapid Control of DD-Protein Levels & Stability

Next, we compared the rate of RNAi-mediated silencing of an endogenous gene to the rate of degradation achieved through fusion of a protein of interest to the DD. When HeLa cells were transfected with siRNA against lamin A/C, a nes-sential cytoskeletal protein commonly used as a control in RNAi experiments, a decrease in protein levels was observed after 24 hours. In contrast, cells stably expressing DD-YFP show nearly complete degradation of the fusion protein within 4 hours of removal of Shield1, illustrating that fusion of the DD to a protein of interest dramatically reduces its stability in cultured cells (Figure 3).

Predictable Regulation of Protein Levels

To confirm that the DD is a ligand-sensitive mutant of FKBP, we subjected a population of NIH 3T3 cells stably expressing DD-YFP to various concentrations of Shield1 over the course of one week (Figure 4). The ProteoTuner Systems’ dose-dependent control is exemplified by the proximity of the observed fluorescence levels to values predicted from the dose-response experiment shown in Figure 2A.

Shield1-Dependent Control of Cellular Phenotypes

We next sought to correlate changes in cellular behavior with the Shield1-depen-dent stabilization of a constitutively active mutant of Cdc42 (Q61L) fused to the DD. Expression of this small GTPase causes well-characterized changes in cellular morphology (1). Shield1-treated populations of cells expressing DD-Cdc42 (Q61L) displayed the predicted change in morphology. This change was reversible, as removal of Shield1 resulted in a fibroblast-like morphology indistinguishable from that of mock-treated transduced cells (Figure 5).

Conclusions

Techniques that target gene function at the level of DNA and mRNA are versatile and powerful strategies for perturbing the protein products encoded by specific genes. However, experimental approaches to regulate proteins directly are limited, especially in mammalian cells. We have developed a “single ligand-single domain” system that allows conditional small-molecule control of protein stability. It is based on the fusion of any protein of interest to a ligand-binding domain (the DD tag) that is engineered to induce degradation in the absence of its ligand. Binding of the Shield1 ligand to this destabilizing domain stabilizes the fusion protein and shields it from degradation, protecting the protein of interest. This system is capable of conferring Shield1-dependent stability to a wide variety of DD fusion proteins, thus introducing a new technique for controlling gene function, directly at the protein level.

Acknowledgements

The work described in this technical note was originally published in Cell (2).

References
Rapid Control of Protein Level in the Apicomplexan *Toxoplasma gondii*

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Analysis of gene function in apicomplexan parasites is limited by the absence of reverse genetic tools that allow easy and rapid modulation of protein levels. The fusion of a ligand-controlled destabilization domain to a protein of interest enables rapid and reversible protein stabilization in *T. gondii*. This allows an efficient functional analysis of proteins that play a dual role during host cell invasion and/or intracellular growth of the parasite.

**Introduction**

Although conditional mutagenesis to study the function of vital genes has been achieved in apicomplexan parasites, the current major limitation is the slow kinetics of the expression switch, which requires several hours to days of downregulation until the phenotypic consequences can be analyzed (1).

Rapid regulation of protein abundance has recently been demonstrated for mammalian cells. Selective stabilization is achieved by reversible binding of a synthetic ligand to a destabilization domain (DD) fused to a protein of interest. The DD tag, based on a mutant version of the rapamycin-binding protein FKBP12, mediates fast proteasomal degradation in the absence of Shield1. However, when the membrane-permeable ligand Shield1 binds to the DD tag, it protects the fusion protein from proteasomal degradation, rapidly increasing the amount of protein in a cell (2).

To determine whether ProteoTuner technology can be applied in apicomplexan parasites, we used *T. gondii* as a model system. We evaluated the system successfully in *T. gondii* by linking the DD to the N-terminus of yellow fluorescent protein (YFP). The DD-YFP fusion protein was detected in as soon as 20 minutes and in less then 1 hour after induction in intra- and extracellular parasites, respectively (data not shown).

Functional Characterization with the ProteoTuner System

Focusing on *T. gondii*-specific proteins, we turned our attention to a conditional mutant for the motor protein TgMyoA which has previously been established and functionally described in detail, using a tetracycline-inducible system (3). Hence, we chose TgMyoA as a candidate protein to evaluate the potential of the DD-fusion protein system (i.e., the ProteoTuner system) for the regulation and functional characterization of essential parasite proteins. We generated stable parasites expressing TgMyoA, tagged at its N-terminus with the DD and a myc tag (DD-MyoA), under control of the strong constitutive promoter p5RT70 (4).

A similar observation has been reported upon regulated overexpression of TgMyoA using a tetracycline-inducible repression system, and it has been speculated that the observed downregulation of endogenous TgMyoA occurs posttranscriptionally (5). However, the use of the DD fusion system allowed us to discover that endogenous TgMyoA is downregulated posttranscriptionally and not posttranscriptionally. It thus appears that ectopic overexpression of TgMyoA results in competition for interaction partners for endogenous TgMyoA, such as the myosin light chain (TgMLC) that stabilizes the motor complex (6). The fusion protein was nonfunctional; however, “tagging” a protein can always cause interference with the function of the protein, and is not due to the DD sequence itself.

Generating Knockout Phenotypes with ProteoTuner Technology

A general approach often used to examine the functions of essential genes is to determine the phenotypic consequences of overexpression of dominant negative alleles. We used the DD-fusion system to characterize the function of a small G protein, Rab11A, by Shield1-dependent stabilization of a dominant negative version. We generated a dominant negative version of TgRab11A (N126I), which inactivates

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**Figure 1. Functional analysis of essential proteins using the ProteoTuner System.** Immunofluorescence and Western blot analyses were performed on *T. gondii* parasite strains expressing DD-MyoA (Panel A) and DD-Rab11A (Panel B). Both visualization methods confirm that the DD fusion proteins are stabilized in the presence of Shield1. Immunofluorescence analyses were performed 4 hr after induction with Shield1, using monoclonal anti-myc (clone 9E10, Sigma) and either anti-Gap45 (7) or polyclonal anti-IMC1 (8). Scale bar, 10 mm. Western blot analyses using these antibodies were performed on the same parasite strains, grown in the presence or absence of Shield1 for 48 hr (DD-MyoA) or for two hr (DD-Rab11A) before analysis.

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<td>DD-Rab11A</td>
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Rapid Control of Protein Level in the Apicomplexan *Toxoplasma gondii*...continued

![Graph showing quantification of invasion and replication of parasite strain DD-Rab11ADN.](image)

**Figure 2.** Quantification of invasion and replication of parasite strain DD-Rab11ADN. Total number of parasitophorous vacuoles was determined. Mean values of three independent experiments ± s.d. are shown (−/+; parasites treated with Shield1 after invasion; +/+; parasites treated with Shield1 before and after invasion). Asterisks indicate a significant difference in total invasion between parasites not treated with Shield1 and parasites treated with Shield1 before and after invasion (P < 0.01, two tailed Student’s t-test).

the GTPase domain. Upon stable transfection of DD-Rab11A<sub>ON</sub>, we isolated clonal parasites in which DD-Rab11A<sub>ON</sub> was detectable 2 hours after addition of 1 μM Shield1 (Figure 1, Panel B). In growth assays, we detected normal plaque formation when compared to RH wild-type (RHwt) parasites in the absence of Shield1. However, in the presence of inducer we did not detect growth of the parasite, demonstrating that expression of this dominant negative G protein is deleterious for parasite growth.

To assess whether Rab11A might have different roles in intra- and extracellular parasites, we took advantage of the rapid induction kinetics of the DD-fusion system. We preincubated extracellular parasites for 20 minutes in the presence or absence of Shield1, allowed them to invade host cells for 3 hours, and then removed the remaining parasites. In addition, we incubated intracellular parasites in the presence or absence of Shield1 for 16 hours, and then analyzed invasion and replication. Whereas RHwt parasites do not show any invasion or replication defect upon treatment with Shield1, stabilization of DD-Rab11A<sub>ON</sub> in extracellular parasites reduced the ability of the parasites to invade the host cell by ~85% when compared to parasites not treated with Shield1 (Figure 2). This indicates a role of Rab11A in invasion of the host cell and in the intracellular propagation (replication or maturation) of the parasite. These results suggest that Rab11A has an essential function in both extra- and intracellular parasites, and shows that ProteoTuner technology can be used to dissect the role of a protein in both extra- and intracellular parasites.

**ProteoTuner Technology is Applicable to Apicomplexan Parasite Systems**

Although the DD system can be applied in *T. gondii*, some proteins may not be functional when fused to a tag, such as the DD. Another possible limitation might be that not all fusion proteins can efficiently target the proteasome. However, the rapid induction kinetics of this system allows specific modulation of protein levels in extra- and intracellular parasites, and will allow a detailed, functional, and highly precise dissection of specific processes during the life cycle of the parasite. Analysis of protein functions is often performed with the help of dominant negative mutants. Using the DD system, it is now possible to apply this strategy in *T. gondii* and possibly *P. falciparum* (9), which may find broad application in research on apicomplexans.

**References**