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Targeted Chemical-Genetic Regulation of Protein Stability In Vivo

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SUMMARY

Loss- and gain-of-function transgenic models are powerful tools for understanding gene function in vivo but are limited in their ability to determine relative protein requirements. To determine cell-specific, temporal, or dose requirements of complex pathways, new methodology is needed. This is particularly important for deconstructing metabolic pathways that are highly interdependent and cross-regulated. We have combined mouse conditional transgenics and synthetic posttranslational protein stabilization to produce a broadly applicable strategy to regulate protein and pathway function in a cell-autonomous manner in vivo. Here, we show how a targeted chemical-genetic strategy can be used to alter fatty acid metabolism in a reombination and small-molecule-dependent manner in live behaving transgenic mice. This provides a practical, specific, and reversible means of manipulating metabolic pathways in adult mice to provide biological insight.

INTRODUCTION

Metabolic pathways are highly interconnected and interdependent. They also exhibit remarkable plasticity that can be easily mistaken for or complemented by redundancy. Understanding these complexities requires specific temporal and reversible pathway perturbation. This is often done using rather non-specific pharmacology. Ideally, one could use small molecules to selectively and potently alter enzyme activity in an allele-specific manner by means that would require little knowledge of the reaction mechanism. The technique would therefore be straightforward and broadly applicable and would occur posttranslationally.

There is great interest in the development of small molecules that regulate the function of user-engineered proteins (Bishop et al., 2000; Haruki et al., 2008; Matsuzawa et al., 2005; McGinness et al., 2006; Robinson et al., 2010), particularly in the context of intact organisms (Chen et al., 2005; Karpova et al., 2005; Liu et al., 2007; Stankunas et al., 2003). Recently, there has been considerable progress in the development of small stabilizing or destabilizing protein domains that interact with well-defined inert small molecules (Banaszynski et al., 2006, 2008; Bonger et al., 2011; Iwamoto et al., 2010; Kanke et al., 2011; Nishimura et al., 2009). Natural or synthetic small-molecule ligands are chosen that interact with specific proteins to either promote or inhibit their rapid posttranslational degradation (Janse et al., 2004). Fusion of a protein of interest to these domains creates a small-molecule-regulated protein whose function is dose-dependent and reversible. Here, we have combined ligand-inducible protein stabilization with genetically tractable recombination-mediated transgene expression to enable the targeted small-molecule regulation of enzyme activity in live mice.

In order to utilize this strategy to study metabolites in vivo, we developed a transgenic vector that allows the small-molecule regulation of malonyl-CoA decarboxylase (MLYCD) after Cre-mediated recombination. MLYCD was chosen because its substrate, malonyl-CoA, represents a central metabolic node in fatty acid biochemistry. Malonyl-CoA is the product of the rate-setting step in de novo fatty acid metabolism, is the substrate for fatty acid synthesis and elongation, and allosterically inhibits the rate-setting step in fatty acid beta-oxidation (Wolfgang and Lane, 2006). Therefore, exerting control over malonyl-CoA through MLYCD has broad experimental utility. This approach is not limited to MLYCD but is broadly applicable to other enzymes and genes.

RESULTS

Design of a Small-Molecule-Regulated Malonyl-CoA Decarboxylase

In order to produce dose-dependent, small-molecule-regulated posttranslational stabilization, Wandless et al. constructed a modified FK506 binding protein 12 (FKBP12) that binds to and is reversibly stabilized by a synthetic, biologically inert small molecule, Shield-1 (Banaszynski et al., 2006, 2008; Maynard-Smith et al., 2007). We made several modifications to the FKBP12-based destabilization vectors. A picornavirus PTV1-2A peptide-linked mCherry was fused in-frame after an FKBP-eYFP destabilization fusion protein and then cloned into an MMLV-based selectable retroviral vector (Figure 1A). The 2A peptide allows two proteins to be produced from a single mRNA at a 1:1 stoichiometric ratio, enabling a ratiometric, autonomous measurement of stabilization and localization within cells (Provost et al., 2007; Szymczak et al., 2004). Malonyl-CoA decarboxylase (MLYCD) was then cloned as a C-terminal fusion with FKBP-YFP flanked by two short flexible linkers. Fusion of the mutant FKBP12 to YFP linked to MLYCD allows real-time visualization of Shield-1-induced stabilization. The N-terminal mitochondrial localization domain and C-terminal peroxisomal

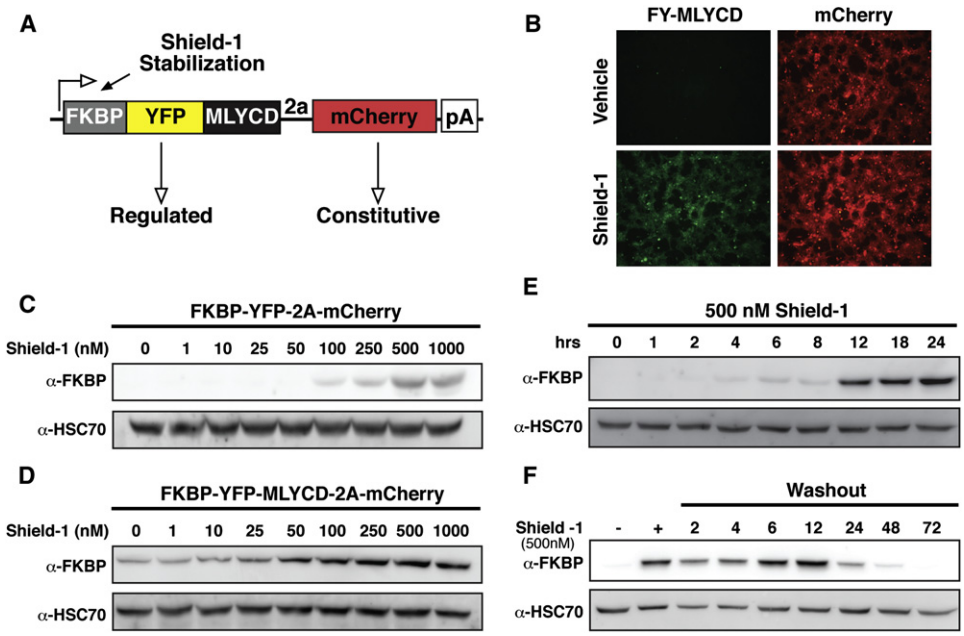

Figure 1. Small-Molecule-Induced Enzyme Stabilization

(A) An FKBP12 destabilization domain that is inducibly stabilized by the small molecule Shield-1 was fused to eYFP and malonyl-CoA decarboxylase (MLYCD). The inducible MLYCE is linked to a constitutively expressed mCherry via a bicistronic viral PTV1-2A peptide.

(B) Epifluorescent images of Shield-1-stabilized MLYCD and constitutive expression of mCherry in HEK293T cells.

(C and D) Western blots of dose-dependent Shield-1-induced stabilization of control (C) and MLYCD (D) HEK293T cells.

(E and F) Time course (E) and washout (F) of stabilization in HEK293T cells.

targeting sequences were removed from MLYCD to facilitate cytoplasmic MLYCD activity (An et al., 2004). Two stable HEK293T stable cell lines were produced that express FKBP12-eYFP-MLYCD-2a-mCherry (FY-MLYCD) or FKBP12-eYFP-2a-mCherry (FYFP) to conduct an initial characterization of this system in vitro. Shield-1 was synthesized as previously reported (Banaszynski et al., 2006; Yang et al., 2000).

In order to verify that the stable cells expressed mCherry constitutively and eYFP in a Shield-1-dependent manner (Figures 1A–1D), stable cell lines were treated with Shield-1 for 16 hr. Addition of Shield-1 induced FYFP and FY-MLYCD in a dose-dependent manner (Figures 1C and 1D). To determine the kinetics of stabilization, stable cells were incubated with Shield-1 and collected at the designated time points. Saturation of stability was achieved 12 hr posttreatment (Figure 1E). Likewise, we conducted a washout experiment to determine the kinetics of destabilization. Stable cells returned to basal stabilization levels 48 hr after Shield-1 removal (Figure 1F). These data show that Shield-1 induced MLYCD stabilization in a dose-dependent and reversible manner.

To demonstrate that the FY-MLYCD fusion protein was enzymatically active, we treated FY-MLYCD stable cells with Shield-1 or vehicle control for 16 hr. Indeed, Shield-1 induced an ~4-fold increase in MLYCD activity (Figure 2A). In a second experiment, increasing amounts of Shield-1 were added to FY-MLYCD cells and then the cells were assayed for ^{3}H-acetate incorporation into lipids, a measure of de novo fatty acid synthesis. Addition of Shield-1 resulted in a dose-dependent suppression of de novo fatty acid synthesis, demonstrating that FY-MLYCD was enzymatically active and its activity was regulated by Shield-1 in a dose-dependent manner (Figure 2B). These experiments show that the fusion of the mutant FKBP12 to MLYCD maintained an active enzyme that can be regulated by Shield-1.

Dual Chemical and Genetic Regulation of Fatty Acid Metabolism In Vivo

Shield-1 has been shown previously to be active when injected into mice, either intravenously or intraperitoneally, in a tumor xenograft model to regulate secreted fusion proteins in vivo

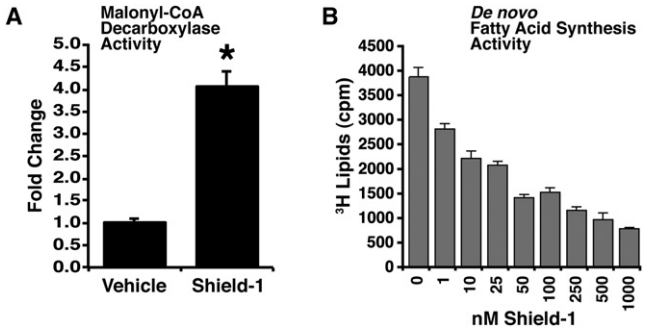

Figure 2. Shield-1-Regulated Malonyl-CoA Decarboxylase Activity

(A) Shield-1 induces MLYCD activity.

(B) Shield-1 suppresses de novo fatty acid synthesis in a dose-dependent manner as measured by ^{3}H-acetate incorporation into lipids.

Error bars represent the SEM.

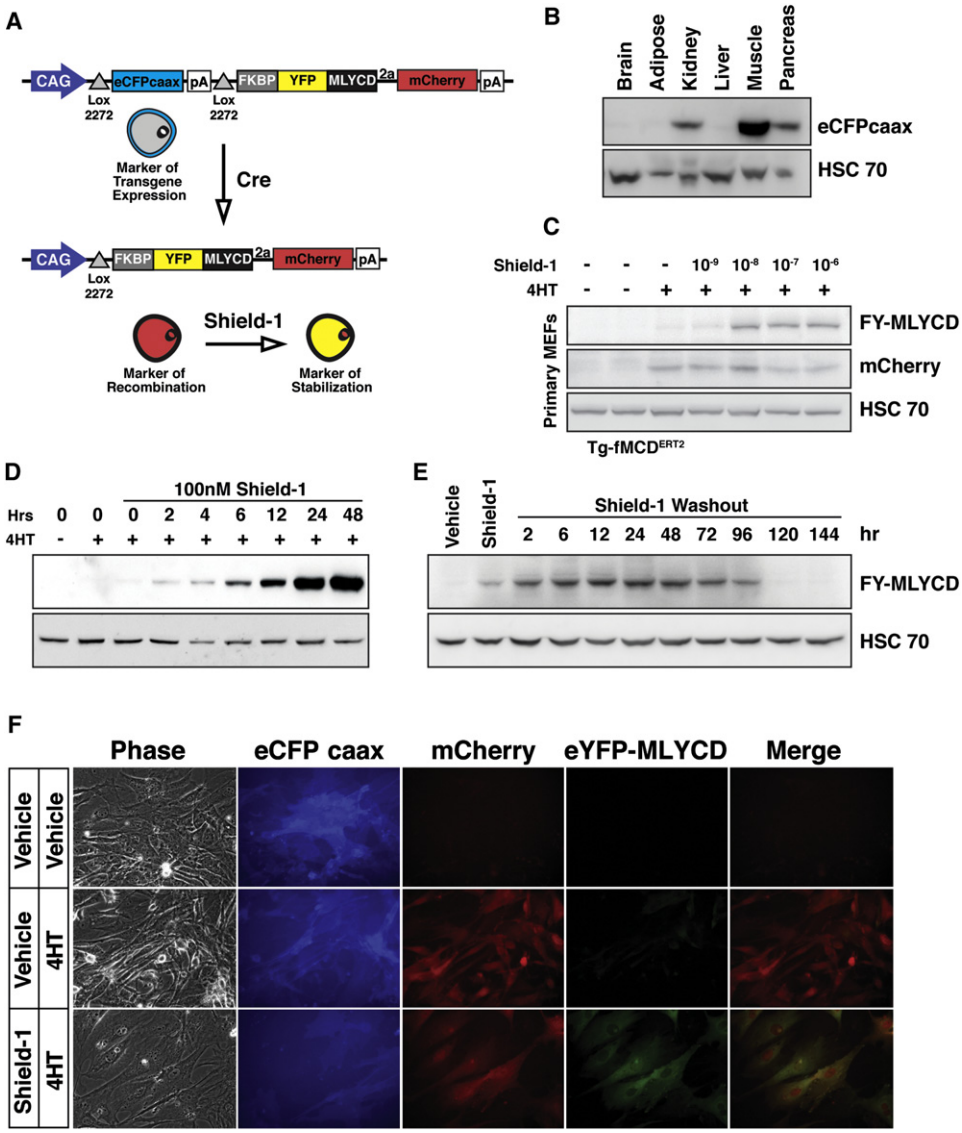

Figure 3. Dual Chemical Regulation of Protein Stability

(A) The destabilization cassette was cloned downstream of a Lox2272 eCFPcaax Stop cassette driven by the ubiquitous CMV-enhanced chicken beta actin promoter (CAG). Cre-mediated recombination between the Lox sites excises the eCFPcaax Stop cassette and expresses the FKBP12-YFP-MLYCD-2A-mCherry cassette.

(B) Differential eCFP tissue expression in transgenic mice as shown by Western blot.

(C) Western blot of MEFs from double-conditional Tg-fMCD^{ERT2} mice. Isolated MEFs were treated with 500 nM 4-hydroxytamoxifen (4HT) and Shield-1 or vehicle control and blotted for FKBP12 (stabilization), mCherry (recombination), and HSC 70 (loading control).

(D and E) Time course (D) and washout (E) stabilization of MLYCD.

(F) Epifluorescent images of tamoxifen-inducible Shield-1-stabilized primary MEFs.

(Banaszynski et al., 2008). Given the success of Shield-1 in mice, we proceeded to modify this technology into a genetically tractable and targeted in vivo mammalian expression system to affect metabolism in live behaving mice. The vector was designed to enable the conditional targeted expression of a Shield-1-regulated cassette in transgenic mice. A lox2272-flanked eCFPcaax stop cassette was cloned into a ubiquitous mammalian expression vector (Reamy and Wolfgang, 2011). Lox2272 was chosen because it does not recombine with traditional loxP sequences used in most conditional knockout (KO) mice (Araki et al., 2002). The FY-MLYCD transgene was cloned downstream of the floxed stop cassette (Figure 3A). FY-MLYCD is produced only after Cre-mediated excision of the eCFP stop cassette. Transgenic mice expressing Lox2272-eCFPcaax pA Lox2272-FKBP12-eYFP-MLYCD-2a-mCherry (Tg-fMCD) were produced and characterized for eCFP expression by Western blot and direct epifluorescent visualization in cryosections. CFP expression was the highest in the muscle, pancreas, and

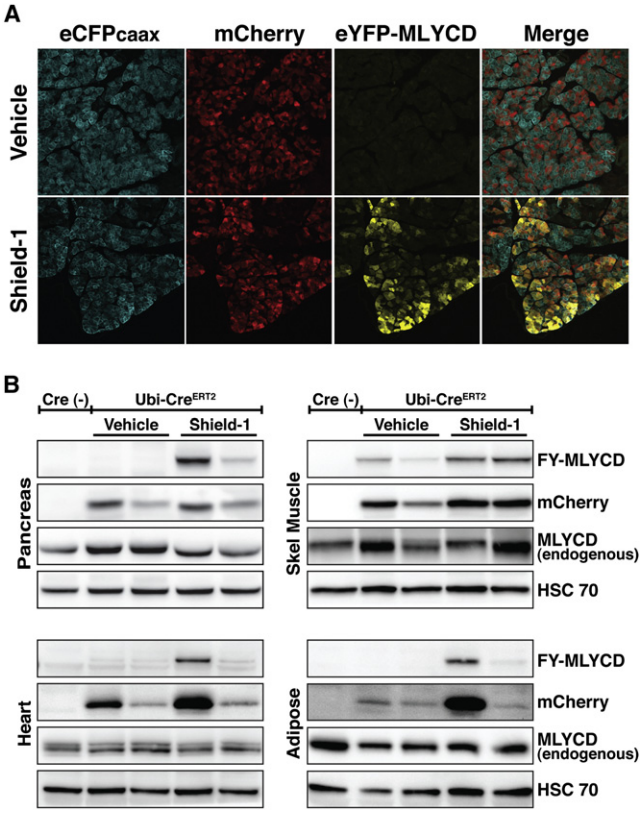

Figure 4. Chemical Genetic Regulation of MLYCD In Vivo

(A) Fluorescent confocal images of Shield-1 stabilization in the pancreas of conditionally regulated Tg-fMCD^{ERT2} mice. Animals were injected with tamoxifen (200 μg/kg), followed by Shield-1 (10 mg/kg) or vehicle control.

(B) Western blots of FY-MLYCD stabilization by Shield-1 in the pancreas, skeletal muscle, heart, and adipose tissue of Tg-fMCD^{ERT2} mice.

kidney with little to no expression in the brain and liver (Figure 3B). These mice were then crossed to mice that ubiquitously express a tamoxifen-inducible Cre recombinase, CRE^{ERT2} (Ruzankina et al., 2007) to produce double-transgenic mice (Tg-fMCD^{ERT2}) in order to initially characterize the Shield-1-inducible system in multiple tissues. These Shield-1-regulated transgenic mice can be bred to any transgenic Cre-recombinase-expressing mouse to achieve tissue specificity or temporal regulation with inducible Cre mice, or both. The flexibility of having dual inducible systems is particularly beneficial in cases where the induction of genes is desired in both a cell-specific and temporal manner. This transgenic vector can be easily tailored to meet individual needs.

Primary mouse embryonic fibroblasts (MEFs) were derived from double-transgenic mice. The MEFs exhibited membranous eCFP expression as a marker for transgene expression. The addition of 4-hydroxy-tamoxifen resulted in recombination and expression of mCherry (marker for recombination), but not of FY-MLYCD (Figures 3C and 3F). Addition of Shield-1 resulted in the expression of cytoplasmic FY-MLYCD (Figure 3F) in a dose-dependent manner (Figure 3C). We conducted kinetic studies to determine the stabilization of FY-MLYCD after 4-hydroxy-tamoxifen and Shield-1 addition. Peak stabilization was reached at 24 hr (Figure 3D), while washout studies showed that MEFs returned to basal stabilization levels 96 hr after Shield-1 removal (Figure 3E). These results demonstrate that MLYCD is dually regulated by Cre-mediated recombination and Shield-1 ex vivo.

Next, we tested this dual regulatory system in vivo. Tg-fMCD^{ERT2} animals were injected four times with 200 mg/kg intraperitoneal (i.p.) tamoxifen over 1 week to induce recombination of the eCFPcaax domain in vivo. After a 48 hr rest period, mice received three i.p. injections of Shield-1 at 10 mg/kg at 12 hr intervals. Transgene expression was assessed by the expression of membranous eCFP (Figure 4A). Recombination was seen in vivo as the expression of untargeted mCherry. Stabilization was visualized by the expression of cytoplasmic eYFP. Little to no expression of eYFP can be seen in the absence of Shield-1 (Figure 4A). Destabilization occurs robustly in vivo, as almost no expression was seen in the absence of Shield-1. mCherry and eYFP expression fully overlapped. No cells were observed expressing eYFP in the absence of mCherry expression. Shield-1 stabilized FY-MLYCD in tissues that express the transgene, including the pancreas, skeletal muscle, heart, and adipose tissue (Figures 4B–4E). The Tg-fMCD^{ERT2} mice exhibited dual regulation of FY-MLYCD in multiple tissues.

Finally, we tested the ability of Tg-fMCD^{ERT2} mice to alter the oxidation of fatty acids in live mice. Two genotypes were chosen, Tg-fMCD^{ERT2} and control Tg-fMCD mice. Tamoxifen was administered to all mice as described above to mediate recombination and expression of the FYMLYCD transgene in Tg-fMCD^{ERT2} mice. Mice were then given either Shield-1 or vehicle as above. The mice were then assayed individually for their ability to fully oxidize radiolabeled palmitate to CO_2. Shield-1 had no effect on control mice, however, Shield-1 induced an ~3-fold increase in fatty acid oxidation in the fed state (Figure 5). This is approximately the increase in fatty acid oxidation seen during prolonged fasting (Wolfgang et al., 2006). Here, we were able to achieve robust changes in the metabolism of fatty acids in live mice using an inert small molecule while simultaneously using wild-type littermates to control for any potential off-target effects of the chemical. These experiments provide the proof of principle for a broadly applicable means to alter protein and metabolic function in vivo.

Tissue-Specific and Dose-Dependent Small-Molecule Regulation of Malonyl-CoA Decarboxylase In Vivo

The ubiquitous tamoxifen-inducible Cre-recombinase-expressing mouse used above has the advantage of temporal (chemical) and ubiquitous control of recombination. These mice were useful for monitoring basic pharmacodynamics of stabilization in multiple tissues, as well as permitting mosaic recombination to serve as an additional internal cell-autonomous control (Figure 4A). This approach is not ideal, however, for dissecting tissue-specific effects of metabolic pathways in the context of whole-animal physiology. To test this system in a tissue-specific context, we crossed our transgenic mice (Tg-fMCD) to mice expressing Cre recombinase from the human alpha-skeletal muscle actin promoter (Miniou et al., 1999) (Tg-fMCD^{skel}). The expression of the muscle-specific Cre recombinase resulted in the deletion of eCFPcaax in muscle without deletion in nonparenchymal tissue, as expected (Figure 6A). This strategy resulted

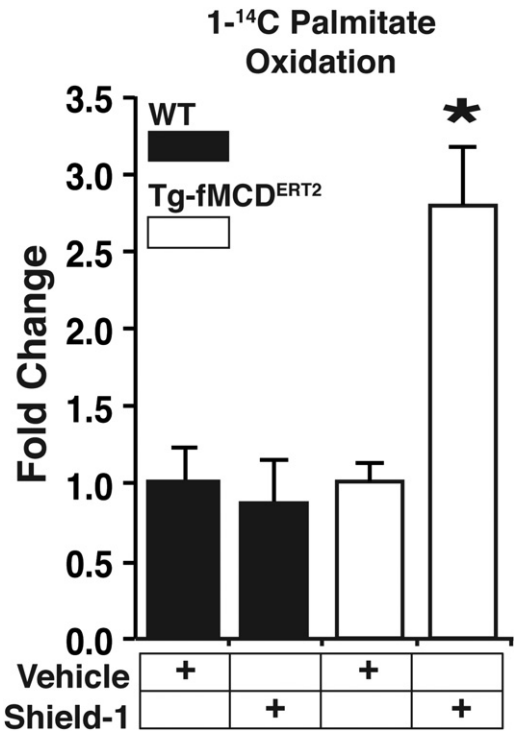

Figure 5. Chemical-Genetic Regulation of Fatty Acid Metabolism In Vivo

Live mouse in vivo oxidation of 1-^{14}C labeled palmitic acid to $^{14}CO_2$ for 45 min. n = 3/group, *p < 0.01. Error bars represent the SEM.

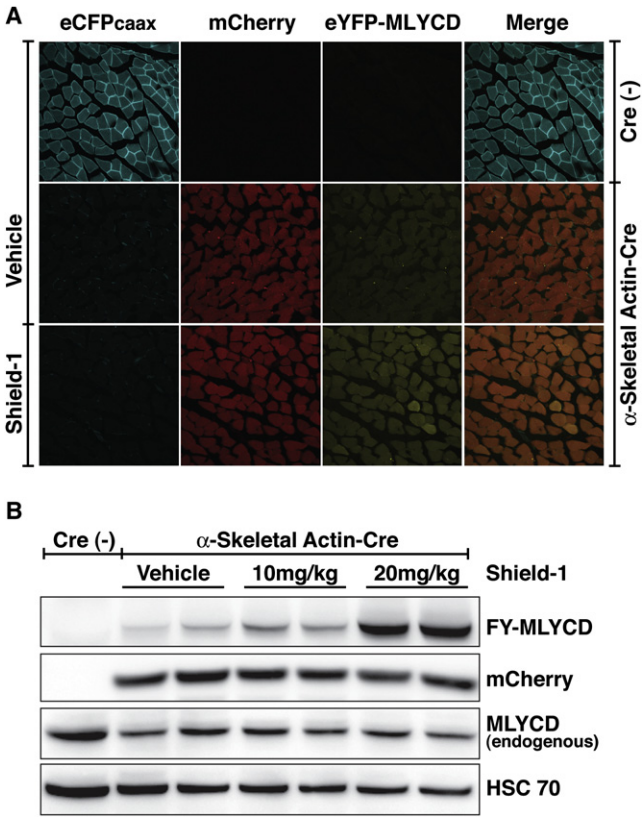

Figure 6. Tissue-Specific and Dose-Dependent Regulation of Protein Stability In Vivo

(A) Images of Shield-1 stabilization in the skeletal muscle of conditionally regulated Tg-fMCD^{skel} mice by fluorescent confocal microscopy. Animals were injected with Shield-1 (20 mg/kg) or vehicle control.

(B) Western blot of Shield-1 dose-dependent stabilization in the skeletal muscle of Tg-fMCD^{skel} and control mice. Animals were injected with Shield-1 (10 mg/kg or 20mg/kg) or vehicle control.

in a more uniform expression of the transgene as assessed by mCherry expression. Again, the administration of Shield-1 to Tg-fMCD^{skel} mice resulted in the induction of eYFP-MLYCD that increased with an increasing dose of Shield-1 (Figure 6B). These experiments show the utility of this method for modifying protein stability in a tissue-specific and dose-dependent manner in live mice.

DISCUSSION

Here, we have combined inducible protein stabilization with Cre-mediated conditionally targeted mouse transgenics to produce a broadly applicable and straightforward chemical-genetic strategy to affect metabolism in vivo. Chemical biology and chemical genetic techniques have been developed to probe the function of proteins in situ in a protein-/gene-specific manner (Bishop et al., 2000; Chen et al., 2005; Haruki et al., 2008; Karpova et al., 2005; Liu et al., 2007; Matsuzawa et al., 2005; McGinness et al., 2006; Robinson et al., 2010; Stankunas et al., 2003). The use of designer small molecules that can acutely but specifically control the function of targeted, but not native, proteins have advantages in basic science over classical pharmaceutical approaches. The specificity of small molecules for their targets is inversely proportional to their popularity. In other words, all small molecules have off-target effects that are sometimes not appreciated decades after their introduction. In the clinic this can be advantageous or catastrophic (Ito et al., 2010). In basic science, these effects can be misleading in the absence of target-specific control experiments in knockout mice, which are rarely feasible. Designer drug-target interactions while not useful in the clinic, can be more exquisitely controlled in a laboratory setting.

The transgenic approach we have designed contains several built-in controls to allow direct visualization of stabilization, recombination, and cellular localization simultaneously. The addition of lox2272 sites makes this approach ideal for adding back wild-type or mutant proteins that have been conditionally knocked out using the more common loxP recombination sites. Lox2272, while still being recognized by Cre recombinase, does not efficiently recombine with LoxP, thus mitigating genomic rearrangement. Ideally one could temporally add back a protein into the same tissue it was deleted from and control its function via Shield-1-induced stabilization or destabilization. This approach would be ideal for lethal null alleles or highly compensated pathways. Furthermore, other orthogonal methods can be used in conjunction with this system to achieve multiple modes of regulation in vivo (Iwamoto et al., 2010; Nishimura et al., 2009; Rossi and Blau, 1998).

This simple recombination-based system has the advantage of rapid development, cost effectiveness, and versatility compared to more laborious knockin strategies. The production of mice expressing transgenes is relatively straightforward and accessible to most researchers. Transgenesis combined with recombination-mediated transgene activation additionally takes advantage of the large number of publically available tissue-specific CRE-expressing mouse lines. Therefore, one can target gain-of-function or gene replacement to specific cell types to better dissect tissue-specific roles of ubiquitous genes. Making knockin mice enables the endogenous expression of genes but does not make it possible to discern tissue-specific effects. Tissue-specific promoters would satisfy this as well but are not versatile in directing transgenes to different cell types. The methodology here is beneficial in cases where one may need to produce several groups of mice to examine, for example, the relative role of enzymes in multiple tissues.

Low-abundance, structurally similar metabolites can be onerous to functionally elucidate within the context of mouse physiology, particularly when defining cause-and-effect relationships. The technique described here has the potential to intimately dissect metabolic pathways in living mice to better understand the relationship between metabolites and metabolic pathways and disease in distinct cell types. For example, acetyl-CoA carboxylases alpha (ACACA) and beta (ACACB) have been assigned differential roles in directing fatty acid metabolism, but these roles have become unclear of late (Abu-Elheiga et al., 2001; Harada et al., 2007; Olson et al., 2010; Savage et al., 2006), presumably due to compensation by these closely related enzymes. Altering specific metabolites in a cell-specific and temporal manner will offer a more precise and nuanced understanding of how these enzymes and pathways contribute to metabolic phenotypes.

SIGNIFICANCE

This mode of posttranslational control over protein stability has the advantage of quick kinetics, dose responsiveness, and reversibility similar to that of small-molecule pharmacology, with the added benefit of allele specificity. This simple, single-vector approach to making double-conditional transgenic mice provides a flexible platform to more precisely refine protein function in vivo. Furthermore, the reaction mechanism does not need to be known, and large-scale chemical screening does not need to be preformed. Therefore, new proteins or structurally similar protein families can be targeted to understand their function in mouse physiology in vivo.

EXPERIMENTAL PROCEDURES

Constructs

The FKBP12 destabilizing domain construct was fused to eYFP and a murine malonyl-CoA decarboxylase cDNA. Several modifications were made to the MLYCD cDNA prior to fusion with FKBP12-YFP: the mitochondrial and peroxisomal targeting signals were removed with the addition of flexible GSG linkers to the N and C termini of MLYCD. Next, a viral 2A bicistronic peptide was fused in frame linked to the red fluorescent protein mCherry to follow cellular localization. The construct and control (lacking MLYCD) were cloned into the retroviral vector, pLPCx (Clonetech), by standard methods.

Generation of HEK293 Stable Cell Lines Expressing FKBP12-YFP-MLYCD-2A-mCherry

HEK293T cells were grown in DMEM containing 10% FBS and 1% pen/strep (Invitrogen) at 37°C in a humidity-controlled chamber at 5% CO_2. To produce stable cell lines, HEK293T cells were infected with the retroviral constructs. Stable cell lines were produced by antibiotic selection with puromycin.

Generation of Transgenic Mice

A lox2272-flanked eCFP caax STOP SV40 polyA cassette was produced by PCR using standard methods and cloned into the pCAG vector (addgene #13787) (Matsuda and Cepko, 2007; Reamy and Wolfgang, 2011). The FKBP12-YFP-MLYCD-2A-mCherry transgene was cloned downstream of a Lox 2272 CFP STOP Lox2272 cassette driven by the ubiquitous CMV-enhanced chicken beta actin promoter (CAG). The transgenic vector was injected into pronuclei of fertilized zygotes (B6SJLF1) at the Johns Hopkins University School of Medicine Transgenic Mouse Core Facility. Transgenic founders and germline transmitters were identified by testing for the expression of CFP by PCR genotyping. We crossed the resulting Tg-fMCD transgenic mouse to a transgenic line that expresses a CRE^{ERT2} inducible by tamoxifen (B6;129S-Tg(UBC-cre/ERT2)1Ejb/J) (Ruzankina et al., 2007) to study the temporal regulation of MLYCD expression (referred to as Tg-$fMCD^{ERT2}$) or to mice expressing Cre from a muscle-specific promoter (B6.Cg-Tg(ACTA1-cre)79Jme/J) (Miniou et al., 1999) (referred to as Tg-$fMCD^{skel}$). Cre-mediated excision of eCFP is confirmed by genotyping PCR. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and under the approval of the Johns Hopkins Medical School Animal Care and Use Committee.

Assay for De Novo Fatty Acid Synthesis

Stably transfected HEK293T cells expressing FY-MLYCD were plated at a density of 4×10^5 cells in a 24 well plate (n = 6) and stimulated with Shield-1 or vehicle at increasing concentrations for 18 hr. Cells are labeled with 1.0 μCi of [^{3}H] acetic acid acetate (Perkin Elmer) for an additional 2 hr. Total lipids are extracted with chloroform/methanol and counted via liquid scintillation.

Malonyl-CoA Decarboxylase Assay

A fluorimetric assay that measures the formation of acetyl-CoA from malonyl-CoA, coupled with citrate synthase and malate dehydrogenase, was used to determine MCD activity in stable HEK293 T cells expressing FY-MLYCD (Saha et al., 2000). Cells were plated at a density of 1×10^6 cells in a 10 cm plate, then stimulated with Shield-1 or vehicle for 24 hr. Cells were harvested with an extraction buffer (0.1 M Tris, pH 7.4, containing 1 mM dithiothreitol (DTT), protease, and PhosStop inhibitors cocktail (Roche)) followed by a brief sonication. Cells were pelleted by centrifugation at 13,000 × rpm (30 min at 4°C) and supernatants were transferred to new Eppendorf tubes. Protein estimation was determined using the Pierce BCA Protein Assay Kit, and 0.5 mg of protein was used to carry out a partial purification of MCD by $(NH_4)_2SO_4$. To the lysates, 3.8 M $(NH_4)_2SO_4$ was slowly added on ice with vortexing, until 60% saturation was reached. The mixture was allowed to precipitate overnight at 4°C followed by centrifugation at 13,000 × rpm (60 min at 4°C). The pellets were resuspended in 0.1 M Tris-HCl (pH 8.0). MCD activity was measured using a Synergy MX multimode microplate reader (BioTek Instruments, Inc.) with excitation and emission wavelengths set to 340 and 460, respectively. Reaction mixtures of 140 μl containing 0.1 M Tris-HCl (pH 8.0), 0.5 mM DTT, 10 mM L-malate, 0.5 mM NAD, and 17 units of malate dehydrogenase were incubated for 7 min at 37°C in a 96 well black flat-bottom assay plate. A baseline-fluorescence measurement was followed by the addition of citrate synthase (3.14 units), succeeded by an additional 2 min incubation. Malonyl-CoA (0.3 mM) was added, followed by 20 μl of the $(NH_4)_2SO_4$-purified sample fraction. The incubation was allowed to continue for an additional 7 min before a second measurement for the rate of formation of NADH was taken.

In Vivo Fatty Acid Oxidation

In vivo oxidation of 1-^{14}C palmitic acid was measured in mice by i.p. injection of labeled palmitate (1 μCi) bound to fatty-acid-free bovine serum albumin, as previously reported (Reamy and Wolfgang, 2011; Wolfgang et al., 2006). Mice were individually housed in metabolic chambers and expired $^{14}CO_2$

was collected by bubbling the collected gas through 1 N NaOH. An aliquant of NaOH was measured by scintillation counting every 15 min for 45 min.

Western Blots

Whole tissues or stable HEK293T cells were harvested and total protein was extracted with sonication in extraction buffer (1% (v/v) Nonidet P-40 in TBS (50 mm Tris-HCl, pH 7.5, 150 mm NaCl) with protease and PhosSTOP phosphatase inhibitors cocktail (Roche). Cellular debris was pelleted at 13,000 × rpm (30 min at 4°C). Protein concentration was estimated using the Pierce BCA Protein Assay Kit. Soluble proteins (25 or 50 μg) were separated using Bio-Rad Tris Glycine SDS-PAGE gels (8%) or Invitrogen BisTris SDS-PAGE gels (4%–12%). Proteins were transferred to nitrocellulose membranes, blocked in 3% nonfat milk and detected by immunoblotting with antibodies to FKBP12 (Thermo Scientific), dsRED (Clonetech), MLYCD (abcam), and Heat shock Protein 8 (HSC-70) (Santa Cruz Biotechnology). HRP was detected using Immobilon Western Chemiluminescent substrate (Millipore Corporation, Billerica, MA) and the FlourChem Western blot imaging system (Cell Biosciences).

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