Using the ProteoTuner™ System to Directly Control the Level of a Secreted Protein and to Fine-Tune the Rescue of a Knockout Phenotype

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*The **ProteoTuner™ Systems** use a new technology to control the level of your protein of interest directly, rather than manipulating protein expression at the level of DNA or mRNA. To further characterize the system, we have demonstrated the nontoxic nature of Shield1. We also showed that the ProteoTuner system can be used on secreted proteins as well as on cytosolic proteins. Finally, we used the ProteoTuner System to rescue a knockout phenotype, demonstrating the tunable nature of the ProteoTuner Systems.*

The **ProteoTuner System** allows you to stabilize or degrade a protein of interest in the cell in a ligand-dependent, tunable, and reversible fashion. The system consists of a vector that encodes the ProteoTuner destabilization domain (DD) upstream of a multiple cloning site, and a supply of the DD's stabilizing ligand, **Shield1.** The DD is a 12 kDa (107 amino acid) tag, based on a mutated FKBP protein. When a protein of interest is expressed as a fusion with the DD tag, it is destabilized and rapidly degraded in the cell by proteasomes. However, when the small (750 Da), membrane-permeant ligand Shield1 is added to the culture medium, it binds reversibly and specifically to the DD tag and protects the DD-tagged protein from degradation so that the protein accumulates rapidly in the cell (1).

No Detectable Side Effects

ProteoTuner technology has been used successfully in a variety of cell lines and organisms (1–6). Recently, Wandless and colleagues used microarray analysis to look for perturbations in gene expression in response to different concentrations of Shield1. Very few genes exhibited appreciable changes in RNA levels after treatment with 1 µM or 10 µM Shield1. None of the changes were observed at a concentration of 100 nM Shield1 (2). Consistent with these findings, in a separate paper, these authors reported that Shield1 did not influence therapeutic outcomes in tumor-bearing mice, and that Shield1-treated mice maintained normal body weight, activity, and feeding behaviors (3).

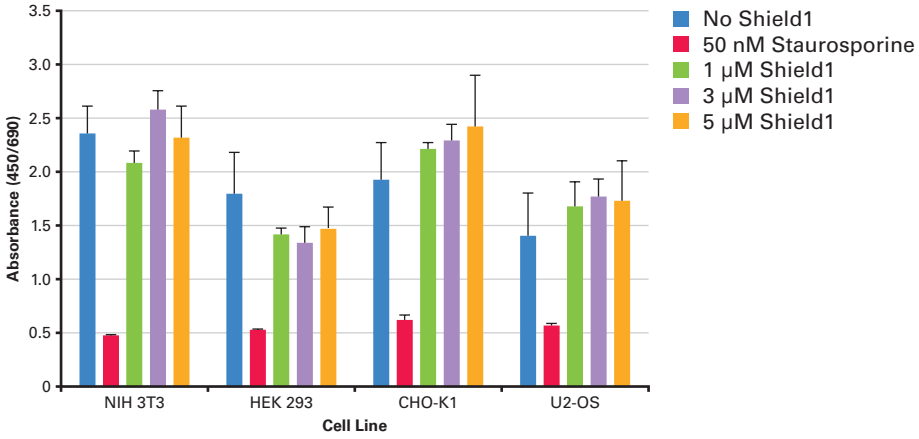

Figure 1. Shield1 does not affect viability, even at 5–10 times the recommended concentration. Each indicated cell type (1 x 10^4 cells/well of a 96-well plate) was treated with 0, 1, 3, or 5 µM Shield1. Staurosporine (50 nM), a known apoptosis inducer, was used as a negative control. 48 hr later, cell proliferation was measured using the Premixed WST-1 Cell Proliferation Reagent.

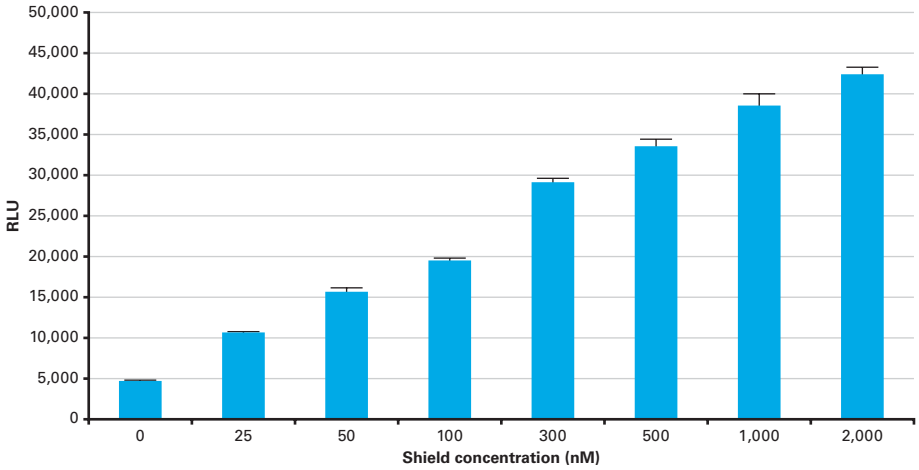

Figure 2. Shield1 has a stabilizing effect on secreted *Metridia* luciferase protein, implying a similar mechanism for Shield1-based stabilization of secreted and cytosolic proteins. Shield1 stabilizes secreted proteins in a tunable manner. Cells were transiently transfected with the DD-*Metridia* luciferase construct, and treated with increasing concentrations of Shield1. Six hr later, media samples were collected and the amount of secreted DD-*Metridia* luciferase in the media was determined using the Ready-To-Glow Secreted Luciferase Reporter Assay. RLU = relative light units.

To further confirm these recent reports, we examined the effect of increasing concentrations of Shield1 on the commonly used cell lines NIH-3T3, HEK 293, CHO-K1, and U2OS. Potential toxicity was monitored using the **Premixed WST-1 Cell Proliferation Reagent.** Shield1 did not perturb cell proliferation, even when the cells were treated with five to ten times the concentration of Shield1 recommended for use in tissue culture cells (Figure 1). Together with the previously published results, these data indicate that Shield1 is generally free of toxic and off-target effects.

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Suitable for Secreted Proteins

The ProteoTuner System's ability to control cytosolic protein levels has already been demonstrated. The system has also recently been tested with secreted proteins (3). We also examined the ProteoTuner System's effects on another secreted reporter protein, *Metridia* luciferase, using the **Ready-To-Glow™ Secreted Luciferase Reporter System.** We first created a construct encoding secreted *Metridia* luciferase tagged with the DD inserted between the N-terminal signal peptide and the actual *Metridia* luciferase. This was necessary in order to allow the protein to enter the secretory pathway.

The cells were transfected with this construct, treated with different concentrations of Shield1, and the amount of secreted DD-*Metridia* luciferase in the media supernatant was monitored according to the Ready-To-Glow assay protocol. The assay readout reflected the ability of Shield1 to stabilize the secreted DD-*Metridia* luciferase protein. The concentration of Shield1 in the culture medium was directly related to the bioluminescence we measured (Figure 2), indicating that Shield1 can stabilize DD-tagged secreted proteins in a Shield1 concentration-dependent manner. However, the extent of stabilization for a secreted protein may be slightly lower than that of DD-tagged cytosolic proteins.

A higher concentration of Shield1 (2 µM) was required to achieve maximum stabilization of this secreted protein during its passage through the endoplasmic reticulum, whereas for cytosolic proteins, near-complete stabilization is achieved in the range of 500–1,000 nM. But as shown in Figure 1, Shield1 concentrations of 5 µM did not affect the cells' viability (Figure 1).

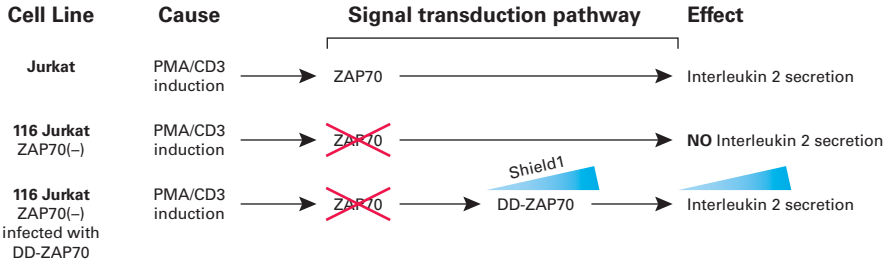

Figure 3. Tunable rescue of the ZAP70 signal transduction pathway in the ZAP70 deficient 116 Jurkat cell line.

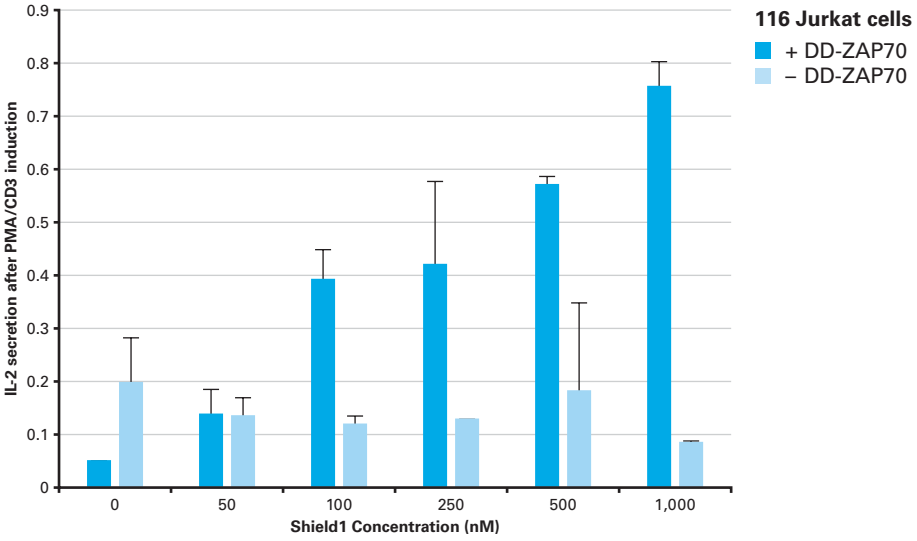

Figure 4. Rescue of the ZAP70-dependent signaling pathway with the ProteoTuner system. The level of rescue is proportional to the amount of ZAP70 that is rescued. 116 Jurkat cells (ZAP70-negative) were infected or mock-infected with a construct for DD-ZAP70, cultured in varying concentrations of Shield1, and induced with PMA and CD3. The extent of recovery was measured by IL-2 secretion into the culture media, via an ELISA assay.

Tunable Rescue of a Knockout Phenotype

Knockout models are powerful tools for protein function studies. However, rescuing a phenotype by reintroducing the "knocked-out" protein in a tunable, regulated manner can provide even more details about the protein's function. Therefore, we used the ProteoTuner system to reintroduce the DD-tagged ZAP70 protein into a ZAP70 knockout cell line (116 Jurkat), and to control the degree of rescue in a Shield1-dependent manner. ZAP70 (Zeta-chain-associated protein kinase 70) is a tyrosine kinase that is naturally expressed in T cells and natural killer cells. It plays a critical role in the initiation of T-cell signaling, and ultimately, IL-2 secretion (Figure 3).

We used the **Lenti-X™ ProteoTuner System** and the **RetroNectin® Reagent** to transduce 116 Jurkat cells with a construct encoding DD-ZAP70, and achieved a transduction rate of up to 90% in these difficult-to-transduce cells. Increasing concentrations of Shield1 stabilized DD-ZAP70 and we were able to rescue ZAP70-dependent signaling upon PMA/CD3 induction, in a tunable manner (Figure 4). The extent of rescue reflected by the amount of IL-2 in the culture media

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was directly proportional to the Shield1 concentration, and thereby to the amount of stabilized DD-ZAP70 in the cell. This indicates that the ZAP70 signaling pathway is not an on/off switch, but can be modulated via ZAP70 expression. Without Shield1, the intracellular level of DD-ZAP70 in transduced cells is so low that it cannot rescue the signal transduction pathway. This is reflected by a low IL-2 level, comparable to that of uninfected 116 Jurkat cells.

A powerful & versatile system

The ProteoTuner systems can be used for a wide range of proteins and types of experiments. Since Shield1 is nontoxic, you can be confident that any changes you observe are specifically due to your manipulation of the protein of interest. Furthermore, the ProteoTuner systems can be used to tune not only the abundance of cytosolic proteins, but that of secreted proteins as well. We have successfully used the systems to fine-tune the reintroduction of a protein of interest into a knockout system, allowing quick and precise control over the degree of rescue.

References

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3. Banaszynski, L. A. *et al.* (2008) *Nature Med.* **14**(10):1123–1127.
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5. Berdeaux, N. *et al.* (2007) *Nature Med.* **13**(5): 597–603.
6. Herm-Götz, A. *et al.* (2007) *Nature Meth.* **4**(12):1003–1005.

Product	Size	Cat. No.
ProteoTuner System	each	632172
ProteoTuner IRES2 System	each	632168
Retro-X ProteoTuner System	each	632171
Retro-X ProteoTuner IRES System	each	632167
Lenti-X ProteoTuner System	each	632173
Lenti-X ProteoTuner Green System	each	632175
Shield1*	60 µl	631037
	200 µl	631038
	500 µl	632189
Lenti-X HT Packaging System	20 rxn	632160
	40 rxn	632161
Premixed WST-1 Cell Proliferation Reagent	2,500 rxns	630118
Ready-To-Glow Secreted Luciferase Reporter System	100 rxns	631730
	500 rxns	631731
	1,000 rxns	631732
RetroNectin Reagent	0.5 mg	TAK T100A
	5 x 0.5 mg	TAK T100B

* The number of reactions depends on the concentration of Shield1 used. At the maximum suggested concentration (1,000 nM), 60 µl = 30-plus reactions in a six-well plate.