Molecular Cell, Vol. 12, 1615–1624, December, 2003, Copyright ©2003 by Cell Press

Conditional Protein Alleles Using Knockin Mice and a Chemical Inducer of Dimerization

Technique

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Summary

We have developed a general method of making conditional alleles that allows the rapid and reversible regulation of specific proteins. A mouse line was produced in which proteins encoded by the endogenous *glycogen synthase kinase-3* β (*GSK-3*β) gene are fused to an 89 amino acid tag, FRB*. FRB* causes the destabilization of GSK-3β, producing a severe loss-of-function allele. In the presence of C20-MaRap, a highly specific, nontoxic, cell-permeable small molecule, GSK-3βFRB* binds to the ubiquitously expressed FKBP12 protein. This interaction stabilizes GSK-3βFRB* and restores both protein levels and activity. C20-MaRap-mediated stabilization is rapidly reversed by the addition of an FKBP12 binding competitor molecule. This technology may be applied to a wide range of FRB*-tagged mouse genes while retaining their native transcriptional control. Inducible stabilization could be valuable for many developmental and physiological studies and for drug target validation.

Introduction

The development of homologous recombination in mice has greatly advanced our understanding of mammalian development and physiology (Thomas and Capecchi, 1990). More recently, the use of Cre recombinase-mediated deletion at specific times and in specific tissues has refined the basic approach, allowing a more detailed analysis of gene function (reviewed in Nagy, 2000). However, these techniques are not reversible and are often too slow to allow direct causal relationships to be deduced between a gene and the biochemical consequences of its deletion. Small molecule inhibitors, by acting directly on proteins, provide rapid inactivation and therefore fine temporal control of gene function. In addition, halting drug treatment or the addition of competitor molecules can reverse pharmacological inhibition. Reversible regulation allows for the study of gene function during specific developmental windows, akin to temperature shift experiments performed on temperature-sensitive alleles in poikilothermic organisms. For example, the small molecules FK506 and cyclosporin A have been used to define the execution point for *calcineurin* during vasculogenesis (Graef et al., 2001). Unfortunately, chemical genetic studies in animals remain limited by the lack of specific inhibitors for most targets and by the poor pharmacologic profiles of many existing small molecules.

An ideal conditional allele system would combine the high specificity and versatility of genetics with the rapid and reversible nature of small molecule-mediated inhibition. Several methods have been developed that combine chemistry with genetics to provide drug-dependent regulation of tagged or mutated proteins. One innovative method involves the mutation of kinases such that they retain their normal activity but become sensitive to an otherwise benign inhibitor (Bishop et al., 2000). Recently, this concept was used to produce transgenic mice expressing a chemically sensitive CaMKII in the brain (Wang et al., 2003). In another approach, proteins are expressed as fusions to steroid binding domains, which causes them to be encapsulated by HSP-90 until they are released by the addition of an appropriate hormone (reviewed in Picard, 2000). In a third method, N-end rule proteolysis of proteins fused to a modified dihydrofolate reductase can be controlled using methotrexate (Johnston et al., 1995; Levy et al., 1999). None of these approaches has yet been applied to an endogenous mouse gene locus. Additional methods used to conditionally remove gene function include targeted protein degradation (Zhou et al., 2000), ribozymes, antisense RNA, and the emerging technology of RNA interference (RNAi) (reviewed in McManus and Sharp, 2002). However, new technologies with faster kinetics and broader applicability are needed.

On the basis of the principle that induced proximity regulates many intracellular processes, we have developed methods of regulating the activity of proteins by inducing their association using small molecules (Spencer et al., 1993). Among other applications, chemical-induced dimerization has been used to regulate cell membrane receptors (Spencer et al., 1993; Pruschy et al., 1994), nonreceptor tyrosine kinases (Spencer et al., 1995), death inducers (Belshaw et al., 1996b; Spencer et al., 1996), exchange factors (Holsinger et al., 1995), GTPases (Castellano et al., 1999), and transcription factors (Belshaw et al., 1996a; Ho et al., 1996; Rivera et al., 1996). To date, however, we and others have been unable to devise a method that would allow dimerization-dependent loss-of-function studies. One approach we have favored is a system whereby dimerization would regulate protein stability or degradation. Such a method would have the potential to be broadly applied and would allow the creation of fast-acting, small molecule-sensitive loss-of-function alleles.

In this report, we show that a nontoxic, membrane-permeable rapamycin-derivative, C20-methallylrapamycin (C20-MaRap) can be used to stabilize proteins fused to a destabilizing polypeptide, FRB*. Using homologous recombination in ES cells, we targeted the serine-threonine kinase *GSK-3*β to produce a conditional loss-of-function *GSK-3*β^{FRB*} allele. In cells derived from these

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knockin mice, GSK-3βFRB* protein levels and activity are rapidly and reversibly controlled using C20-MaRap and a competitor molecule. Our results provide a method for regulating the products of an endogenous murine gene.

Results

C20-Methallylrapamycin: A Potent, Nontoxic Rapalogue

Rapamycin binds the 12 kDa FK506 binding protein, FKBP12 (Harding et al., 1989; Siekierka et al., 1989), to form a composite surface that interacts with and inhibits FKBP12-rapamycin-associated protein (FRAP) (Brown et al., 1994; Sabatini et al., 1994). The minimal region of FRAP sufficient for FKBP12-rapamycin binding is an 89 amino acid domain termed FRB, for FKBP-rapamycin binding (Chen et al., 1995; Liberles et al., 1997). FRB can be used as a tag to make fusion proteins that retain the ability to heterodimerize with FKBP12 in the presence of rapamycin. Rapamycin arrests growth and proliferation of many cell types (reviewed in Jacinto and Hall, 2003) by inhibiting protein translation through p70 S6-kinase (Kuo et al., 1992) and other proteins. Embryos from pregnant mice treated with rapamycin fail to develop a telencephalon, phenocopying a mouse FRAP mutant, *flat top*, (Hentges et al., 2001) and precluding the use of rapamycin in developmental studies. To overcome these undesired teratogenic effects of rapamycin, chemically modified rapamycin analogs (or rapalogues) have been synthesized that do not bind the wild-type FRB domain (Liberles et al., 1997). These rapalogues, such as C16-methallylrapamycin (C16-MaRap), contain bulky chemical substitutions, "bumps," at the FRB binding interface of rapamycin. A compensatory "hole" created at the rapamycin binding site of certain FRB point mutants restores binding specificity to these modified rapalogues. FRB* is one such FRB mutant that contains three point mutations: K2095P, T2098L, and W2101F (Liberles et al., 1997).

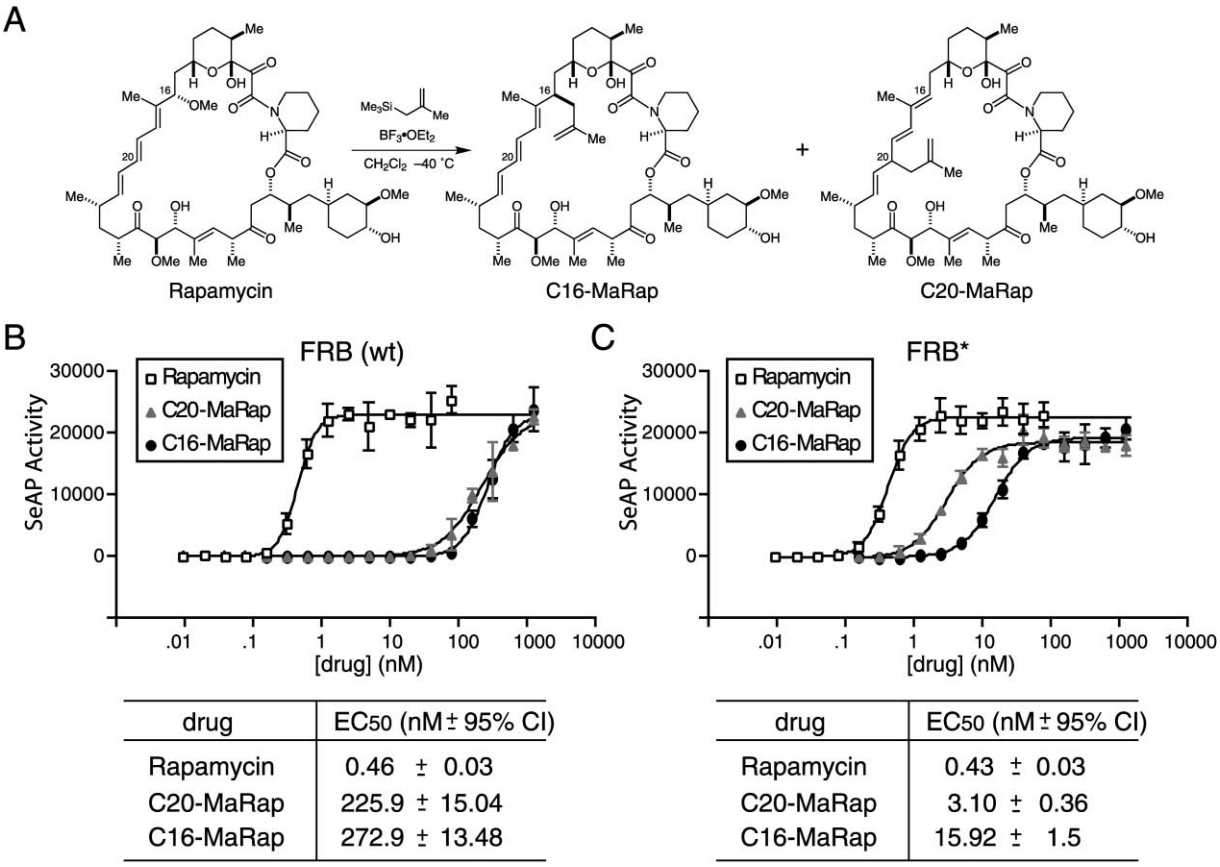
We modified the published methods for the synthesis of C16-MaRap (Liberles et al., 1997) to isolate both C16 and C20-methallylrapamycin (C20-MaRap) (Figure 1A) (for details see http://crablab.stanford.edu/synthesisofmarap.htm). We tested the ability of both of these bumped rapalogues to activate transcription by recruitment of chimeric transcriptional activation and DNA binding domains (Ho et al., 1996; Rivera et al., 1996). In these assays, FKBP fused to the GAL4 DNA binding domain and FRB or FRB* fused to the activation domain of VP16 were cotransfected into COS-1 cells along with a GAL4-dependent secreted alkaline phosphatase (SeAP) reporter plasmid. Addition of rapamycin or rapalogues caused heterodimerization of the fusions and activation of reporter gene expression. By titrating the drugs on transfected cells, we found that C20-MaRap bound FRB with low efficacy (EC_{50} = 225.9 ± 15.04 nM) but retained activity toward FRB* (EC_{50} = 3.10 ± 0.36 nM) (Figures 1B and 1C). In contrast, rapamycin was highly effective on both FRB (EC_{50} = 0.46 ± 0.03 nM) and FRB* (EC_{50} = 0.43 ± 0.03 nM). Like C20-MaRap, C16-MaRap exhibited selective binding for FRB* (EC_{50} = 15.92 ± 1.5 nM) versus FRB (EC_{50} = 272.9 ± 13.48 nM) but was not as effective as C20-MaRap on either substrate. The disruption of the rigid triene bond region from C17 to C22 may increase the flexibility of C20-MaRap, improving its ability to fit within the mutant FRB* binding pocket.

FRB* Directs Fusion Proteins to Degradation

GSK-3β participates in many signaling pathways, including negative roles in Wnt, insulin, and NF-AT signaling (reviewed in Doble and Woodgett, 2003; Beals et al., 1997). Its numerous roles make dissecting the separate functions of GSK-3β difficult by conventional genetics. We expressed GSK-3β fused to FRB* with the intention of conditionally regulating GSK-3β using an inducible dimerization system that will be described elsewhere. However, we found that GSK-3βFRB* was expressed at a lower level than an otherwise identical GSK-3βFRB fusion or cotransfected unmodified GSK-3β in transiently transfected mouse embryonic fibroblasts (MEFs) (Figure 2A). Treating the transfected cells with the protein translation inhibitor cycloheximide (CHX) revealed that GSK-3βFRB*, unlike GSK-3βFRB, was rapidly degraded (Figure 2A). Interestingly, when rapamycin or C20-MaRap was added to the cells, GSK-3βFRB* expression was restored to normal (Figure 2A). We investigated whether FRB* conferred similar reversible instability to other fusion proteins by expressing a collection of eight FRB* fusion proteins in MEFs in the presence or absence of C20-MaRap (Figure 2B). Despite their dissimilarities in molecular weight, subcellular site of action, and structure, the levels of each FRB* fusion protein increased between 3.5- and 100-fold upon drug addition (data not shown). These results suggest that FRB* has the general property of conferring reversible instability to fusion proteins.

To understand the minimal requirements for conditional protein stability, we repeated the assay with GSK-3βFRB#. FRB# contains only the T2098L substitution present in FRB* and is otherwise wild-type. GSK-3βFRB#, like GSK-3βFRB*, is rapidly degraded while being stabilized by rapamycin (Figure 2A). From these data, we conclude that the T2098L mutation may be the major cause of FRB*-mediated instability. As this mutation introduces a hydrophobic residue in a region that would be otherwise surface exposed (Choi et al., 1996), proteins fused to FRB* or FRB# may become partially unfolded, leading to the destabilization and eventual degradation of the fusion protein.

To further characterize FRB*-mediated destabilization, we expressed each of FRB, FRB*, and FRB# as GST fusions in *E. coli*. The thermal stability of the purified fusion proteins was determined using circular dichroism spectroscopy (CD). Freshly isolated GST-FRB variants did not unfold or aggregate at 4°C as determined by the characteristic appearance of the CD spectra (Figure 2C). The melting temperature (T_m) of each protein was determined by measuring CD absorbance at 230 nm, a wavelength which is in the β sheet absorbing region. This wavelength was chosen because FRB is small and entirely composed of an α-helical bundle (Choi et al., 1996). Therefore, the contribution of FRB to the absorbance in this region is minimal, and monitoring T_m at this wavelength reports primarily on the stability of GST. Both GST-FRB* and GST-FRB# had a T_m approximately 5°C



drug	EC50 (nM ± 95% CI)
Rapamycin	0.46 ± 0.03
C20-MaRap	225.9 ± 15.04
C16-MaRap	272.9 ± 13.48

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Rapamycin	0.43 ± 0.03
C20-MaRap	3.10 ± 0.36
C16-MaRap	15.92 ± 1.5

Figure 1. Synthesis and Potency of C16 and C20 MaRap

(A) Chemical synthesis and structure of C16- and C20-methallylrapamycin. For details see http://crablab.stanford.edu.

(B and C) MaRap exhibits selective affinity for FRB*. SeAP reporter activity levels induced by dimerization of either FRB-VP16 (left side) or FRB*-VP16 (right side) to $FKBP_3$-GAL4DBD were measured in quadruplicate at the indicated concentrations of rapamycin, C16-MaRap, or C20-MaRap, and plotted on a graph using GraphPad Prism (GraphPad Software). Error bars represent 95% confidence intervals. EC_{50} values were determined by fitting a sigmoidal curve to the data using GraphPad Prism and are presented at ±95% confidence intervals.

lower than GST fused to wild-type FRB (Figure 2D). These results are consistent with the model that the T2098L mutation destabilizes FRB structure. This instability is subsequently transmitted to the entire fusion protein and likely triggers the destruction of FRB* or FRB# fusion proteins in cells.

The preceding results demonstrate that FRB* confers two characteristics to fusion proteins: it provides the ability to dimerize to FKBP12 in the presence of the nontoxic rapalogue, C20-MaRap, and it causes the protein to be destabilized and degraded. Importantly, this directed degradation is blocked by dimerization to FKBP12 using C20-MaRap. Dimerization may "lock" FRB* in a folded state, energetically stabilized by interactions with FKBP12 and the rapalogue (Figure 2E). These dual features of FRB* suggested a general means of producing conditional alleles based on small molecule-induced stabilization.

Construction of an FRB* Allele of Murine GSK-3β

With the goal of understanding the complex developmental roles of GSK-3 proteins, we created an FRB* knockin allele of *GSK-3*β to permit conditional regulation of GSK-3β at the protein level. A selectable plasmid was constructed with a neomycin resistance selection cassette flanked by loxP sites placed in the last intron of *GSK-3*β and an FRB*HA sequence placed immediately before the stop codon of *GSK-3*β (Figure 3A). Homologous recombination in ES cells was used to target this construct to the endogenous *GSK-3*β locus. Mice were derived from correctly targeted cells, and the neomycin resistance cassette was removed by crossing these mice with mice expressing the Cre recombinase under the ubiquitous β-actin promoter (Lewandoski et al., 1997). As a result, the only genomic changes in the derived mouse line were a single loxP site in the last intron of *GSK-3*β and the FRB*HA sequence inserted at the extreme 3′ end of the *GSK-3*β coding region. This design was employed to ensure minimal disruption of *GSK-3*β regulatory elements. Indeed, *GSK-3*β^{FRB^*} mRNA was present at normal levels (data not shown). Furthermore, a C-terminal FRB* fusion was chosen to prevent interference with the N-terminal serine 9 phosphorylation site of GSK-3β. Additionally, the crystal structure of GSK-3β (Dajani et al., 2001; ter Haar et al., 2001) predicted that a C-terminal FRB* tag would not interfere with GSK-3β's structure or GSK-3β's own dimerization. GSK-3βFRB* retained the ability to inhibit NF-AT-depen-

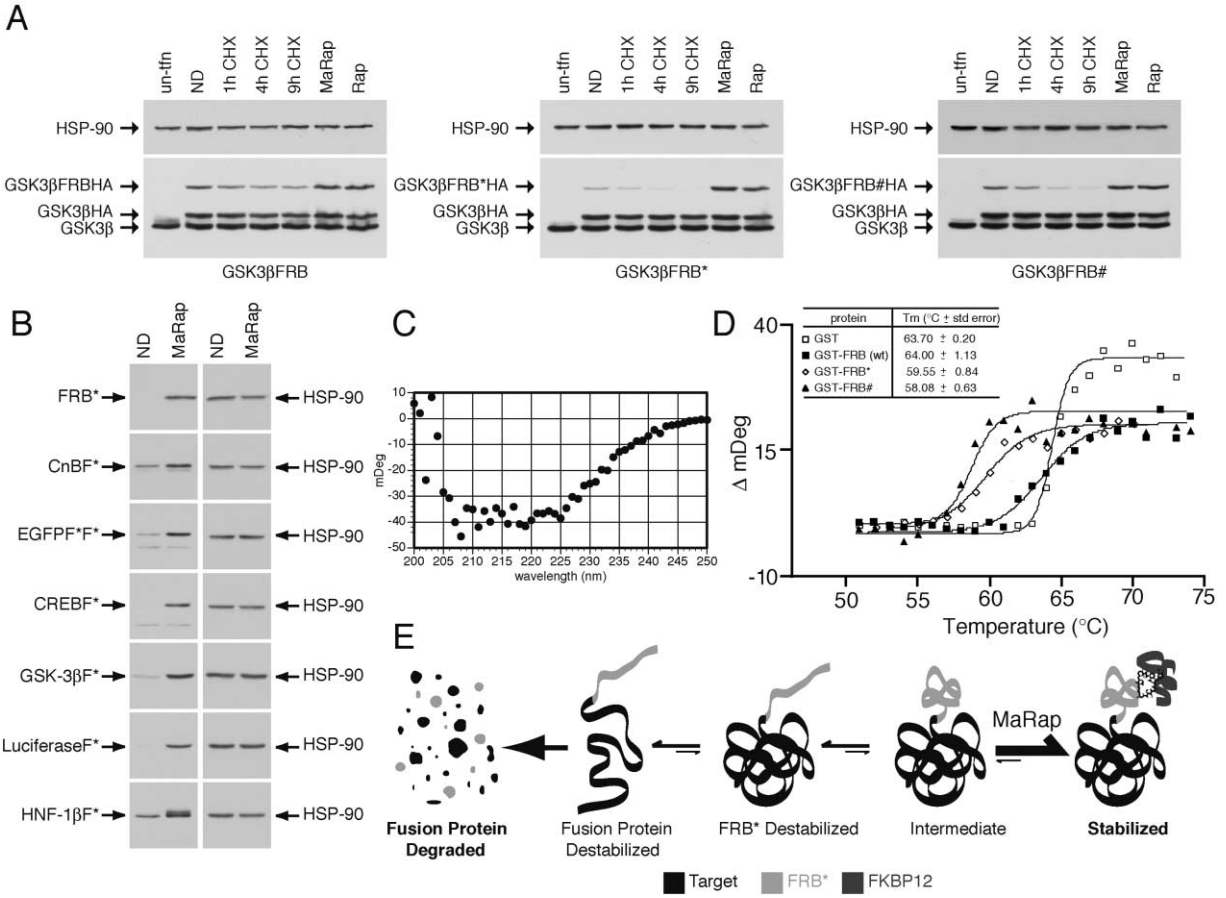

Figure 2. FRB* Destabilizes Fusion Proteins

(A) Destabilization is primarily a result of the T2098L mutation present in FRB*. Wild-type MEFs were cotransfected with an equimolar mixture of GSK-3β expressing plasmid and one of GSK-3βFRB (left panel), GSK-3βFRB* (middle panel), or GSK-3βFRB# (right panel) expressing plasmids. The transfections were split into multiple wells and treated with cycloheximide (CHX) for the periods shown, as well as with rapamycin or C20-MaRap for 36 hr where indicated. The cells were lysed in RIPA buffer, and protein expression was detected by Western blotting with an anti-GSK-3β antibody. The transfected proteins contain hemagglutinin (HA) epitope tags. HSP-90 and endogenous GSK-3β are used as loading controls.

(B) FRB*-mediated reversible destabilization can be widely applied. The indicated FRB* fusion proteins were expressed in wild-type MEFs by transfection of corresponding plasmids. Transfections were split into two plates and either left untreated (ND) or treated with 200 nM C20-MaRap for 36 hr. Expression of the FRB* fusion proteins was detected by Western blotting lysates with an anti-FRB antibody.

(C and D) The T2098L mutation reduces the melting temperature of GST-FRB fusion proteins. Recombinant GST-FRB, GST-FRB*, and GST-FRB# proteins were prepared from transformed *E. coli* and subjected to circular dichroism spectroscopy. (C) The absorbance spectra of GST-FRB* indicates the presence of β sheets and α helices. (D) The change in absorbance at 230 nM normalized to standard conditions over increasing temperature was determined and charted for each of GST, GST-FRB(wt), GST-FRB*, and GST-FRB#.

(E) An equilibrium model of inducible stabilization.

dent transcription in transient tranfection assays in Jurkat T cells and could still be phosphorylated at serine 9 (data not shown).

Although *GSK-3*β^{+/FRB^*} mice were viable and fertile, most *GSK-3*β^{FRB^*/FRB^*} mice died immediately following birth, being present at normal Mendelian ratios at embryonic day E18.5 (K.S. and G.R.C., unpublished data). Although there were no obvious defects to explain this phenotype, the mice did exhibit a complete cleft of the secondary palate (K.S. and G.R.C., unpublished data). As a result, the occasional homozygous pup that did not die immediately after birth failed to suckle and died within 12 hr of birth. In our laboratory, this phenotype is indistinguishable from that of *GSK-3*$\beta^{KO/KO}$ and *GSK-3*β^{KO/FRB^*} mice on outbred backgrounds. We examined the expression of GSK-3βFRB* in heterozygous mice and found GSK-3βFRB* levels to be greatly reduced relative to wild-type protein in all tissues examined. Heart and thymus expression levels are shown in Figure 3B. This result is consistent with the FRB*-directed degradation of GSK-3βFRB* seen in transfection experiments and with the severe loss-of-function phenotype of *GSK-3*β^{FRB^*/FRB^*} mice.

GSK-3βFRB* Is Inducibly and Reversibly Stabilized in MEFs

We prepared MEFs containing the *GSK-3*β^{FRB^*} allele. Heterozygous cells expressed greatly reduced levels of GSK-3βFRB*, consistent with its low level of expression in adult tissues. GSK-3βFRB* was degraded at an en-

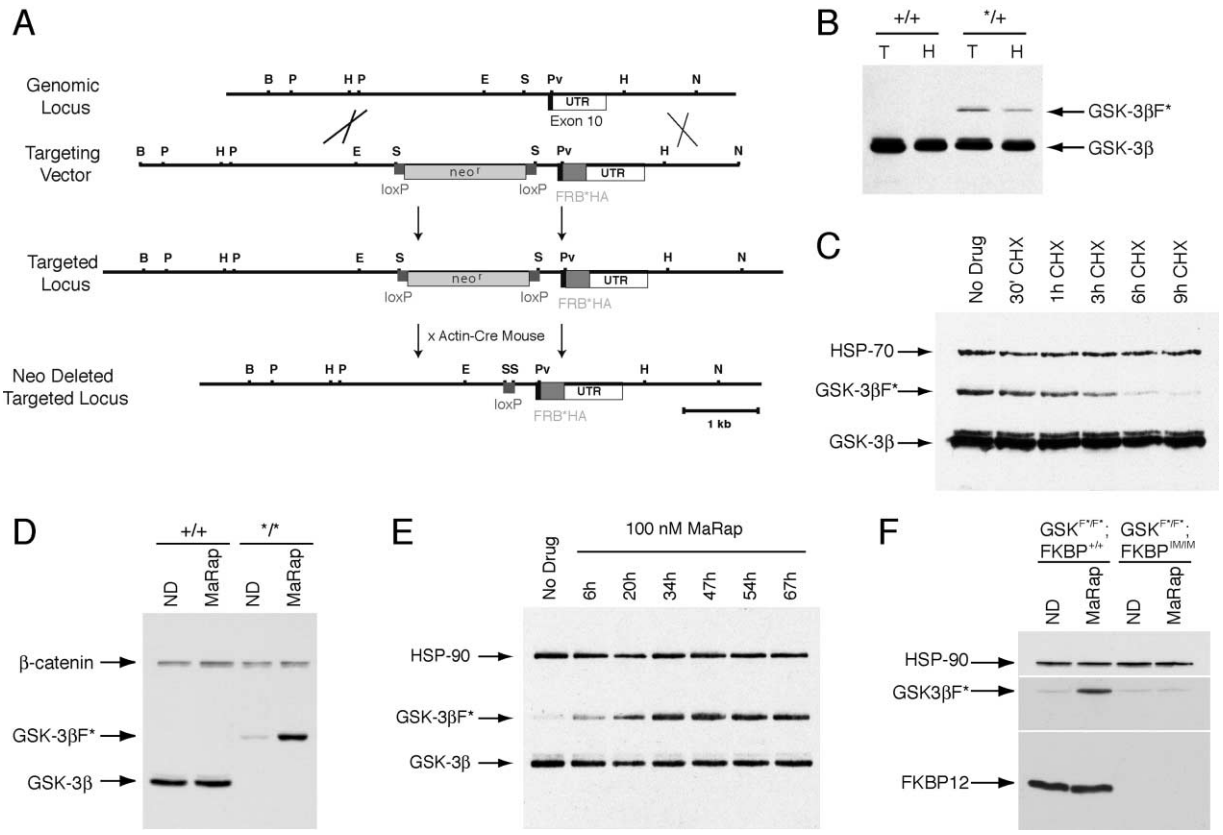

Figure 3. Inducible Stabilization of GSK-3βFRB*

(A) The *GSK-3β*^{FRB*} knockin mouse. A schematic of the strategy employed to target the C terminus of endogenous murine *GSK-3β* with an FRB* tag. Exon 10 is the last exon of *GSK-3β* containing translated mRNA. The darker box represents the coding portion of the exon while UTR indicates the untranslated region.

(B) Heterozygous *GSK-3β*^{FRB*} mice express reduced levels of GSK-3βFRB* relative to protein produced by the wild-type allele. Ten micrograms of thymus (T) or heart (H) RIPA lysate was run on an SDS-PAGE gel, and the expression level of GSK-3β was determined by Western blotting.

(C) GSK-3βFRB* is destabilized in fibroblasts. Heterozygous MEFs were treated with cycloheximide (CHX) for the indicated periods. The expression level of GSK-3β and GSK-3βFRB* in 10 μg of protein lysate from each condition was detected by Western blotting using an anti-GSK-3β antibody. The blot is overexposed to allow visualization of GSK-3βFRB* at all time points. HSP-70 protein levels serve as a loading control.

(D) GSK-3βFRB* is fully stabilized upon C20-MaRap treatment in fibroblasts. MEFs of the indicated genotypes were left untreated (ND) or were treated for 36 hr with 100 nM C20-MaRap (MaRap). Protein lysates were prepared, and the expression of GSK-3β proteins was determined by Western blotting. β-catenin expression is used as a loading control.

(E) GSK-3βFRB* expression is restored to wild-type levels with C20-MaRap treatment. Heterozygous *GSK-3β*^{FRB*} MEFs were treated with 100 nM C20-MaRap for the indicated periods. GSK-3β expression was detected by Western blot analysis of cell lysates. HSP-90 expression serves as the loading control.

(F) GSK-3βFRB* stabilization requires FKBP12 binding. Homozygous *GSK-3β*^{FRB*} MEFs expressing wild-type FKBP12 or carrying an allele of *FKBP12* that does not express detectable FKBP12 (*FKBP*^{IM}) were left untreated (ND) or were treated with 100 nM C20-MaRap for 36 hr. GSK-3βFRB*, FKBP12, and HSP-90 (loading control) expression was detected by Western blotting with appropriate antibodies.

hanced rate relative to the wild-type GSK-3β protein observed when translation was blocked with CHX (Figure 3C). A 36 hr treatment with C20-MaRap restored GSK-3βFRB* levels in *GSK-3β*$^{FRB*/FRB*}$ cells to those of GSK-3β in wild-type MEFs (Figure 3D). Rapamycin produced an identical effect (data not shown), and neither drug had an effect on wild-type GSK-3β, β-catenin, or HSP-90 expression. We performed a time course of C20-MaRap treatment on heterozygous MEFs to study the rate of inducible stabilization and found that stabilization was complete in approximately 24 hr with half maximal stabilization observed at 8–12 hr (Figure 3E). Importantly, the levels did not increase beyond those of the wild-type protein, suggesting that stabilization is a result of blocking an FRB*-mediated effect, rather than a dominant effect of recruiting FKBP12. However, as expected, the stabilization did require FKBP12 binding, as indicated by the complete loss of C20-MaRap-induced stabilization in fibroblasts that are doubly homozygous for *GSK-3β*^{FRB*} and an *FKBP12* allele, *FKBP12*^{IM}, that lacks detectable FKBP12 expression (Figure 3F).

FK506 is a small molecule that binds to FKBP12 with high affinity to form a complex that interacts with and inhibits the phosphatase calcineurin (Liu et al., 1991). We reasoned that FK506 could be used to reverse inducible stabilization by competing with C20-MaRap for binding to FKBP12. We stabilized GSK-3βFRB* in heterozygous MEFs and then treated the cells across a time course

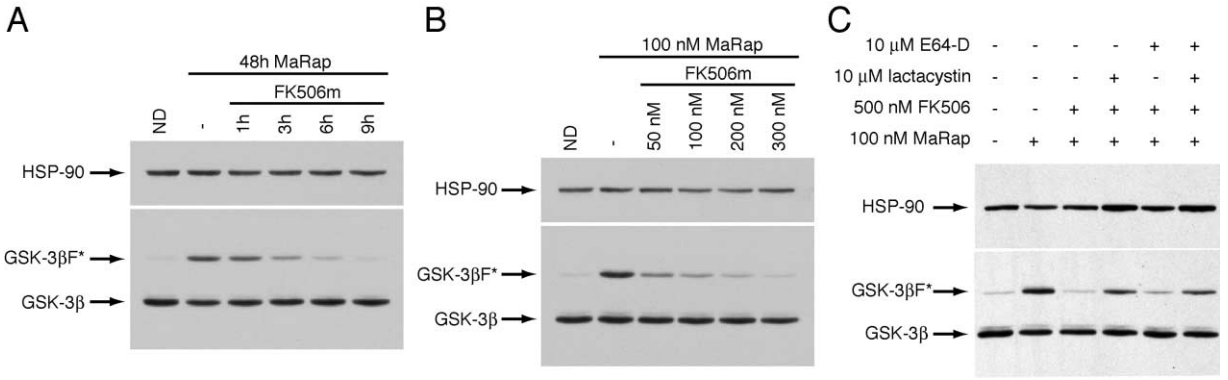

Figure 4. GSK-3βFRB* Stabilization Is Reversible and Mediated by the Proteasome

(A) FK506M rapidly reverses C20-MaRap induced stabilization of GSK-3βFRB*. Heterozygous *GSK-3β*^{FRB*} MEFs were left untreated (ND) or were treated with 100 nM C20-MaRap for 36 hr. Subsequently, 300 nM FK506M was added for the indicated periods in the continued presence of C20-MaRap. Lysates were prepared and analyzed for GSK-3β and GSK-3βFRB* expression by Western blotting. HSP-90 serves as a loading control.

(B) The relative concentration of C20-MaRap to FK506M produces graded stabilization. This is similar to (A), except the MEFs were supplemented with the indicated concentrations of FK506M for 9 hr following 100 nM C20-MaRap treatment for 36 hr.

(C) FRB* targets GSK-3βFRB* for proteasomal degradation. Heterozygous *GSK-3β*^{FRB*} MEFs were treated with the indicated combinations of small molecules. In each case that C20-MaRap was used, the cells were first stabilized for 36 hr with C20-MaRap and then lactacystin and/or E-64D was added for 30 min prior to the addition of FK506 to reverse stabilization for the final 9 hr before harvesting. As before, GSK-3β, GSK-3βFRB*, and HSP-90 levels were detected by Western blotting. HSP-90 expression is a loading control.

with a derivative of FK506 called FK506M that does not bind calcineurin but retains the ability to bind FKBP12 (Spencer et al., 1993). Following FK506M treatment, GSK-3βFRB* was rapidly degraded, returning to steady-state low levels in less than 9 hr (Figure 4A). Inducible stabilization is therefore readily reversible. The use of different relative concentrations of FK506M to C20-MaRap produced a gradient of steady-state GSK-3βFRB* levels (Figure 4B). As such, the ratio of the two drugs can be used to titer GSK-3βFRB* activity and produce an allelic series.

FRB*-Mediated Degradation Is Directed by the Proteasome

To determine the mechanism underlying FRB*-mediated degradation, we tested whether degradation occurred in the presence of specific protease inhibitors. GSK-3βFRB* was stabilized to normal levels in heterozygous MEFs before the stabilization was reversed by the addition of FK506 to the culture media. When the stabilization was reversed in the presence of lactacystin (Figure 4C) or MG132 (data now shown), two proteasomal inhibitors, GSK-3βFRB* remained largely stabilized. E-64D, an inhibitor of lysosomal cysteine proteases, did not protect GSK-3βFRB* from degradation. Therefore, FRB*-mediated destabilization results, at least in part, in proteasomal degradation. However, we did not detect ubiquitination of GSK-3βFRB* (data not shown), indicating that its proteasomal targeting may be ubiquitin independent or that the life of ubiquitinated intermediates is very short.

GSK-3β Activity Is Restored by Inducible Stabilization

We performed GSK-3β immunoprecipitation-kinase assays on fibroblast lysates to test whether inducible stabilization restored GSK-3β activity. Immunoprecipitated wild-type GSK-3β effectively incorporated radiolabeled ATP into the GSP peptide from glycogen synthase, but not into the GSA peptide, which contains a serine-alanine mutation at the GSK-3 phosphorylation site (Figure 5A). Destabilized GSK-3βFRB* isolated from untreated *GSK-3β*$^{FRB*/FRB*}$ MEFs contained less than 10% of normal GSK-3β kinase activity. However, GSK-3βFRB* immunoprecipitated from C20-MaRap-treated *GSK-3β*$^{FRB*/FRB*}$ cells had an over 7-fold increase in activity and approximately 60% of the activity of wild-type cells. Furthermore, the stabilized GSK-3βFRB* remained bound to FKBP12 during the kinase assay, indicating that FKBP12-C20-MaRap binding does not inhibit GSK-3β's activity or substrate access (Figure 5B). GSK-3βFRB* stabilized in *GSK-3β*$^{FRB*/FRB*}$ MEFs showed a similar activity to that present in identically prepared and treated *GSK-3β*$^{+/KO}$ MEFs (Figure 5A). Since *GSK-3β*$^{+/KO}$ cells and mice appear completely normal, we anticipate that inducible stabilization could recover sufficient levels of GSK-3β activity to restore the kinase's normal functions.

Because GSK-3β regulates a wide variety of signaling pathways, transcription factors, and cell cycle regulators, we expected to find multiple cellular phenotypes in *GSK-3β*$^{FRB*/FRB*}$ fibroblasts that we could study using inducible stabilization. However, as reported with *GSK-3β*$^{KO/KO}$ MEFs (Hoeflich et al., 2000), we found no defects in cell growth or β-catenin levels in the *GSK-3β*$^{FRB*/FRB*}$ MEFs (data not shown). Additionally, we found no defect in NF-AT activity or in the rate of endogenous NF-ATc1 rephosphorylation following pharmacologic calcineurin inhibition in *GSK-3β*$^{FRB*/FRB*}$ or *GSK-3β*$^{KO/KO}$ MEFs (data not shown). Furthermore, unlike Hoeflich et al. (2000), we did not detect a significant defect in NF-κB signaling in response to TNF-α, or enhanced cell death in response to TNF-α and CHX (data not shown). This discrepancy may be due to different methods used during fibroblast preparation. The lack of phenotypes is likely due to the presence of functionally redundant *GSK-3α*.

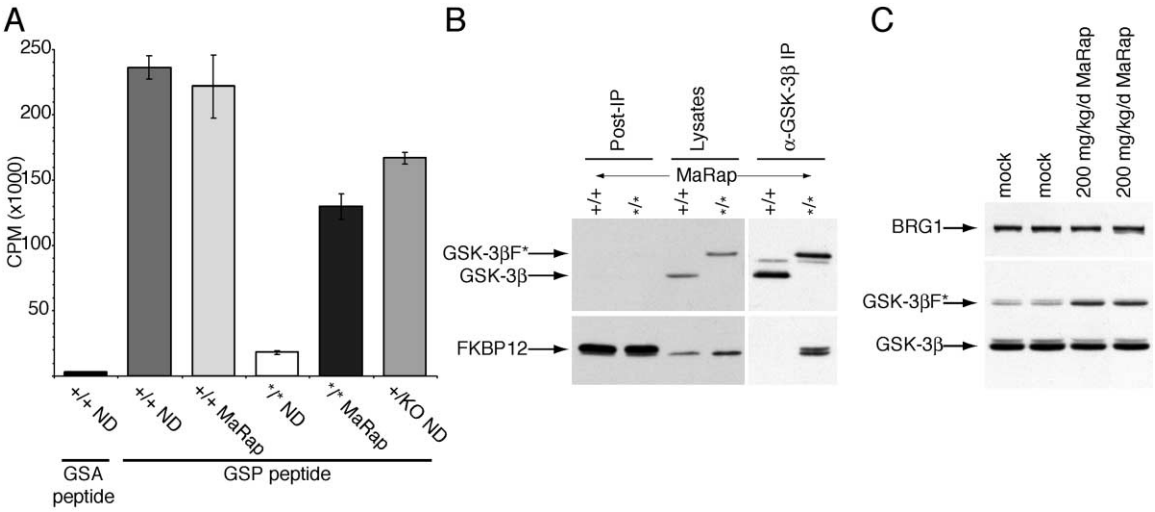

Figure 5. GSK-3βFRB* Activity Is Restored upon Inducible Stabilization, and GSK-3βFRB* Can Be Inducibly Stabilized In Vivo

(A and B) GSK-3β or GSK-3βFRB* was immunoprecipitated from MEFs of the indicated genotypes and drug treatments and used in [γ-^{32}P]ATP kinase assays using the GSP substrate peptide from glycogen synthase or, in the first lane, the alanine-mutated negative control GSA peptide. Each assay was performed in triplicate, and error bars represent one standard deviation.

(B) GSK-3βFRB* has kinase activity when bound to FKBP12. A portion of the IP reactions and post-IP supernatant from an IP-kinase assay were saved for Western blot analysis. The levels of GSK-3β, GSK-3βFRB*, and FKBP12 were detected in each condition by Western blotting using respective antibodies. Two micromolars of recombinant FKBP12 was added to each IP to ensure that GSK-3βFRB* would remain bound to FKBP12-C20-MaRap.

(C) GSK-3βFRB* is inducibly stabilized in embryos in vivo. A heterozygous *GSK-3β^{FRB*}* mouse, impregnated by a heterozygous male and carrying E10.0 embryos, was mock-injected or injected with C20-MaRap over a 36 hr period before the embryos were isolated at E11.5. The anterior portion of heterozygous embryos was lysed in RIPA buffer, and the expression of GSK-3β and GSK-3βFRB* was determined by Western blotting. BRG1 expression serves as a loading control.

The amino acid sequence of GSK-3α is highly similar to GSK-3β (82% identity), and GSK-3α can replace GSK-3β for most of its biological functions that have been tested. Therefore, conditional biological studies of GSK-3 signaling functions in fibroblasts and probably other cell types using GSK-3βFRB*-inducible stabilization will require the background removal of *GSK-3α*.

GSK-3βFRB* Can Be Stabilized In Utero

To be used as a conditional allele system in whole animal developmental studies, we must be able to deliver C20-MaRap to the embryos of pregnant mice. If successful, we would be able to use C20-MaRap and FK506M in concert to study the functions of GSK-3βFRB* or other knockin tagged proteins during very precise developmental windows. To test whether C20-MaRap can be delivered to embryos, E10.0 pregnant heterozygous *GSK-3β^{FRB*}* mice (crossed to heterozygous male), were injected with 200 mg/kg/day of C20-MaRap. The injection course consisted of three intraperitoneal (i.p.) injections repeated every 12 hr, followed by the harvesting of the embryos at E11.5. Neither the mother nor the embryos were overtly affected by this drug course. In contrast, rapamycin, similarly injected at 10 mg/kg/day, produced "flat top" embryos due to a failure of telecephalon outgrowth, demonstrating the necessity of using nonteratogenic rapalogues. Protein lysates were prepared from the C20-MaRap-treated embryos and from stage-matched embryos from a mock-injected mother and analyzed by Western blotting. GSK-3βFRB* protein levels were partially restored in embryos from the C20-MaRap-treated mothers (Figure 5C). Full stabilization was not observed, likely because the concentrations of C20-MaRap remained low in embryos (<25 nM), as measured by organic extraction and biological activity testing using the transcriptional switch assay (data not shown). New rapalogues that combine both the ability of C20-MaRap to bind FRB* and the excellent pharmacokinetics of rapamycin will be required to provide more effective stabilization in vivo.

Discussion

Conditional alleles that act at the protein level have proven invaluable for dissecting complicated biological events because they provide rapid and reversible regulation. For example, the classic temperature-sensitive allele experiments of Hartwell and Nurse led to fundamental discoveries of the cell cycle (Hartwell et al., 1970; Nurse et al., 1976). Unfortunately, such approaches have not been previously validated in a mouse system. An ideal system for making conditional alleles in mice could be broadly and readily applicable to many targets and would allow the rapid and reversible regulation of the target while avoiding nonspecific background effects. Toward this goal, we report the development of a technology that allows the inducible stabilization of FRB* fusion proteins using a chemical inducer of dimerization. We apply this method to endogenous murine *GSK-3β*, producing a chemically sensitive allele of an endogenous mouse gene. By combining gene targeting, which is highly specific and can be used on nearly any mouse

gene with small molecules which provide rapid protein-level regulation, this technology fulfills many of the characteristics of an ideal conditional allele system.

Conditional protein alleles are particularly suited for developmental studies because existing gene deletion technologies do not provide sufficient temporal control to dissect separate roles of a gene during a series of rapid development events. Inducible stabilization of GSK-3βFRB* is complete within 24 hr of C20-MaRap treatment in MEFs (Figure 3E), and may even be faster in rapidly dividing cells in vivo. By subsequently reversing inducible stabilization with FK506M, gene function can be conditionally and predictably abated during precise developmental periods. FK506M released GSK-3βFRB* from FKBP12 and caused GSK-3βFRB* to be degraded with a $t_{1/2}$ of approximately 2 hr (Figure 4A and data not shown). Reapplying a rapalogue and restabilizing the targeted protein would allow the determination of temporal windows when the targeted gene is required for its various functions. For example, a rapalogue and FK506M could be used in concert with the *GSK-3*β^{FRB*} mice to determine the period when GSK-3β activity is required for viability at birth. Although rapamycin and other rapalogues have good pharmacokinetics, allowing their use in whole animal studies (Granger et al., 1995; Yatscoff et al., 1995), C20-MaRap is not as effective in vivo as it is in cell culture. The poor pharmacokinetics of C20-MaRap are probably related to its short half-life in serum (K.S., J.H.B., and G.R.C., unpublished data) and to its high affinity for cytochrome P450 (J.E.G. and G.R.C., unpublished data). As higher doses of C20-MaRap are not feasible by i.p. injections or several other drug delivery methods that we have attempted, rapalogues exhibiting better pharmacokinetics are required for further in vivo studies. We are actively screening for such a compound that could be used on FRB*-tagged knockin target genes.

One major advantage of the inducible stabilization conditional allele system is that when it is desirable to remove gene function for an extended period of time, the researcher simply needs to cease drug delivery. For example, if the researcher is interested in the long-term adult effects of removing gene function but needs to bypass a developmental requirement, the stabilizing drug only needs to be applied during that early developmental window to allow the mouse to survive to adulthood. This "drug-on" nature of our system is particularly useful for drug target validation studies. Protein stability can be controlled in the adult in a reversible manner, and the level of activity can be titrated to achieve a therapeutic window. Because our system is genetically based, it can be readily combined with various mouse disease models to test whether removing the function of the gene will cause or prevent disease phenotypes. Additionally, the effects of removing gene function can be assayed even after the disease is apparent or at different stages of disease severity. *GSK-3*β has been hypothesized to have roles in several disease conditions, including type II diabetes, bipolar disorder, and neurodegenerative disease. The *GSK-3*β^{FRB*} allele could be valuable for validating the clinical use of GSK-3β targeting therapeutics in mouse models of human disease.

The utility of conditional protein alleles is not limited to developmental or target validation studies. Most physiological events, including cell cycle progression, apoptosis, axonal outgrowth, synapse formation, and lymphocyte activation, are also dynamic and would therefore benefit from protein targeting. Furthermore, many of these processes can be readily studied in cell, tissue, or organ culture systems, where we can effectively deliver C20-MaRap. Importantly, conditional protein alleles can be used to minimize or define secondary effects. Cells or tissues can be cultured to an appropriate initiation point before the target protein is inactivated. This feature is especially valuable for genomic or proteomic experiments in which indirect effects can obscure the identification of direct targets of the protein whose function is removed. Additionally, inducible stabilization is not limited to conditional loss-of-function studies. The ability to rapidly stabilize and/or destabilize expressed proteins could be useful for both basic research applications and to provide small molecule-regulated gene therapeutics.

Experimental Procedures

C20-MaRap Synthesis

Rapamycin (400 mg) and trimethylmethallylsilane (2.3 ml) were dissolved in dichloromethane (30 ml) and cooled to −40°C. Boron trifluoride etherate (3.38 ml) was added in one portion, and the reaction was stirred at −40°C and monitored by analytical HPLC. The reaction was quenched after 5 hr with aqueous saturated sodium bicarbonate and warmed to room temperature, and the organic products were extracted into dichloromethane. Silica-gel chromatography (2:1 to 1:1 to 1:3 hexanes: ethyl acetate) gave 201 mg of MaRap as a mixture of three isomers by analytical HPLC: C20-(*R* or *S*)-methallylrapamycin (isomer A), C20-(*R* or *S*)-methallylrapamycin (isomer B), and C16-(*R*)-methallylrapamycin (isomer C). The compounds were purified by reverse-phase HPLC using a ternary solvent system. HPLC solvents and the column (Waters Xterra M8 phenyl 5 μm, 19 × 100 mm column) were preheated in coiled tubing using a 50°C water bath, with a flow rate of 17 ml/min. The mobile phase consisted of an isocratic mixture of nine parts of 80% CH_3OH/H_2O and one part of 20% CH_3CN/H_2O. The desired product, C20-MaRap (63 mg, t_r = 11.7 min) was isolated along with the other C20-MaRap diastereomer (isomer A, t_r = 10.5 min) and C16-(*R*)-MaRap (isomer C, t_r = 14.5 min). The two C20-MaRap diastereomers lack the characteristic UV absorbance pattern of the rapamycin triene (λ_{max} = 278 nm). C20-MaRap was characterized using ^{1}H NMR and mass spectrometry. ESI-MS ($C_{54}H_{83}NO_{12} + Na^+$) expected m/z 960 and observed m/z 960.5. For a detailed protocol for producing MaRap, see http://crablab.stanford.edu/synthesisofmarap.htm.

Reporter Assays

COS-1 cells were electroporated with 2 μg each of the following expression plasmids (Ho et al., 1996): pG_5IL2SX (which drives transcription of bacterial SeAP through GAL4 DNA binding elements), pBJ5-GF3E (which expresses the GAL4 DNA binding domain fused to three copies of human FKBP12), and pBJ5-FRBVE (which expresses an FRB fusion with the herpes simplex virus VP16 activation domain). A FRB*VP16 fusion was also used selectively. Transfected cells were distributed equally to 96-well plates and were incubated at 37°C for 24 to 36 hr before media was supplemented with drug in a dilution curve in which each point is represented in quadruplicate. Cells were incubated at 37°C for an additional 24 hr followed by a 2 hr treatment at 65°C to inactivate endogenous phosphatases. Each well was supplemented with 100 μl of 1 mM methylumbelliferrylphosphate dissolved in 2 M diethanolamine buffered to pH 10.0 with carbonate and incubated at 37°C for 16 hr. Fluorescence was measured with a Spectra Max Gemini XS (Molecular Devices) plate reader at 355 nm transmission and 455 nm emission using Softmax Pro software.

Mouse Genetics

A selectable plasmid was constructed containing mouse genomic DNA from the 3′ end of the *GSK-3β* locus, where a loxP-flanked neomycin-resistance cassette was cloned into the last intron, and an FRB*HA sequence was inserted in place of the normal stop codon. This plasmid was electroporated into TC1 ES cells, and drug resistant lines were screened for correct targeting using Southern blots of restriction digested DNA and radiolabeled probes outside of each targeting arm. Mice were derived from the targeted ES cells mice using standard techniques. The neomycin-resistance cassette was deleted by crossing germline transmitting mice with mice expressing the Cre recombinase under the β-actin promoter (Lewandoski et al., 1997). Deletion of the cassette was confirmed by PCR. The final derived mouse line was screened for the absence of the Cre transgene and maintained on a CD1 outbred background.

Cell Culture

MEFs were prepared from E14.5–E16.5 embryos of indicated genotypes by eviscerating the embryos in HBSS, treating the remaining tissue with 0.2% collagenase and trypsin, and plating the cells in DMEM containing 10% FCS, antibiotics, and 0.1 mM β-mercaptoethanol. Once established, the MEFs were cultured using standard techniques and treated with various small molecules as indicated.

Western Blotting

Cell lysates were prepared in RIPA buffer (10 mM Tris [pH 7.2], 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, protease inhibitor cocktail [Invitrogen Life Technologies]) and then separated using standard SDS-PAGE before being transferred to PVDF (Millipore) using standard Western blotting procedures. The anti-GSK-3β antibody (used at 1:2500) the anti-HSP-90 antibody (1:2500), and the anti-FKBP12 antibody (1:2500), were purchased from BD Transduction Labs. The anti-β-catenin antibody (1:2000) and anti-HSP-70 antibody (1:1000) were purchased from Santa Cruz Biotechnology. The anti-BRG1 antibody (1:1000) was previously described (Khavari et al., 1993). The anti-FRB antibody was prepared in our laboratory using recombinant GST-FRB as an antigen. In all cases, Western blots were incubated overnight with the appropriate primary antibodies in TBST (10 mM Tris 8.0, 150 mM NaCl, 0.1% Tween 20) containing 10% dry milk and then washed three times with TBST. The blots were subsequently incubated for 30 min with HRP-conjugated secondary antibodies from Jackson Immunoresearch, washed three more times with TBST, and then developed using standard ECL reagents (Amersham Biosciences) and Kodak XAR film.

MEF Transfections

MEFs were transfected using FuGENE-6 (Roche Applied Science). Plasmid DNA (5 μg per 6 cm dish) was mixed in a 1:2 ratio with the FuGENE-6 reagent in Opti-MEM (Invitrogen Life Technologies) before being applied to the cells growing in DMEM/10% FCS and containing C20-MaRap where appropriate. After 12 hr, the cells were split to 6-well plates or 6 cm dishes before further drug treatments to ensure each well contained an equivalently transfected population.

Circular Dichroism Spectroscopy

FRB variants were amplified by PCR and ligated into pGEX2T as in-frame fusions to generate pGEX-FRB(wt), pGEX-FRB*, and pGEX-FRB#. These vectors were used to transform chemically competent *Escherichia coli* BL21 cells to derive the strains: GST-FRB(wt), GST-FRB*, and GST-FRB#. Proteins were induced by the addition of IPTG and purified using glutathione-sepharose beads (Amersham Biosciences) by standard techniques. Pooled washes from the final elution steps were dialyzed against CD buffer (10 mM Tris-HCl [pH 7.2], 100 mM NaCl, 1 mM DTT). Protein concentrations were determined by absorbance at 280 nm. Purity was >80% by SDS-PAGE. Dialyzed samples were diluted to 0.25 mg/ml in CD buffer. Freshly prepared samples were used as significant precipitation was observed after prolonged storage at −80°C or 4°C under these buffer conditions. The circular dichroism spectra of diluted samples was monitored on an AVIV 62A DS spectrometer. For T_m determinations, ellipticity was monitored at 230 nm while temperature was varied from 50°C to 75°C with 1°C steps. Equilibration time was 1 min. Nonlinear regression fits were performed using GraphPad Prism (GraphPad Software). The reported values are the mean of two independent experiments.

Immunoprecipitation-Kinase Assays

MEFs were treated as indicated and then lysed in mild lysis buffer (20 mM Tris 7.2, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 5% glycerol, 100 nM C20-MaRap, 100 nM microcystin-LR [Calbiochem], and protease inhibitor cocktail [Calbiochem]). Four hundred micrograms of lysate was buffer-exchanged into immunoprecipitation (IP) buffer (10 mM Tris 7.2, 150 mM NaCl, 1 mM EGTA, 100 nM C20-MaRap, 100 nM microcystin, and protease inhibitor cocktail), supplemented with 2 μM purified recombinant FKBP12, and prepared using standard GST fusion purification methods. IP reactions were incubated at 4°C for 1.5 hr with 125 ng of anti-GSK-3β, followed by a 2 hr incubation with 20 μl of a 1:4 dilution of Protein G Sepharose Fast Flow (Amersham Biosciences) in Sepharose CL-4B (Amersham Biosciences). GSK-3β activity was assayed in kinase buffer (20 mM Tris 7.2, 10 mM $MgCl_2$, 1 mM DTT, 100 nM C20-MaRap, 1 mM peptide, 0.5 mM ATP) by measuring [γ-^{32}P]ATP (Amersham Biosciences) incorporation into the phospho-glycogen-synthase peptide-2 (GSP) or the negative control glycogen synthase peptide-2[Ala 21] (GSA) (Upstate). The reactions were spotted on P81 paper (Whatman), washed with 125 mM phosphoric acid, and measured by liquid scintillation.

Whole Animal Studies

GSK-3β$^{+/FRB*}$ mice were intercrossed and the date of vaginal plug observation was set as E0.5. At E9.5, the stage of embryonic development was confirmed by ultrasonography (Chang et al., 2003) and mice were weighed. Drug administration commenced at E10.0. C20-MaRap was dissolved as a stock in *N,N*-dimethylacetamide (DMA) and was diluted into a delivery vehicle containing 10% polyethylene glycol 400, 17% polyoxyethylene sorbitan monooleate, and 10% DMA to a final concentration of 100 mg/kg. One hundred microliters was immediately injected intraperitoneally to pregnant females with injections repeated twice more at 12 hr intervals to E11.5 when mice were sacrificed, and embryos and organs were dissected and frozen in $N_{2(l)}$. Tissues anterior to the embryonic forelimbs of individual embryos were used for preparation of protein extracts with the remainder used to determine the concentration of drug and for genotyping.

Acknowledgments

We thank Kelvin Neu for creative suggestions, Feng Chen for producing the *FKBP^{IM}* allele, Ann Kuo and Lei Chen for valuable technical assistance, Stuart Schreiber and Steve Diver for assistance with the C20-MaRap synthesis, Jim Woodgett for providing *GSK-3β* knockout mice, Susan McConnell for providing *β-actin Cre* mice, and Ariad Pharmaceuticals for generous gifts of rapamycin and an FRB# containing plasmid. We are indebted to members of the Crabtree laboratory for experimental advice and stimulating discussions. K.S. is a Howard Hughes Medical Institute predoctoral fellow. J.E.G. is funded by a Helen Hay Whitney Foundation postdoctoral fellowship. G.R.C. is an Investigator with the Howard Hughes Medical Institute. This work was additionally funded by National Institutes of Health grants CA39612 to G.R.C. and GM68589 to T.J.W.

Received: June 16, 2003
Revised: September 23, 2003
Accepted: September 25, 2003
Published: December 18, 2003

References

Beals, C.R., Sheridan, C.M., Turck, C.W., Gardner, P., and Crabtree, G.R. (1997). Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. Science *275*, 1930–1934.

Belshaw, P.J., Ho, S.N., Crabtree, G.R., and Schreiber, S.L. (1996a). Controlling protein association and subcellular localization with a synthetic ligand that induces heterodimerization of proteins. Proc. Natl. Acad. Sci. USA *93*, 4604–4607.

Belshaw, P.J., Spencer, D.M., Crabtree, G.R., and Schreiber, S.L. (1996b). Controlling programmed cell death with a cyclophilin-cyclosporin-based chemical inducer of dimerization. Chem. Biol. *3*, 731–738.

Bishop, A.C., Ubersax, J.A., Petsch, D.T., Matheos, D.P., Gray, N.S., Blethrow, J., Shimizu, E., Tsien, J.Z., Schultz, P.G., Rose, M.D., et al. (2000). A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature *407*, 395–401.

Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S., and Schreiber, S.L. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature *369*, 756–758.

Castellano, F., Montcourrier, P., Guillemot, J.C., Gouin, E., Machesky, L., Cossart, P., and Chavrier, P. (1999). Inducible recruitment of Cdc42 or WASP to a cell-surface receptor triggers actin polymerization and filopodium formation. Curr. Biol. *9*, 351–360.

Chang, C.P., Chen, L., and Crabtree, G.R. (2003). Sonographic staging of the developmental status of mouse embryos in utero. Genesis *36*, 7–11.

Chen, J., Zheng, X.F., Brown, E.J., and Schreiber, S.L. (1995). Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. Proc. Natl. Acad. Sci. USA *92*, 4947–4951.

Choi, J., Chen, J., Schreiber, S.L., and Clardy, J. (1996). Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. Science *273*, 239–242.

Dajani, R., Fraser, E., Roe, S.M., Young, N., Good, V., Dale, T.C., and Pearl, L.H. (2001). Crystal structure of glycogen synthase kinase 3 β: structural basis for phosphate-primed substrate specificity and autoinhibition. Cell *105*, 721–732.

Doble, B.W., and Woodgett, J.R. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. J. Cell Sci. *116*, 1175–1186.

Graef, I.A., Chen, F., Chen, L., Kuo, A., and Crabtree, G.R. (2001). Signals transduced by Ca^{2+}/calcineurin and NFATc3/c4 pattern the developing vasculature. Cell *105*, 863–875.

Granger, D.K., Cromwell, J.W., Chen, S.C., Goswitz, J.J., Morrow, D.T., Beierle, F.A., Sehgal, S.N., Canafax, D.M., and Matas, A.J. (1995). Prolongation of renal allograft survival in a large animal model by oral rapamycin monotherapy. Transplantation *59*, 183–186.

Harding, W.M., Galat, A., Uehling, D.E., and Schreiber, S.L. (1989). A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. Nature *341*, 758–760.

Hartwell, L.H., Culotti, J., and Reid, B. (1970). Genetic control of the cell-division cycle in yeast. I. Detection of mutants. Proc. Natl. Acad. Sci. USA *66*, 352–359.

Hentges, K.E., Sirry, B., Gingeras, A.C., Sarbassov, D., Sonenberg, N., Sabatini, D., and Peterson, A.S. (2001). FRAP/mTOR is required for proliferation and patterning during embryonic development in the mouse. Proc. Natl. Acad. Sci. USA *98*, 13796–13801.

Ho, S.N., Biggar, S.R., Spencer, D.M., Schreiber, S.L., and Crabtree, G.R. (1996). Dimeric ligands define a role for transcriptional activation domains in reinitiation. Nature *382*, 822–826.

Hoeflich, K.P., Luo, J., Rubie, E.A., Tsao, M.S., Jin, O., and Woodgett, J.R. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature *406*, 86–90.

Holsinger, L.J., Spencer, D.M., Austin, D.J., Schreiber, S.L., and Crabtree, G.R. (1995). Signal transduction in T lymphocytes using a conditional allele of Sos. Proc. Natl. Acad. Sci. USA *92*, 9810–9814.

Jacinto, E., and Hall, M.N. (2003). Tor signalling in bugs, brain and brawn. Nat. Rev. Mol. Cell Biol. *4*, 117–126.

Johnston, J.A., Johnson, E.S., Waller, P.R.H., and Varshavsky, A. (1995). Methotrexate inhibits proteolysis of Dihydrofolate reductase by the N-end rule pathway. J. Biol. Chem. *270*, 8172–8178.

Khavari, P.A., Peterson, C.L., Tamkun, J.W., Mendel, D.B., and Crabtree, G.R. (1993). BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription. Nature *366*, 170–174.

Kuo, C.J., Chung, J., Fiorentino, D.F., Flanagan, W.M., Blenis, J., and Crabtree, G.R. (1992). Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. Nature *358*, 70–73.

Levy, F., Johnston, J.A., and Varshavsky, A. (1999). Analysis of a conditional degradation signal in yeast and mammalian cells. Eur. J. Biochem. *259*, 244–252.

Lewandoski, M., Meyers, E.N., and Martin, G.R. (1997). Analysis of Fgf8 gene function in vertebrate development. Cold Spring Harb. Symp. Quant. Biol. *62*, 159–168.

Liberles, S.D., Diver, S.T., Austin, D.J., and Schreiber, S.L. (1997). Inducible gene expression and protein translocation using nontoxic ligands identified by a mammalian three-hybrid screen. Proc. Natl. Acad. Sci. USA *94*, 7825–7830.

Liu, J., Farmer, J.D., Jr., Lane, W.S., Friedman, J., Weissman, I., and Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell *66*, 807–815.

McManus, M.T., and Sharp, P.A. (2002). Gene silencing in mammals by small interfering RNAs. Nat. Rev. Genet. *3*, 737–747.

Nagy, A. (2000). Cre recombinase: the universal reagent for genome tailoring. Genesis *26*, 99–109.

Nurse, P., Thuriaux, P., and Nasmyth, K. (1976). Genetic control of the cell division cycle in the fission yeast Schizosaccharomyces pombe. Mol. Gen. Genet. *146*, 167–178.

Picard, D. (2000). Posttranslational regulation of proteins by fusions to steroid-binding domains. Methods Enzymol. *327*, 385–401.

Pruschy, M.N., Spencer, D.M., Kapoor, T.M., Miyake, H., Crabtree, G.R., and Schreiber, S.L. (1994). Mechanistic studies of a signaling pathway activated by the organic dimerizer FK1012. Chem. Biol. *1*, 163–172.

Rivera, V.M., Clackson, T., Natesan, S., Pollock, S., Amara, J.F., Keenan, T., Magari, S.R., Phillips, T., Courage, N.L., Cerasoli, F., Jr., et al. (1996). A humanized system for pharmacologic control of gene expression. Nat. Med. *2*, 1028–1032.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S.H. (1994). RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell *78*, 35–43.

Siekierka, J.J., Hung, S.H.Y., Poe, M., Lin, C.S., and Sigal, N.H. (1989). A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. Nature *341*, 755–757.

Spencer, D.M., Wandless, T.J., Schreiber, S.L., and Crabtree, G.R. (1993). Controlling signal transduction with synthetic ligands. Science *262*, 1019–1024.

Spencer, D.M., Graef, I., Austin, D.J., Schreiber, S.L., and Crabtree, G.R. (1995). A general strategy for producing conditional alleles of Src-like tyrosine kinases. Proc. Natl. Acad. Sci. USA *92*, 9805–9809.

Spencer, D.M., Belshaw, P.J., Chen, L., Ho, S.N., Randazzo, F., Crabtree, G.R., and Schreiber, S.L. (1996). Functional analysis of Fas signaling in vivo using synthetic inducers of dimerization. Curr. Biol. *6*, 839–847.

ter Haar, E., Coll, J.T., Austen, D.A., Hsiao, H.M., Swenson, L., and Jain, J. (2001). Structure of GSK3beta reveals a primed phosphorylation mechanism. Nat. Struct. Biol. *8*, 593–596.

Thomas, K.R., and Capecchi, M.R. (1990). Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. Nature *346*, 847–850.

Wang, H., Shimizu, E., Tang, Y.P., Cho, M., Kyin, M., Zuo, W., Robinson, D.A., Alaimo, P.J., Zhang, C., Morimoto, H., et al. (2003). Inducible protein knockout reveals temporal requirement of CaMKII reactivation for memory consolidation in the brain. Proc. Natl. Acad. Sci. USA *100*, 4287–4292.

Yatscoff, R.W., Wang, P., Chan, K., Hicks, D., and Zimmerman, J. (1995). Rapamycin: distribution, pharmacokinetics, and therapeutic range investigations. Ther. Drug Monit. *17*, 666–671.

Zhou, P., Bogacki, R., McReynolds, L., and Howley, P.M. (2000). Harnessing the ubiquitination machinery to target the degradation of specific cellular proteins. Mol. Cell *6*, 751–756.