METHODS

GSK-3β mutant mice Generation of drug-dependent *GSK-3β*^{FRB*} mice was described previously[6]. Conventional mutant alleles of *GSK-3* (*GSK-3β*$^{-}$) were a kind gift from Dr. James Woodgett and have been described previously[5]. For timed pregnancies, the date of observation of a vaginal plug was considered embryonic day 0.5 and the stage of gestation was confirmed by ultrasound[22]. Genotyping was performed as previously described[5, 6]. All of the experiments shown were performed in outbred CD-1 mice, however, we saw the same cleft palate phenotype in *GSK-3β*$^{-/-}$ mice and observed neither hepatic defects nor midgestational lethality. We found identical phenotypes in our inbred *GSK-3β*$^{FRB*/FRB*}$ mice (produced in 129J ES cells and backcrossed to C57BL/6J) but then chose to pursue our experiments in an outbred background due to breeding strategies.

Bone Preparations Mice were harvested at indicated time points, skinned, eviscerated, fixed and stored in 95% ethanol. Alcian blue and Alizarin red staining of bones were performed according to established protocols.

Drug treatments Rapamycin (sirolimus) was resuspended in a stock solution at 20 mg/ml in N,N-dimethylacetamide (DMA) and stored at –20°C until use. Pregnant dams were treated with subcutaneous injections every 12 hours during the time periods indicated. Each injection consisted of 5 mg/kg rapamycin diluted in 200 µl of injection vehicle (10% polyethylene glycol 400, 17% Tween-80). For vehicle controls, animals were injected with 200 µl of injection vehicle (every 12 hours either for two days or continuously from e13.5 to e18.5) and displayed no phenotypic abnormalities.

Western blots Tissue samples were harvested in cold PBS, lysed in RIPA and stored at –80°C. Western blots were run according to standard protocols. Primary antibodies used: GSK-3 antibody (1:1000 dilution, Santa Cruz Biotechnology, catalog #sc-7291), HSP90 antibody (1:1000 dilution, BD Transduction Laboratories, catalog #610418). Secondary antibody: goat anti-mouse HRP-conjugated (1:5000 dilution, Pierce).

Reporter Assays/Pharmacokinetics Quantitation of rapamycin in treated embryos was performed as previously described using a rapamycin-dependent secreted alkaline phosphatase system [6]. In brief, tissue samples were dissected and immediately frozen at –80°C until further analysis. Tissues were then weighed, aliquoted into a fresh eppendorf tube, homogenized in PBS, and extracted three times with ethyl acetate. Ethyl acetate solution was then separated on a silica gel column and dried in a speedvac. Pellet was resuspended in 4 µl ethanol, diluted in 400 µl of media, and added in serial dilutions to COS-1 cells transfected with transcriptional switch components as described.

Cell Death and Cell Proliferation Embryos were harvested at the indicated stages, fixed overnight in 4% paraformaldehyde in PBS and processed for cryosectioning according to standard methods. Twelve micron sections were cut and stained with the In Situ Cell Death Kit (Roche) according to the manufacturer's protocol or with anti-phoshorylated Histone H3 antibody (Upstate Biotechnology, 1:500 dilution) followed by goat-anti rabbit IgG-568 (Molecular Probes, 1:300 dilution) according to standard protocols. Cell counts from palatal/tongue samples were performed by examining three adjacent sections from three positions (front to back) of the tongue for each embryo. Three sections from each animal were examined for cell counts in the sternum.

Supplementary Figure S1: Reversible Protein Stabilization

a. A mutant FK506-rapamycin binding (FRB*) domain confers instability on fusion proteins. In the presence of rapamycin or rapamycin analogues, FRB*-tagged glycogen synthase kinase-3β (GSK-3β) dimerizes with endogenous FK506 binding proteins (FKBPs), thus stabilizing the fusion protein and restoring protein levels and activity.

b. Gene targeting strategies such as traditional knockouts and cre-mediated loss of function alleles are irreversible.

c. In the absence of drug, FRB*-tagged proteins mimic traditional knockout alleles. Addition of rapamycin stabilizes the protein and restores function. Because the FRB*-tagged allele is a knockin, we are restoring protein function in endogenous spatial and temporal contexts. After cessation of drug treatment, the FRB* fusion protein is destabilized and degraded, removing active protein. Thus, inducible stabilization is reversible.

d. Stabilization of GSK-3βFRB* *in vivo.* Western blotting of brain tissue from embryos treated *in utero* from e15.5 to e17.0. Embryos were sacrificed seven hours after the last treatment and dissected. Samples from wild type (lanes 1-2), heterozygous (lanes 3-4), and mutant littermates (lanes 5-6) are shown. In mutant animals, GSK-3βFRB* is stabilized. For comparison, note amounts of GSK-3βFRB*, top band in lanes 5 and 6, compared to GSK-3β (bottom band, lanes 1 and 2 (100% of wildtype) and lanes 3 and 4 (50% of wildtype). GSK-3α serves as a loading control.

e. Stabilization of GSK-3β is undetectable within 15 hours after the last injection. Western blotting of brain tissue from embryos treated with 4 doses of rapamycin *in utero*. Samples were collected 41 hours after last injection (lane 1), 27 hours after last injection (lane 2) and 15 hours after last injection (lane 3). GSK-3α and HSP-90 serve as loading controls.

f. Stabilization of GSK-3βFRB* in the sternum. Western blotting of sternal extracts from embryos treated either with vehicle control or with 5mg/kg rapamycin *in utero* from e16.0 to e17.0 (3 doses). Embryos were sacrificed seven hours after the last treatment and dissected. In each case, a wild-type sample (lanes 1 and 5), heterozygous samples (lanes 2 and 6) and homozygous *GSK-3β*$^{FRB*/FRB*}$ animals are shown (lanes 3-4 and lanes 7-8). In vehicle controls (lanes 1-4), there is no detectable GSK-3βFRB* protein. In rapamycin treated animals, GSK-3βFRB* protein can be detected (lanes 5-8, top band).

g. In all experiments described, pregnant dams were injected subcutaneously with 4 doses of rapamycin (5 mg/kg each). Treatments were begun at the indicated time points and continued every 12 hours afterwards. Representative litter pictured was treated from e14.5 to e16.0 and harvested at e18.5. This litter included two mutant animals (overtly indistinguishable from wildtype). All animals are alive and show good colour and muscle tone.

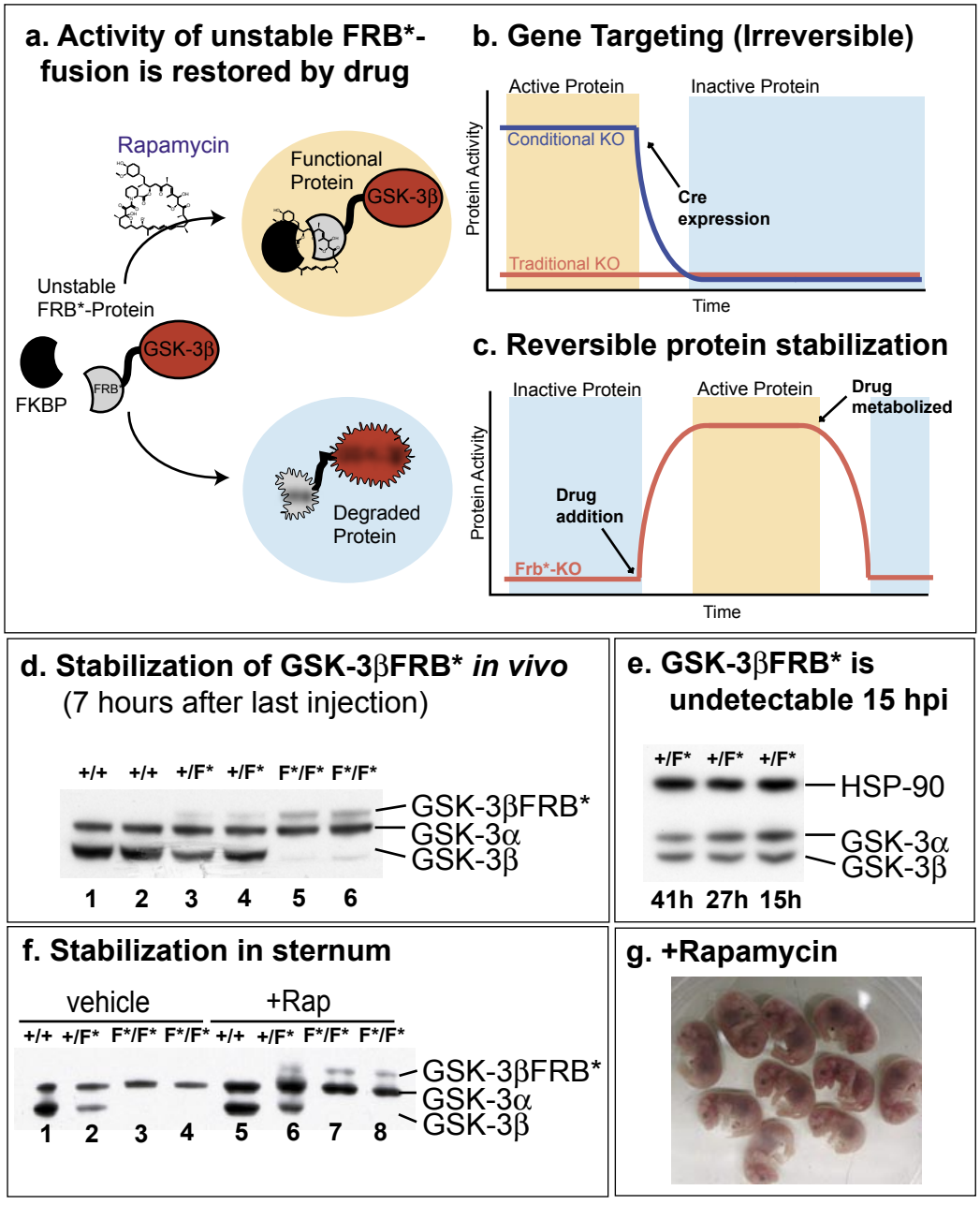
Supplementary Figure 2: Cell Proliferation and Cell Death

a. Wildtype and mutant animals showed comparable amounts of mitosis. (Error bars = s.d.) Animals were sectioned coronally at stage e13.0 (prior to palate closure) and e15.5 (after palate closure and before sternal fusion). Sections were then stained for phosphorylated-Histone H3 to mark mitotic cells. Positive cells in the tongue, palate and sternum were counted. Increase in positive cells in the e13.0 mutant palate is not statistically significant (*p=0.02).

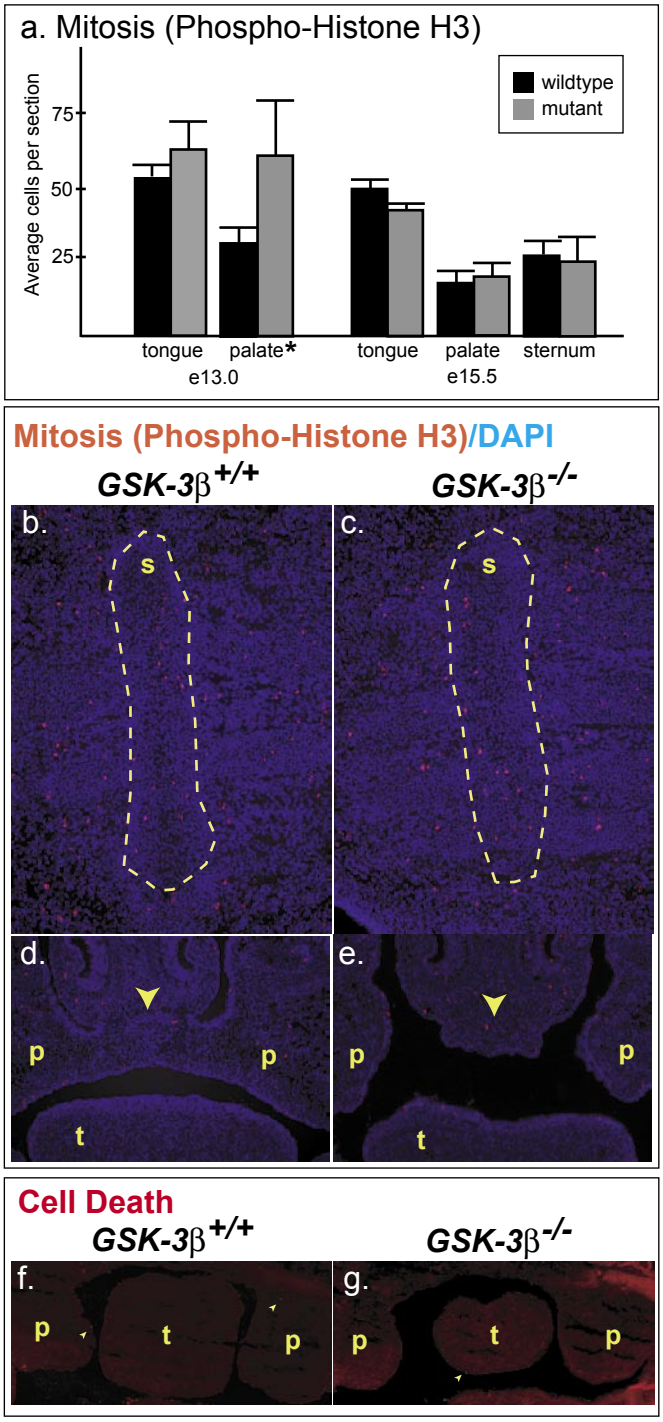
b-c. At e15.5, wildtype (b) and mutant (c) animals show similar levels of phosphorylated Histone H3 (stained in red) in the sternum (outlined with dashed line). S=sternum; Nuclei (DAPI stain) are stained in blue.

d-e. At e15.5, wildtype (d) and mutant (e) animals show similar levels of phosphorylated Histone H3 (marked in red) in the palatal shelves (p) and the tongue (t). The midline is marked with a yellow arrowhead; Nuclei (DAPI stain) is marked in blue.

f-g. Pictured here at e13.0, wildtype (f) and mutant (e), there is minimal cell death in the tongue (t) and palatal shelves (p) at all stages examined. The few positive cells are marked with yellow arrowheads.



Supplementary Figure S1



Supplementary Figure S2