A General Chemical Method to Regulate Protein Stability in the Mammalian Central Nervous System

Mari Iwamoto,1 Tomas Björklund,2 Cecilia Lundberg,3 Deniz Kirik,2 and Thomas J. Wandless1,*
1Department of Chemical and Systems Biology, 318 Campus Drive, Stanford University, Stanford, CA 94305, USA
2Brain Repair and Imaging in Neural Systems, Department of Experimental Medical Science, BMC D11, Lund University, 22184 Lund, Sweden
3CNS Gene Therapy Unit, Wallenberg Neuroscience Center, Department of Experimental, Medical Science, MBC A11, Lund University, 22184 Lund, Sweden
*Correspondence: wandless@stanford.edu
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SUMMARY

The ability to make specific perturbations to biological molecules in a cell or organism is a central experimental strategy in modern research biology. We have developed a general technique in which the stability of a specific protein is regulated by a cell-permeable small molecule. Mutants of the Escherichia coli dihydrofolate reductase (ecDHFR) were engineered to be degraded, and, when this destabilizing domain is fused to a protein of interest, its instability is conferred to the fused protein resulting in rapid degradation of the entire fusion protein. A small-molecule ligand trimethoprim (TMP) stabilizes the destabilizing domain in a rapid, reversible, and dose-dependent manner, and protein levels in the absence of TMP are barely detectable. The ability of TMP to cross the blood-brain barrier enables the tunable regulation of proteins expressed in the mammalian central nervous system.

INTRODUCTION

Numerous perturbation techniques have been developed to study gene function in biological systems. The Cre-Lox technology is a useful approach to inactivate genes in mice. Tissue- or cell-specific promoters can be used to drive Cre expression thus providing a degree of temporal and spatial control to the disruption of targeted genes (Gaveriaux-Ruff and Kieffer, 2007). However, this approach is not reversible, and identifying a suitable promoter can be difficult. Strategies to conditionally regulate the transcription of genes (e.g., the tetracycline-regulated transactivator) are widely used to control gene expression (Furth et al., 1994). Protein levels can also be regulated by targeting the mRNA transcripts using RNA interference (Xia et al., 2002). All of the techniques that target the precursor DNA or mRNA molecules encoding a protein of interest suffer from inevitable delays following the perturbation. Existing protein molecules must be degraded, so the experimental delay is dictated by the biological half-life of the protein under study.

Targeting proteins directly, rather than the DNA or mRNA molecules that encode them, is a more direct and rapid experimental strategy. In the past decade, a number of conditional protein regulation systems have been developed that utilize the cellular degradation machinery. In Varshavsky’s early system a temperature-sensitive mutant of mammalian DHFR (DHFR<sup>ts</sup>) bearing N-terminal arginine residue, a destabilizing residue by the N-end rule, was found to be stable at a permissive temperature of 23°C but unstable at 37°C (Dohmen et al., 1994; Lévy et al., 1999). Addition of methotrexate, a high-affinity ligand for mammalian DHFR, to cells expressing Arg-DHFR<sup>ts</sup> partially inhibited degradation of the protein (Lévy et al., 1999). This was an important demonstration that a small-molecule ligand can stabilize a protein otherwise targeted for degradation in cells.

In 2003, Crabtree and coworkers demonstrated the rapid and reversible regulation of the GSK-3β kinase fused to an unstable triple mutant of the FRB domain (FRB<sup>b</sup>) using the rapamycin-derivative C20-MaRap (Stankunas et al., 2003). MaRap induces a heterodimerization event between the FRB<sup>b</sup>-GSK3β fusion and ubiquitously expressed FKBP12 to stabilize the FRB<sup>b</sup> fusion and restore the function of the fused kinase. The FRB<sup>b</sup>-GSK3β was inducibly stabilized in cells as well as in embryos in vivo (Liu et al., 2007). This system demonstrated that ligand-dependent stability represented an attractive strategy to regulate the function of a specific protein in a complex biological environment.

We previously developed a strategy in which a cell-permeable ligand is used in conjugation with a single genetically encoded domain to regulate any protein of interest. A small domain can be fused to any gene, and a fusion protein is produced as a result of this specific genetic perturbation. Mutants of the human FKBP12 protein were engineered to be metabolically unstable in the absence of its high-affinity ligand, Shield-1 (Banaszynski et al., 2006). We call these mutants destabilizing domains (DDs) and observe that the instability of a DD conferred to any fused partner protein results in degradation of the entire fusion protein by the proteasome. Shield-1 binds to and stabilizes the DD in a dose-dependent manner. The genetic fusion of the DD to the gene of interest ensures specificity, and small-molecule control confers reversibility and dose dependence to protein stability and function.

Building on our experiences with the FKBP-derived DDs, we chose E. coli dihydrofolate reductase (ecDHFR) as a candidate protein to engineer a second destabilizing domain with improved properties. The 159-residue enzyme catalyzes the reduction of dihydrofolate to tetrahydrofolate, a cofactor that is essential for several steps in prokaryotic primary metabolism (Schnell et al., 2004). Numerous inhibitors of DHFR have been developed as drugs (Schweitzer et al., 1990), and one such inhibitor,
trimethoprim (TMP), inhibits ecDHFR much more potently than mammalian DHFR (Matthews et al., 1985). This large therapeutic window renders TMP “biologically silent” in mammalian cells. The specificity of the ecDHFR-TMP interaction, coupled with the commercial availability and attractive pharmacological properties of TMP, makes this protein-ligand pair ideal for development as a DD system as presaged in another context by Varshavsky (Dohmen et al., 1994; Johnston et al., 1995; Lévy et al., 1999).

RESULTS

Screening Strategies to Engineer DDs
To engineer mutants that display ligand-dependent stability, we designed a cell-based screen using yellow fluorescent protein (YFP) as a reporter for ecDHFR stability. The strategy was designed to identify mutants possessing the desired characteristics of a destabilizing domain: low protein levels in the absence of TMP (i.e., low basal stability), large dynamic range, robust and predictable dose-response behavior, and rapid kinetics of degradation. We used error-prone PCR (Atwood-Moore et al., 2005; Zaccolo et al., 1996) to generate libraries of ecDHFR mutants based on the parental sequence (see Figures S1A–S1D available online), as well as two-point mutants (Y100I and G121V) that are known to compromise enzymatic activity (Adams et al., 1991; Cameron and Ben-G121V) as well as YFP fluorescence expression. This population was then treated with 1 or 0.1 \( \mu M \) TMP, where we selected cells that exhibited low levels of YFP fluorescence. The majority of mutants reach basal fluorescence levels within 4 hr, as anticipated from the final step of the screening strategy (Figures S1J and S1K). To investigate the performance of proteolytic activity, cells stably transduced with either the R12Y/Y100I-YFP or YFP-N18T/A19V fusion proteins were cultured in the presence or absence of 10 \( \mu M \) TMP. Cells were also treated with inhibitors of either proteasomal- or lysosome-mediated degradation (Reusch et al., 1999). Only cells treated with the proteasome inhibitor MG132 exhibited reduced degradation of DD-YFP fusion protein upon switching to nonpermissive conditions, suggesting that degradation is mediated by proteasome (Figure S1L).

We have thus far demonstrated that ecDHFR-derived DDs destabilize YFP when fused to the N terminus for which they were screened and identified. In situations where proteins may not functionally tolerate N- or C-terminal fusions, it would be preferable to have destabilizing domains that can regulate protein levels when spliced into a tolerant domain within the protein of interest. To investigate whether the ecDHFR-derived DDs could confer instability in this context, we spliced the sequences for either the R12Y/Y100I-YFP or the N18T/A19V DD between the codons encoding residues 157 and 158 of the YFP gene, which is a site within YFP known to tolerate insertions (Magliery et al., 2005). To accommodate the folding requirements of these globular proteins we tethered the N and C termini of the ecDHFR domain to the YFP splice site using linkers varying from 2 to 19 residues on either side of the ecDHFR domain. The internal R12Y/Y100I domain dramatically decreased YFP fluorescence levels in the absence of TMP, and YFP levels were stabilized upon addition of TMP (Figure 1C). Although the N18T/A19V DD is strongly destabilizing when fused to the C terminus of YFP, this mutant was not as effective as the R12Y/Y100I mutant when spliced internally (Figure S1M).

FKBP- and ecDHFR-Derived DDs Are Tunably and Predictably Regulated by Their Respective Ligands
One of the goals of this study was to develop a DD system that, when used in combination with the FKBP-derived DD system, would allow for the tuning of protein turnover in a ligand-dependent manner. We have demonstrated that the ecDHFR-derived DDs, when fused to the N terminus of YFP, are fully stabilized by 1 \( \mu M \) TMP and display improved dynamic range. The R98H/F103S mutant is stabilized 100-fold upon treatment with TMP compared to a 31-fold increase in YFP levels for the Y100I mutant (Figure 1A). The enhanced dynamic range is largely the result of more effective destabilization under nonpermissive conditions. The C-terminal DD screen produced mutants with wide dynamic ranges of YFP expression (between 63- and 93-fold) that are stabilized by lower concentrations of TMP (Figure 1B). All of the DDs caused drastic destabilization of the YFP protein in the absence of TMP. YFP fluorescence, measured by analytical flow cytometry, was barely above the autofluorescence of untransduced NIH 3T3 cells (Figure S1G).

We next investigated the kinetics of destabilized domain rescue and degradation. YFP fluorescence levels of each of the DDs increased at a similar rate upon treatment with TMP (Figures S1H and S1I). To measure the rates of protein degradation upon switching to nonpermissive conditions, mutants were stabilized with TMP for 24 hr at which time TMP was removed from the culture media and cells were assayed for YFP fluorescence. The majority of mutants reach basal fluorescence levels within 4 hr, as anticipated from the final step of the screening strategy (Figures S1J and S1K). To investigate the performance of proteolytic activity, cells stably transduced with either the R12Y/Y100I-YFP or YFP-N18T/A19V fusion proteins were cultured in the presence or absence of 10 \( \mu M \) TMP. Cells were also treated with inhibitors of either proteasomal- or lysosome-mediated degradation (Reusch et al., 1999). Only cells treated with the proteasome inhibitor MG132 exhibited reduced degradation of DD-YFP fusion protein upon switching to nonpermissive conditions, suggesting that degradation is mediated by proteasome (Figure S1L).
A previous attempt to regulate CD8α, a transmembrane glycoprotein found on the surface of T cells, resulted in modest levels of regulation at the cell surface (Banaszynski et al., 2006). Because N18T/A19V and R12Y/Y100I ecDHFR are both more potent DDs when fused to the C terminus of YFP, we suspected that the ecDHFR-derived DDs might offer improved regulation of CD8α expression. To test this theory, we fused either the N18T/A19V or R12Y/Y100I DD to the C terminus of CD8α and transduced these fusions into NIH 3T3 cells. Flow cytometry was used to quantitate levels of CD8α at the cell surface, where we observed a significant decrease in CD8α expression in the absence of TMP. Upon treatment of cells with 10 μM TMP for 24 hr, the N18T/A19V fusion showed higher expression of CD8α, and therefore greater dynamic range, than the R12Y/Y100I fusion (Figure 3A). TMP-mediated stabilization for 24 hr followed by withdrawal of the ligand for 24 hr (+/−; in Figure 3A) produced protein levels nearly as low as cells maintained under nonpermissive conditions. Encouraged by these results, we tested the ability of these DDs to destabilize a more structurally complex transmembrane protein, the β2-adrenergic receptor (β2AR) (Cherezov et al., 2007), which is a member of the GPCR family of integral membrane proteins. Cells were stably transduced with either the N18T/A19V or R12Y/Y100I DDs fused to the 3′-end of the β2AR gene, and we observed low levels of β2AR expression in the absence of TMP. Addition of TMP caused protein levels to increase, and TMP-mediated stabilization for 24 hr followed by 24 hr under TMP-free nonpermissive conditions returned β2AR levels to the low background level (Figure 3B).

### Regulation of Transmembrane Proteins

The commercial availability, oral bioavailability, and low cost of TMP make this ecDHFR-derived DD system particularly appealing for the regulation of transmembrane proteins in vivo. While the current studies were performed in NIH 3T3 cells, the ecDHFR-Derived DD system is expected to be applicable to a wider range of cell types, including primary cells and human primary tissues. In conclusion, we have demonstrated that the ecDHFR-Derived DD system is a versatile tool for the orthogonal and independent regulation of two proteins of interest.
responsive integral transmembrane protein, human 2AR, a G protein-coupled receptor, as a significant amount of protein is trafficked to the membrane in the absence of Shield-1 (Banaszynski et al., 2006). When we fuse DHFR-N18T/A19V domain to CD8a, we observe far greater destabilization of CD8a relative to the L106P fusion. Encouraged by this result, we applied the DD technology to the more structurally complex and therapeutically relevant integral transmembrane protein, human β2AR, a G protein-coupled receptor (GPCR). TMP-dependent stability of β2AR was achieved with a 10-fold increase in C24/C24/2AR-DD expression upon addition of the ligand TMP. Characterization of the ecDHFR-derived DDs revealed that the DDs behave very similarly to the FKBP-derived DDs, and the two DD systems work orthogonally as demonstrated in mammalian cells. However, there are notable differences between the two systems. The C-terminal ecDHFR-derived DDs are much more effective at destabilizing the fusion protein than the FKBP-derived L106P DD. Whereas the destabilizing effects of the L106P domain are reduced when fused at the C terminus of YFP, the ecDHFR-derived DDs F103L and N18T/A19V are very successful at reducing the protein levels when fused to the C terminus of YFP, with negligible protein levels observed in the absence of TMP. Fusion of the FKBP-derived L106P DD to the C terminus of a single-transmembrane protein CD8a is only modestly destabilizing, as a significant amount of protein is trafficked to the membrane in the absence of Shield-1 (Banaszynski et al., 2006). When we fuse DHFR N18T/A19V domain to CD8a, we observe far greater destabilization of CD8a relative to the L106P fusion. Encouraged by this result, we applied the DD technology to the more structurally complex and therapeutically relevant integral transmembrane protein, human β2AR, a G protein-coupled receptor (GPCR). TMP-dependent stability of β2AR was achieved with a 10-fold increase in β2AR-DD expression upon addition of the ligand. GPCRs represent an important class of drug targets for the pharmaceutical industry and currently 30% of marketed drugs act on this family of proteins (Hopkins and Groom, 2002). GPCRs play key roles in biological processes such as cellular metabolism, cell growth,
inflammation, and neuronal signaling. Extensive research has taken place in the past decades to understand the function and pathways involved with these membrane proteins. These new ecDHFR-derived DDs may prove to be important tools for probing the functional roles of this large and therapeutically important family of cell surface receptors.

Another important advantage of using the ecDHFR-derived DD system is that the ligand is commercially available, inexpensive, and possesses good pharmacological characteristics. The drug can be administered orally, a less invasive and more convenient method of drug administration than IP injection or IV administration. TMP-dosed rats presented no adverse effects such as loss of body weight or diarrhea during this study, even when dosed at 0.4 mg/ml (twice the dose used to regulate transgenes) for 6 weeks (data not shown). TMP crosses the blood-brain barrier (Barling and Selkon, 1978), allowing the conditional regulation of specific proteins within the central nervous system of living rodents. TMP also passes the placental barrier (Schulz, 1972), with teratogenicity observed only when rats are given very high concentrations of TMP. Many genes misregulated in diseases such as cancer exhibit embryonic lethal phenotypes in knockout mouse models. For example, the disruption of genes involved in angiogenesis such as vascular endothelial growth factor or vascular endothelial receptor tyrosine phosphatase results in vascular defects, leading to embryonic lethality (Carmeliet et al., 1996; Dominguez et al., 2007) and precluding study of the roles of these proteins in tumor angiogenesis. The DHFR-derived DD system may be a suitable alternative for in vivo regulation of proteins that are required during embryonic development. Pregnant mice carrying fetuses encoding genes targeted with the ecDHFR-derived DD can be administered TMP during gestation to maintain protein levels necessary for proper development of the embryos.

**Figure 3. Regulation of Transmembrane Proteins**

(A) The ecDHFR mutants N18T/A19V and R12Y/Y100I were fused to the 3' end of the CD8a gene and were stably transduced into NIH 3T3 cells. Cells were divided into three pools, and the first population (−) was mock-treated, and the second population (+) was treated with 10 μM TMP for 24 hr. The third population (+/−) was treated with 10 μM TMP for 24 hr and then washed with media and cultured for 24 hr in the absence of TMP. Live cells were probed with a FITC-conjugated anti-CD8a antibody and assayed by flow cytometry. Data are presented as the average MFI ±SD and normalized against the average MFI of untransduced NIH 3T3 cells that were also stained with antibody.

(B) The N18T/A19V and R12Y/Y100I DDs were fused to the 3' end of the β2AR gene and these fusions were stably expressed in NIH 3T3 cells. As a positive control, unfused β2AR was stably expressed and analyzed alongside both fusions. The cells were divided into three pools as described in (A) and analyzed by flow cytometry. All β2AR genes encoded an N-terminal FLAG epitope. Live cells were probed with Alexa Fluor 488-labeled M1 anti-FLAG antibody, which only recognizes the epitope expressed at the cell surface.

**Figure 4. Regulating DD-YFP and YFP-DD in Rat Brain**

Adult rats received an injection of recombinant lentivirus (rLV) encoding R12Y/Y100I-YFP in the left striatum and YFP-N18T/A19V in the right striatum. Immunohistochemistry of rat brain tissue 3 weeks post rLV injection in animals that received (A) no TMP and (B) 0.2 mg/ml TMP in drinking water. rLV-ecDHFR R12Y/Y100I-YFP with (C) no TMP (D) 0.2 mg/ml TMP. The 40 μm coronal slices were probed with GFP antibody, followed by incubation in biotinylated secondary antibody. The tissues were visualized with avidin-biotin-peroxidase complex using 3,3-diaminobenzidine as chromogen. Scale bar in (A) represents 100 μm, and insets represent 20 μm.

Another important advantage of using the ecDHFR-derived DD system is that the ligand is commercially available, inexpensive, and possesses good pharmacological characteristics. The drug can be administered orally, a less invasive and more convenient method of drug administration than IP injection or IV administration. TMP-dosed rats presented no adverse effects such as loss of body weight or diarrhea during this study, even when dosed at 0.4 mg/ml (twice the dose used to regulate transgenes) for 6 weeks (data not shown). TMP crosses the blood-brain barrier (Barling and Selkon, 1978), allowing the conditional regulation of specific proteins within the central nervous system of living rodents. TMP also passes the placental barrier (Schulz, 1972), with teratogenicity observed only when rats are given very high concentrations of TMP. Many genes misregulated in diseases such as cancer exhibit embryonic lethal phenotypes in knockout mouse models. For example, the disruption of genes involved in angiogenesis such as vascular endothelial growth factor or vascular endothelial receptor tyrosine phosphatase results in vascular defects, leading to embryonic lethality (Carmeliet et al., 1996; Dominguez et al., 2007) and precluding study of the roles of these proteins in tumor angiogenesis. The DHFR-derived DD system may be a suitable alternative for in vivo regulation of proteins that are required during embryonic development. Pregnant mice carrying fetuses encoding genes targeted with the ecDHFR-derived DD can be administered TMP during gestation to maintain protein levels necessary for proper development of the embryos.
The rapid switch-like behavior of the DD system makes it a powerful conditional perturbation method, and the ability to tunably regulate protein levels with a high degree of predictability makes the destabilizing domains valuable in broader contexts. For instance, a knowledge of the proteins comprising a signaling network is necessary but not sufficient for a full understanding of the behavior of the system. Researchers can utilize the DD systems to study how molecules comprising a signaling network are “wired” together through intermolecular interactions or diffusible second messengers by perturbing one member of a circuit and observing the effects of this perturbation on the other members of the circuit. This form of sensitivity analysis, performed on the proteins of an intact signaling circuit in a living cell, will enable researchers to deconvolute the interactions between signaling proteins and will ultimately lead to quantitative models of the signaling circuit that can both explain as well as predict the consequences of perturbations.

Thus far, all of the systems described above operate in a way where the protein level is maintained in the presence of ligand. Recently a conditional protein stability technology was developed by which proteins are rapidly depleted in the presence of a protein of interest, its instability is conferred to the fused partner protein. This system represents a promising approach for using a ligand to destabilize (rather than stabilize) targeted proteins, and obviates the need to continuously administer the ligand until the desired experimental window. Depending on the system used the cost of maintenance dosing could be prohibitive, not to mention the effect of long-term administration of ligand to the animals. While the auxin-based system has the advantage of lacking off-target effects in mammalian cells, this system requires two genetic elements rather than one: researchers must introduce a transgene encoding the F-box protein, TIR1, as well as a second gene encoding the Aid-tagged gene of interest. A system requiring only a single genetic manipulation would be more desirable. Ideally, fusion of a single DD to a targeted protein would confer ligand-dependent stability, but in the opposite sense of the current DDs.

SIGNIFICANCE

We have developed a general method to regulate stability of a specific protein in a rapid, reversible, and tunable manner using a commercially available small molecule. Mutants of E. coli dihydrofolate reductase (ecDHFR) protein were engineered to be degraded when expressed in mammalian cells. When this destabilizing domain (DD) is fused to a protein of interest, its instability is conferred to the fused protein, resulting in rapid degradation of the entire fusion protein by the proteasome. Trimethoprim (TMP) is a high-affinity ligand for ecDHFR that stabilizes fusion proteins in a dose-dependent manner, and protein levels in the absence of TMP are negligible. This ecDHFR-TMP DD system works orthogonally to the existing FKBP/Shld1-based DD system, allowing simultaneous regulation of two proteins independently. The ecDHFR-DD system successfully regulates transmembrane proteins that were difficult to target with the FKBP-DD, such as a GPCR protein, beta2-adrenergic receptor. We have shown a successful regulation of transgenes expressed in rat brain by oral administration of TMP. The ability of TMP to cross the blood-brain barrier (BBB) enables the tunable regulation of proteins expressed in cells found within the mammalian central nervous system. Finally, TMP passes the placental barrier, possibly allowing tunable regulation of genes essential in embryonic development. The DHFR-derived DD system may prove useful in dissecting the role of genes involved in cancer and other diseases in vivo that previously were considered difficult due to essentiality of the genes in embryonic development. Controlling protein function directly using a small-molecule ligand is a more attractive approach than targeting precursor DNA or mRNA, as direct targeting of protein is rapid and is not limited by the intrinsic half-life of the targeted proteins.

EXPERIMENTAL PROCEDURES

ecDHFR Library Generation

Diversity in the ecDHFR sequence was generated using a combination of error-prone PCR (Atwood-Moore et al., 2005) and nucleotide analog mutagenesis (Zaccolo et al., 1996). Three ecDHFR genes were used as templates: parental, Y100I, and G121V (see Figure S1 for a description of the parental sequence). For each of these three templates, three mutagenesis conditions were used to generate diversity. Condition set A utilized the nonnatural nucleotides 8-oxo-dGTP and dPTP to encourage nucleotide misincorporation. Condition set B utilized 4 ng template, 0.5 μM of each oligonucleotide primer, 3.75 units of Taq polymerase, 5 mM MgCl₂, 0.2 mM MnCl₂, 0.4 mM dNTPs, and an excess 0.2 mM of dATP and dCTP. Condition set C was identical to B, but dGTP and dTTP were used in excess instead of dATP and dCTP.

Library Screening and Validation

Calculated library diversity was 3 x 10⁷ members for the ecDHFR-YFP library and 1.5 x 10⁸ members for the YFP-ecDHFR library. Screening was performed as described in the text. Upon completion of the fourth sort the cells were allowed to recover in media, and genomic DNA was obtained using QIAGEN DNeasy kit. To isolate individual ecDHFR mutants from the pool of genomic DNA, the ecDHFR gene was PCR-amplified using primers that annealed to regions just outside the 5’ and 3’ end of the ecDHFR gene. PCR products were then subcloned into the pB BMN YFP plasmids and transformed into E. coli Top10 cells. For the N-terminal ecDHFR-DD library, 48 randomly chosen clones were further characterized in mammalian cells. The plasmid DNA was isolated from bacteria and stably transduced into NIH 3T3 cells using the retroviral expression system described below. The cells were dosed with solvent or 10 μM TMP (1:1000 dilution of 10 mM stock in DMSO), and only the cells that displayed low YFP fluorescence in the absence of TMP and high levels of YFP expression in the presence of TMP were sequenced. Thirty-six clones were similarly isolated and validated from the C-terminal DD library. Sequence information is provided in Tables S1 and S2.

Cell Culture, Transfections, and Transductions

The NIH 3T3 cell line was cultured in DMEM supplemented with 10% donor bovine serum (Invitrogen), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. All stable cell lines were created using the Phoenix ecotropic packaging cell line that was transfected using a standard lipofectamine 2000 protocol. Viral supernatants were harvested 48 hr post-transfection, filtered, and supplemented with 4 μg/ml polybrene. NIH 3T3 cells were incubated with viral supernatant for 4 hr at 37°C. Cells were cultured in growth media for 48 hr to allow for viral integration and then assayed as
described. For fluorescence decay kinetics experiments, TMP was removed from the cells by first harvesting the cells with trypsin and then washing TMP from the cells by repeated centrifugation, aspiration, and resuspension in TMP-free media. Alternatively, washing adherent cells once with TMP-free media followed by incubating cells in media “conditioned” with bacterially expressed wild-type ecDHFR protein at 5 μM concentration is even more effective for switching to nonpermissive conditions.

Flow Cytometry

Twenty-four hours prior to analysis, transduced NIH 3T3 cells were plated and treated with TMP as described. For cell lines expressing fluorescent proteins, cells were removed from the plate using trypsin and resuspended in growth media to avoid aggregation of the cells. Transmembrane protein expressing cell lines were removed from the plate using 2 mM EDTA in 1 x PBS, then treated with fluorescently labeled antibody prior to analysis. Cells were analyzed at the Stanford Shared FACS Facility with 10,000 events represented.

Imaging

Tunability experiment was conducted on Zeiss Axioskop 2 epifluorescence microscope equipped with a QICAM FAST 1394 digital CCD camera.

Rat Models

Lentiviral vectors were constructed using Gateway technology (Invitrogen). The human CMV promoter was placed upstream of the appropriate fusion gene in the lentiviral backbone 2k7neo (Suter et al., 2006). The vectors were produced and then titered using quantitative PCR as described previously (Nielsen et al., 2009). Adult female Sprague-Dawley rats were injected with lentivirus (6 × 10⁷ TU in 2 μl vehicle) was injected into each striatum of adult (225 g) rats (n = 6). rLV encoding the R12Y/Y100I-YFP fusion was injected into the left striatum and rLV encoding YFP-N18T/A19V was injected into the right striatum. The injections were performed in two 1 μl deposits along a single injection tract at the coordinates: anteroposterior = +0.5 mm; mediolateral = ±3.0 mm relative to bregma and dorsoventral = −5.0 and −4.0 mm, calculated from the dorsal surface. The injection flow rate was 0.4 μL/min and the glass capillary was kept in place for 1 min between deposits and 3 min after the second deposit before retraction. Rats were housed at two or three per cage under a 12 hr light:dark cycle with ad libitum access to food and water. Half of the animals received TMP in the drinking water (TMP emulsion 10 mg/ml, with a final concentration of 0.2 mg/ml in water) for 3 weeks, upon which animals were sacrificed by sodium pentobarbital euthanasia and transcardially perfused with paraformaldehyde.

Immunohistochemistry

The fixed brain tissue was cut by freezing microtome into 40 μm coronal slices for free floating immunohistochemical staining for YFP expression. The sections were quenched in 3% H₂O₂ and 10% methanol, preincubated in 5% goat serum, and incubated overnight at room temperature with polyclonal antibody that cross-reacts with YFP.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and one table and can be found at doi:10.1016/j.chembiol.2010.07.009.

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