Broadly Applicable Methodology for the Rapid and Dosable Small Molecule-Mediated Regulation of Transcription Factors in Human Cells

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Ⓢ *Supporting Information*

ABSTRACT: Direct and selective small molecule control of transcription factor activity is an appealing avenue for elucidating the cell biology mediated by transcriptional programs. However, pharmacologic tools to modulate transcription factor activity are scarce because transcription factors are not readily amenable to small molecule-mediated regulation. Moreover, existing genetic approaches to regulate transcription factors often lead to high nonphysiologic levels of transcriptional activation that significantly impair our ability to understand the functional implications of transcription factor activity. Herein, we demonstrate that small molecule-mediated conformational control of protein degradation is a generally applicable, chemical biological methodology to obtain small molecule-regulated transcription factors that modulate transcriptional responses at physiologic levels in human cells. Our establishment of this approach allows for the rapid development of genetically encoded, small molecule-regulated transcription factors to explore the biologic and therapeutic impact of physiologic levels of transcription factor activity in cells.

Transcription factors are attractive targets for pharmacologic regulation because modulating their activity provides a general method to alter physiology and pathology at the level of gene regulation.[1] The absence of prototypical ligand binding sites has encumbered the discovery of small molecules that can selectively modulate transcription factor function.[2] Since transcription factors employ much of their solvent-exposed surface to form large multiprotein:DNA complexes, their activities could potentially be modulated by small molecules that bind to those surfaces and induce or inhibit requisite protein:protein and protein:DNA interactions. Small molecules that target such surfaces are difficult to develop and, with few exceptions (such as nuclear hormone receptors), transcription factors have proven to be intractable drug targets thus far.[3−7]

Transcription factors are also difficult to regulate using genetic approaches. Transcription factor activity can be controlled using tetracycline (tet)-inducible systems, but this approach often results in high nonphysiologic levels of transcription factor activity and substantial basal levels of the tet-inducible protein.[8] Furthermore, tet-inducible regulation requires the incorporation of the tet-repressor in target cells and tissues. A small molecule-regulated intein splicing strategy to activate the transcription factor Gli1 was recently reported, but this approach requires substantial protein engineering to produce a transcription factor inactivated by the intein insert and has a slow activation time scale (12−24 h).[9,10] Other strategies to regulate transcription factor activity are similarly challenged by nonphysiologic levels of activity and/or the requirement for significant engineering of the transcription factor or target cell.[11,12] The difficulties associated with activating transcription factors at physiologic levels have significantly limited our ability to both explore the consequences of dynamic regulation of transcription factor activity in normal physiology and elucidate the therapeutic potential for transcription factor regulation to treat human disease.

We recently showed that conformational control of protein degradation allows dose-dependent control of the basic leucine zipper (bZIP) stress-responsive transcription factor ATF6.[13] In this approach, destabilized domains of mutant proteins (DDs) are fused to transcription factors that are constitutively expressed in cells.[14,15] The DD suppresses transcription factor activity by targeting the entire fusion to proteasomal degradation. This degradation can be dose-dependently inhibited by the addition of a small molecule DD pharmacologic chaperone that binds to the poorly populated, folded DD conformation, stabilizing and increasing intracellular concentrations of the entire fusion protein and thus facilitating transcription factor activation. Here, we explore the potentially broad applicability of this methodology to regulate the activity of transcription factors of multiple structural classes within the physiologically relevant regime.

We initially tested the generality of our approach for transcription factor regulation by fusing an FKBP12 DD[14] to a constitutively active version of the winged-helix transcription factor heat-shock factor 1 (FKBP.cHSF1) (Figure 1A). HSF1 is a stress-responsive transcription factor responsible for activating the cytosolic heat-shock response.[16] FKBP.cHSF1 should be rapidly degraded in untreated cells, whereas the addition of Shield-1, the FKBP12 small molecule pharmacologic chaperone, should stabilize FKBP.cHSF1 (Figure 1A). Consistent
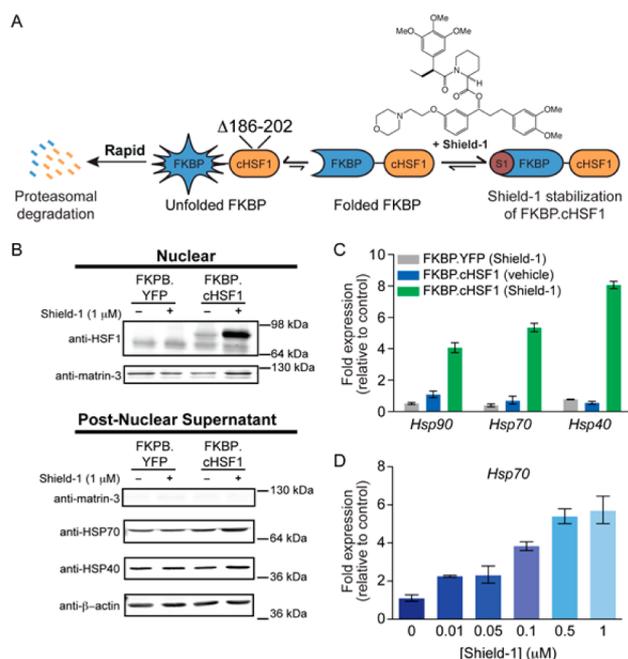
Figure 1. Application of DDs to regulate the activity of the winged-helix transcription factor HSF1. (A) Model showing the Shield-1-dependent regulation of the FKBP.cHSF1 fusion protein. The HSF1 sequence in this fusion protein lacks amino acids 186−202, which renders HSF1 constitutively active. The structure of Shield-1 is shown. (B) Immunoblot of nuclear and postnuclear extracts of HEK293T-REx cells expressing FKBP.YFP or FKBP.cHSF1. Shield-1 (1 μM) or vehicle was added to the indicated cells 18 h prior to harvest. (C) qPCR analysis of Hsp90, Hsp70, and Hsp40 in HEK293T-REx cells expressing FKBP.YFP or FKBP.cHSF1. Shield-1 (1 μM) or vehicle was added 18 h prior to harvest, as indicated. qPCR data are presented as fold-increase relative to vehicle-treated cells expressing FKBP.YFP. (D) qPCR analysis of Hsp70 in HEK293T-REx cells expressing FKBP.cHSF1 treated with increasing concentrations of Shield-1 for 18 h. qPCR data are presented as fold-increase relative to vehicle-treated controls.

with this prediction, we observe a robust, Shield-1-dependent stabilization of FKBP.cHSF1 in nuclear fractions isolated from HEK293T-REx cells expressing FKBP.cHSF1 (Figure 1B). Shield-1-dependent stabilization of FKBP.cHSF1 results in a significant induction of the cHSF1 target genes Hsp90, Hsp70 and Hsp40 (Figure 1C). Importantly, there is no induction of cHSF1 target genes in untreated cells expressing FKBP.cHSF1. Shield-1 also does not induce expression of these genes in cells transfected with our FKBP.YFP control plasmid, indicating that the increased expression requires cHSF1 transcriptional activity (Figure 1B,C). The induction of cHSF1 target genes upon administration of Shield-1 is rapid, demonstrating significant upregulation of Hsp70 mRNA levels in 3 h and reaching maximal induction in <16 h (Figure S1A in Supporting Information). Furthermore, Shield-1 activation of FKBP.cHSF1 allows dose-dependent control over both the levels of the FKBP.cHSF1 protein and the cHSF1 transcriptional program at a range of physiologically relevant levels (Figures 1D and S1B in Supporting Information).

We next evaluated whether DD-transcription factor fusions could similarly be applied to other structural classes of transcription factors. We fused a FKBP12 DD[14] C-terminal to the active, spliced XBP1s bZIP transcription factor (XBP1s.FKBP) − one of the transcription factors activated in
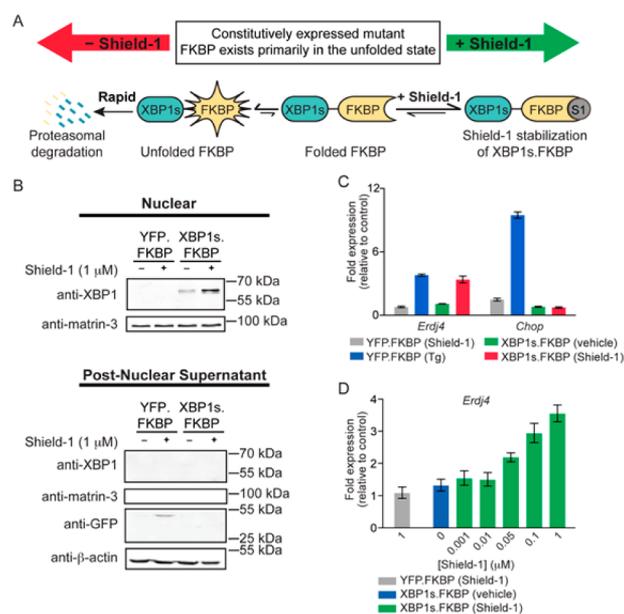
Figure 2. Development and characterization of a small molecule-regulated XBP1s bZIP transcription factor. (A) Model showing the Shield-1 mediated stabilization of XBP1s.FKBP. (B) Immunoblot of nuclear and postnuclear extracts isolated from HEK293T-REx cells expressing YFP.FKBP or XBP1s.FKBP. (C) qPCR analysis of ERdj4 and Chop in HEK293T-REx cells expressing YFP.FKBP or XBP1s.FKBP. Shield-1 (1 μM) or vehicle was added for 18 h prior to harvest. Cells treated with thapsigargin (Tg; 1 μM, 6 h) are shown as a control. qPCR results are presented as fold-increase relative to vehicle-treated cells expressing YFP.FKBP. (D) qPCR analysis of Erdj4 in HEK293T-REx cells expressing XBP1s.FKBP and treated with increasing doses of Shield-1 for 18 h. qPCR data are presented as fold-increase relative to vehicle-treated controls.

the canonical endoplasmic reticulum (ER) unfolded protein response (UPR) (Figure 2A).[17] The addition of Shield-1 to HEK293T-REx cells expressing XBP1s.FKBP stabilizes the entire fusion in nuclear fractions (Figure 2B). Shield-1-dependent stabilization of XBP1s.FKBP increases expression of the XBP1s target gene ERdj4[13] to levels consistent with those observed upon thapsigargin (Tg) treatment, which activates the global, endogenous UPR (Figure 2C). Importantly, we observe no basal induction of ERdj4 in untreated cells expressing XBP1s.FKBP. We also do not observe increased expression of the UPR-induced gene Chop, which is not a target of XBP1s.[13] Thus, Shield-1-dependent stabilization of XBP1s.FKBP increases ERdj4 expression selectively, rather than through stress-dependent, global UPR activation. Furthermore, we observe a Shield-1 dose-dependent increase in ERdj4 expression, demonstrating the capacity to sensitively regulate the XBP1s transcriptional program at physiologically relevant levels (Figure 2D). These results demonstrate that DDs can be applied to regulate the activity of multiple structural classes of transcription factors in cells.

Inhibiting transcription factor activity is also of interest. We evaluated the potential for DD-transcription factor fusions to inhibit transcriptional activity using a dominant negative construct of the UPR-associated transcription factor ATF6 (ATF6(bZIP)), a construct prepared by removing the ATF6 trans-activation domain, Figure 3A).[18] Stress-dependent global UPR activation activates ATF6, which induces gene expression through homodimerization and heterodimerization with XBP1s mediated through the ATF6 bZIP domain.[19] ATF6(bZIP) can
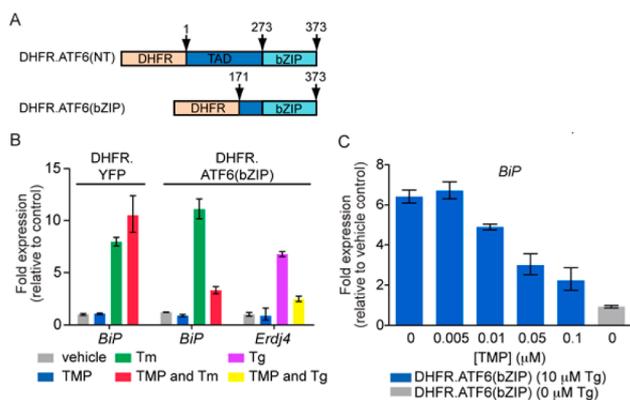
Figure 3. DD regulation of dominant negative ATF6. (A) Domain architecture of dominant negative DHFR.ATF6(bZIP). (B) qPCR of *BiP* and *ERdj4* in HEK293T-REx cells expressing DHFR.YFP or DHFR.ATF6(bZIP) pretreated for 15 h with TMP (10 μM) then challenged with either tunicamycin (Tm; 10 μg/mL; 6 h) or thapsigargin (Tg; 10 μM, 6 h). (C) qPCR of *Bip* in HEK293T-REx cells expressing DHFR.ATF6(bZIP) pretreated with increasing doses of TMP (15 h) then challenged with Tg (10 μM, 6 h).

dimerize with ATF6 or XBP1s and suppress stress-dependent upregulation of UPR target genes induced by either transcription factor.

We prepared a fusion between a DD version of bacterial dihydrofolate reductase (DHFR)[15] and ATF6(bZIP) (DHFR.ATF6(bZIP)). The DHFR DD is stabilized by the addition of the DHFR pharmacologic chaperone trimethoprim (TMP), which should increase levels of DHFR.ATF6(bZIP) and suppress stress-induced expression of XBP1s- and ATF6-selective UPR target genes (e.g., *Erdj4* and *BiP*, respectively[13]). HEK293T-REx cells expressing DHFR.ATF6(bZIP) show no significant impairment in the expression of the UPR target genes *BiP* and *ERdj4* in the absence of TMP (Figure 3B). The addition of the UPR activator tunicamycin (Tm) causes a similar induction of *BiP* in cells expressing DHFR.YFP or DHFR.ATF6(bZIP), indicating that DHFR.ATF6(bZIP) expression in the absence of TMP does not impair stress-dependent increases in UPR target gene expression. Preincubation of cells expressing DHFR.ATF6(bZIP) with TMP sharply attenuates the Tm- or Tg-dependent increase in *BiP* and *ERdj4* expression, respectively, demonstrating efficient TMP-dependent suppression of endogenous ATF6 and XBP1s activity. Furthermore, TMP dose-dependently suppresses Tg-dependent *BiP* expression in cells expressing DHFR.ATF6-(bZIP) (Figure 3C). Thus, these results demonstrate the capacity to sensitively suppress the activity of bZIP transcription factors using the DD approach.

The ability of both DHFR- and FKBP- transcription factor fusions to regulate transcription factor activity suggests that the DD approach could allow for the orthogonal, ligand-dependent regulation of two transcription factors in a single cell. To test this prediction, we transfected the previously reported, active DHFR.ATF6[13] into HEK293T-REx cells expressing FKBP.cHSF1 and monitored the ligand-dependent induction of ATF6 and/or cHSF1 target genes by qPCR (Figure 4A). In these cells, the addition of TMP (which stabilizes DHFR.ATF6) induces expression of ATF6 target genes, but not cHSF1 target genes (Figure 4B). Alternatively, the addition of Shield-1 (which stabilizes FKBP.cHSF1) induces expression of cHSF1 target genes but not ATF6 target genes. The addition
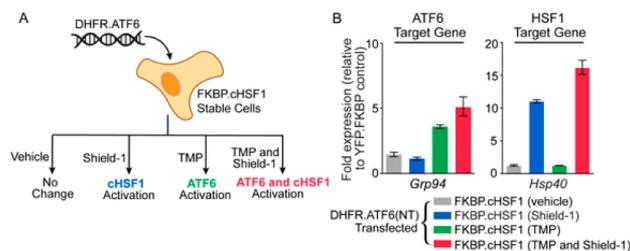
Figure 4. Dual regulation of two DD-transcription factor fusions in a single cell. (A) Illustration showing the incorporation of DHFR.ATF6 into HEK293T-REx cells expressing FKBP.cHSF1. (B) qPCR analyses of *Grp94* and *Hsp40* mRNA levels in cells expressing FKBP.cHSF1 and DHFR.ATF6 treated with Shield-1 (1 μM), TMP (10 μM) or both.

of Shield-1 and TMP induces expression of both sets of genes. No effects on gene transcription were observed in control HEK293T-REx cells expressing DHFR.YFP and YFP.FKBP (see Figure S2). Thus, we can sensitively and orthogonally regulate the activity of two transcription factors with small molecules in a single cell using the DD approach.

Our results demonstrate that DD-dependent regulation of transcription factors is a generally applicable strategy to develop and rapidly implement ligand-regulated transcription factors of multiple structural classes in cells. Furthermore, we show that the regulation of transcription factors using DDs allows for the dosable activation of transcriptional programs to physiologically relevant levels and can be used to control multiple transcription factors in a single cell. These DD-regulated transcription factors can be employed in any cell model system of interest.[13] This highly modular methodology for the DD-dependent regulation of transcription factors provides a general experimental approach that can be applied to explore the consequences of dynamically regulating transcription factor activity in normal physiology and to test the possibility that modulating transcription factor activity can be therapeutically beneficial to treat human disease. These objectives can now be accomplished without the formidable challenges associated with extensive reengineering of transcription factor sequences or identifying potent and selective small molecule modulators of a transcription factor of interest. Currently, we are employing the DD-regulated transcription factors described herein to explore the potential treatment of protein misfolding and aggregation diseases by activating stress-responsive transcription factors in disease models. Our strategy likewise enables research into many other important biologic processes dynamically regulated by diverse transcription factors, such as stem-cell differentiation and development.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information
Detailed experimental protocols for plasmid construction, cell culture, and drug treatment; qPCR analysis of gene expression. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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