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Tissue-selective expression of a conditionally-active ROCK2-estrogen receptor fusion protein

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Abstract

The serine/threonine kinases ROCK1 and ROCK2 are central mediators of actomyosin contractile force generation that act downstream of the RhoA small GTP-binding protein. As a result, they have key roles in regulating cell morphology and proliferation, and have been implicated in numerous pathological conditions and diseases including hypertension and cancer. Here we describe the generation of a gene-targeted mouse line that enables CRE-inducible expression of a conditionally-active fusion between the ROCK2 kinase domain and the hormone-binding domain of a mutated estrogen receptor (ROCK2:ER). This two-stage system of regulation allows for tissue-selective expression of the ROCK2:ER fusion protein, which then requires administration of estrogen analogues such as tamoxifen or 4-hydroxytamoxifen to elicit kinase activity. This conditional gain-of-function system was validated in multiple tissues by crossing with mice expressing CRE recombinase under the transcriptional control of *cytokeratin14* (*K14*), *murine mammary tumor virus* (*MMTV*) or *cytochrome P450 Cyp1A1* (*Ah*) promoters, driving appropriate expression in the epidermis, mammary or intestinal epithelia respectively. Given the interest in ROCK signaling in normal physiology and disease, this mouse line will facilitate research into the consequences of ROCK activation that could be used to complement conditional knockout models.

Introduction

The actomyosin cytoskeleton provides the physical structure that determines cell morphology; dynamic changes in cytoskeleton architecture are responsible for alterations in cell shape, adhesion, migration and division. Members of the Rho family of small molecular weight GTP-binding proteins (Rho GTPases) are key

regulators of cytoskeleton organization (Olson, 2016; Sadok and Marshall, 2014). Filamentous actin (F-actin) fibers associate with myosin motor proteins that hydrolyze ATP to power cytoskeletal contractile force generation (Geeves, 2016). Amongst the most important mediators of Rho GTPase signaling that influence contractile force are the serine/threonine kinases ROCK1 and ROCK2, which phosphorylate myosin regulatory light chains leading to increased myosin ATPase activity and consequent association with F-actin (Geeves, 2016; Julian and Olson, 2014; Rath and Olson, 2012).

There has been considerable interest in the biological functions of the ROCK kinases because of their apparent promise as chemotherapeutic targets in a variety of diseases and pathological conditions. The first described ROCK inhibitor Y27632 was discovered based on its ability to reverse agonist-induced contraction of smooth muscle, including aortic, mesenteric and coronary artery tissue samples, and to lower the blood pressure of hypertensive rats (Uehata *et al.*, 1997). Fasudil is a potent ROCK inhibitor, and has been routinely used in Japan to reduce the risk of cerebral vasospasm associated with subarachnoid hemorrhage (Satoh *et al.*, 2014). It has also been suggested that ROCK inhibitors could be used for cancer therapy to reduce primary tumor growth and to combat metastatic dissemination (Olson, 2008; Rath and Olson, 2012). Recently, a multi-AGC kinase inhibitor AT13148 that has been in Phase I clinical testing was shown to be a potent ROCK inhibitor with anti-tumor activity that also blocked melanoma cell migration and metastasis *in vivo* (Sadok *et al.*, 2015; Yap *et al.*, 2012).

Complete knockouts of *Rock1* or *Rock2* revealed important roles in the movement of epithelial sheets necessary for eyelid and ventral body wall closure and for the closure of umbilical rings (Shimizu *et al.*, 2005; Thumkeo *et al.*, 2005). Mice

that allow for the conditional knockout of *Rock1* or *Rock2* have been generated and used to examine how ROCK1 contributes to specific biological processing including reactive fibrosis in response to heart pressure overload (Zhang *et al.*, 2006) and insulin receptor signaling (Lee *et al.*, 2009). By combining conditional deletion of *Rock1* and *Rock2*, it was determined that together they make essential contributions to cell cycle progression, and have redundant and obligatory roles in non-small cell lung cancer and melanoma (Kümper *et al.*, 2016). These loss-of-function approaches can be very informative about the requirement for ROCK signaling in various contexts, and help to predict and validate the effect of inhibitor administration.

As a complement to loss-of-function systems or the use of pharmacological inhibitors to study ROCK signaling, we constructed conditionally-active forms of ROCK1 and ROCK2 by fusing the kinase domains with the hormone-binding domain of the estrogen receptor (ER) (Croft *et al.*, 2005; Croft *et al.*, 2004; Croft and Olson, 2006a). By treating cells with estrogen analogues including tamoxifen or 4-hydroxytamoxifen (4HT), it is possible to switch on ROCK2:ER fusion protein activity in a time and dose-dependent manner (Croft and Olson, 2006a). This conditional activation system has been used to study ROCK signaling and consequent actomyosin contractility in cell lines *in vitro* and *in vivo* (Croft *et al.*, 2005; Croft *et al.*, 2004; Croft and Olson, 2006a; Croft and Olson, 2006b; Kumar *et al.*, 2012; Sanz-Moreno *et al.*, 2011). The isolated kinase domains used in the ER-fusion proteins are similar to the fragments produced by caspase (Coleman *et al.*, 2001) or granzyme B mediated proteolysis (Sebbagh *et al.*, 2005), as well as examples of truncated protein products that result from somatic mutations in human tumors (Lochhead *et al.*, 2010). To examine the role of ROCK activation *in vivo,* we generated a

genetically engineered mouse line in which expression of a ROCK2:ER fusion protein was driven by the *cytokeratin 14* (*K14*) promoter (Samuel *et al.*, 2009). Conditional activation of ROCK2:ER was sufficient to induce epidermal hyperplasia and the conversion of chemically-induced cutaneous papillomas to invasive carcinomas (Samuel *et al.*, 2011) and also to accelerate the healing of full thickness skin wounds (Kular *et al.*, 2015).

To extend the utility of the conditionally-active ROCK2:ER system for gain-of-function studies including in development, we generated a genetically modified mouse line by targeting the *Hypoxanthine phosphoribosyltransferase* (*Hprt*) locus on the X chromosome with a construct in which ROCK2:ER transgene transcription from an artificial *chicken actin gene* (*CAG*) promoter (Niwa *et al.*, 1991) is dependent on CRE recombinase-mediated removal of a quadruple SV40 polyadenylation sequence transcriptional "stop" cassette flanked by *loxP* sites (Jackson *et al.*, 2001). By crossing with mice expressing CRE recombinase from a *K14* promoter, we found that it was possible in mouse epidermal keratinocytes to induce expression of the ROCK2:ER fusion protein that could then be conditionally activated with 4HT. This genetically modified mouse line will allow future studies aimed at characterizing the effects of ROCK gain-of-function in a tissue-specific manner to complement existing conditional *Rock1* and *Rock2* deletion mice.

Results and Discussion

To establish a conditionally active ROCK2 fusion protein (Figure 1A), a DNA sequence encoding the kinase domain (amino acids 5-553) of human *ROCK2* was joined to that encoding *enhanced green fluorescent protein* (*EGFP*) at the 5' end and an engineered version of the hormone binding domain of the *estrogen receptor* (*ER*)

at the 3' end, modified to include a G525R replacement that greatly reduces binding of 17β estradiol, to prevent activation by circulating estrogenic hormone, while retaining binding of 4HT (Danielian *et al.*, 1993) to allow activation by exogenous ligands. When treated with 4HT, conditional ROCK2:ER activation leads to the phosphorylation of substrates including the regulatory myosin light chains (MLC), the myosin binding subunit (MYPT) of the MLC phosphatase complex and LIM kinases 1 and 2 (LIMK1/2), which collectively results in actomyosin contractile force generation (Croft and Olson, 2006a). As a control for expression of the ROCK2:ER fusion protein that might have additional non-catalytic functions, we also made a kinase-dead version (KD2:ER) that has a single amino acid mutation (K125G) that blocks ATP-binding and kinase catalytic activity (Croft and Olson, 2006a).

HM1 embryonic stem cells lack the promoter and first two exons of the *Hprt* locus on the X-chromosome (Selfridge *et al.*, 1992). By targeting this locus with constructs containing flanking homology arms that include the human *HPRT* promoter and first exon as well as the second and third exon of mouse *Hprt,* it is possible to reconstitute a functional *Hprt* gene that enables positive selection of successful recombinants in hypoxanthine-aminopterin-thymidine (HAT) medium (Bronson *et al.*, 1996). In this way, we were able to specifically target the ROCK2:ER transgene to the *Hprt* locus (Figure 1B).

To allow for conditional expression of ROCK2:ER, the fusion protein coding sequence was cloned downstream of a synthetic promoter (*CAG*) consisting of a cytomegalovirus early enhancer element, a *chicken β-actin* gene promoter with the first exon and intron, and a splice acceptor (SA) from the *rabbit β-globin* gene (Niwa *et al.*, 1991), followed by a loxP-Stop-loxP (LSL) transcriptional stop cassette (Figure 1B). The inclusion of quadruple SV40 derived polyadenylation signals (Stop) within

this cassette leads to the premature termination of transcription, until CRE-mediated recombination of the loxP sites removes the Stop cassette to allow transcription to proceed (Figure 1C). By crossing with mice expressing CRE recombinase from tissue selective promoters, it would be possible to restrict ROCK2:ER expression to targets of choice, thereby enabling for determination of the consequences of ROCK activation in a site-specific manner.

To validate the inducibility of ROCK2:ER expression *in vivo*, *LSL-ROCK2:ER* mice were intercrossed with mice expressing a fusion protein comprising CRE recombinase and the hormone binding domain of the progesterone receptor (PR) under the control of the *K14* promoter (Figure 2A), which allows for conditional CRE activation by ligands including RU486 (Berton *et al.*, 2000) and expression of the ROCK2:ER fusion protein. Subsequent treatment with 4-hydroxytamoxifen would then activate the expressed ROCK2:ER fusion protein (Figure 2A). Polymerase chain reaction (PCR) analysis of DNA isolated from ear skin that had been topically treated with RU486 (2 µg in 20 µl ethanol per ear for 1 week) demonstrates the CRE and ligand-dependent recombination of *LSL-ROCK2:ER* (Figure 2B). In all cases, a *LSL-ROCK2:ER* 575 base pair (bp) PCR product was visible (Figure 2B, top and bottom panels), while a 600 bp CRE:PR transgene product was evident in the 4 positive samples (Figure 2B, middle panel). Only in the presence of both CRE:PR and RU486 treatment was there loxP-Stop-loxP recombination that led to the production of a 500 bp ROCK2:ER band (Figure 2B, lower panel). Furthermore, immunohistochemical (IHC) staining of untreated or RU486 treated ear skin with antibodies against EGFP (Figure 2C) or ROCK2 (Figure 2D) revealed ROCK2:ER expression restricted to the epidermal layer following CRE:PR stimulation with RU486. In mouse skin treated with RU486 to permit ROCK2:ER expression, the

additional topical application of 4HT (330 µg in 20 µl solution in ethanol) resulted in inCREased phosphorylation of the ROCK substrate MYPT1 on T696 (pMYPT1) (Figure 2E). Further confirmation of the CRE-dependent expression of ROCK2:ER was obtained by crossing *LSL-ROCK2:ER* mice with a mouse line that expressed constitutively active CRE from a *K14* promoter (Dassule *et al.*, 2000) (Figure 2F). When keratinocytes were isolated from mouse tails and cultured *in vitro* as we previously described (Samuel *et al.*, 2011), western blotting of cell lysates revealed that ROCK2:ER expression in *LSL-ROCK2:ER* mice was only observed in the presence of *K14-CRE*, similar to the ROCK2:ER expression in the *K14-ROCK2:ER* positive control keratinocytes (Samuel *et al.*, 2009). Similar to previous observations (Croft *et al.*, 2004), treatment with 1 µM 4HT increased the levels of ROCK2:ER protein in *K14-CRE; LSL-ROCK2:ER* and *K14-ROCK2:ER* keratinocytes (Figure 2F), likely through protein stabilization. These results confirm CRE-induced ROCK2:ER expression in *LSL-ROCK2:ER* mice in cytokeratin 14 positive epidermal keratinocytes.

To further validate the tissue-specific inducibility and functionality of ROCK2:ER in target tissues, the *LSL-ROCK2:ER* and *LSL-KD2:ER* mice were separately crossed onto the *Tg(MMTV-CRE)4Mam/J (MMTV-CRE)* (Wagner *et al.*, 1997) and *Tg(Cyp1a1-CRE)1Dwi (Ah-CRE)* (Ireland *et al.*, 2004) strains, which express CRE recombinase within the mammary and intestinal epithelia respectively (Figure 3A, 4A). These crosses yielded the further strains *MMTV-CRE; LSL-ROCK2:ER, MMTV-CRE; LSL-KD2:ER, Ah-CRE; LSL-ROCK2:ER* and *Ah-CRE; LSL-KD2:ER*. To determine whether ROCK2:ER could be expressed and conditionally activated within the mammary epithelium, twice weekly intra-peritoneal injections of tamoxifen (60 mg/kg body weight in corn oil) were administered to

cohorts of 8 week old *LSL-ROCK2:ER, LSL-KD2:ER, MMTV-CRE; LSL-ROCK2:ER* and *MMTV-CRE; LSL-KD2:ER* mice. Mice were sacrificed 24 hours after the final injection and sections derived from formalin-fixed paraffin-embedded mammary tissue were subjected to immunofluorescence analysis, revealing expression of the ROCK2:ER or KD:ER fusion proteins in *MMTV-CRE; LSL-KD2:ER* and *MMTV-CRE; LSL-ROCK2:ER* tissues (Figure 3B), accompanied by enhanced phosphorylation of the ROCK target MYPT1 at T696 in *MMTV-CRE; LSL-ROCK2:ER* mouse mammary epithelium but not in the *MMTV-CRE; LSL-KD2:ER* tissue (Figure 3C).

To determine whether ROCK2:ER could be expressed and conditionally activated within the intestinal epithelium, four intra-peritoneal injections of β-Naphthoflavone (80 mg/kg body weight per injection) were administered to cohorts of 8 week old *Ah-CRE; LSL-KD2:ER* and *Ah-CRE; LSL-ROCK2:ER* mice over 48 hours to induce intestine-specific CRE recombinase expression. Four days following the final injection of β-Naphthoflavone, mice were injected intraperitoneally with tamoxifen (60 mg/kg body weight in corn oil), with a further injection of tamoxifen a week later. Mice were sacrificed 24 hours after the final injection and sections derived from formalin-fixed paraffin-embedded intestinal tissue were subjected to immunofluorescence analysis, revealing enhanced phosphorylation of the ROCK target MYPT1 at T696 in *Ah-CRE; LSL-ROCK2:ER* mouse intestinal epithelium and not in the *Ah-CRE; LSL-KD2:ER* tissues (Figure 4B). Taken together, these results demonstrate that *LSL-ROCK2:ER* mice inducibly express conditionally-active ROCK2 in a tissue-specific manner.

As a test of the consequences of conditional ROCK2 activation in the whole mouse, *LSL-ROCK2:ER* and *LSL-KD2:ER* mice were separately crossed with *Tg(CAG-cre/Esr1*)5Amc (CAG-CRE:ER)* (Hayashi and McMahon, 2002) mice that

undergo CRE-mediated recombination in most tissues in response to tamoxifen treatment (Figure 5A). When mice were administered tamoxifen citrate in their drinking water (100 µg/mL) to induce CRE-mediated recombination and ROCK2:ER fusion protein activation, 6/6 *CAG-CRE:ER; LSL-ROCK2:ER* mice died within 7 days, while all tamoxifen treated *CAG-CRE:ER; LSL-KD2:ER* and untreated *CAG-CRE:ER; LSL-ROCK2:ER* mice survived over this time period (Figure 5B). When brains were dissected and examined histologically, it became apparent that all tamoxifen treated *CAG-CRE:ER; LSL-ROCK2:ER* mice had evidence of cerebral hemorrhagic lesions that were not observed in tamoxifen treated *CAG-CRE:ER; LSL-KD2:ER* (Figure 5C). These results suggest that there would be potential for the *CAG-CRE:ER; LSL-ROCK2:ER* transgenic mouse line to be used as an inducible cerebral stroke model.

Although loss-of-function genetically-modified mice have been useful in determining how specific targets may make essential contributions to various biological processes, there is also considerable value in gain-of-function (GoF) systems. Rather than inferring how a given protein might be involved from a loss of effect, GoF mutants can be used to determine biological roles by observing direct positive effects. We have described a GoF system that allows for the conditional activation of ROCK2 activity in a tissue selective manner, which has a wide-variety of potential applications for the study of tissue homeostasis and function, as well as in models of pathological conditions and diseases including cancer.

Methods

Animal models

Conditional *LSL-ROCK2:ER* and control *LSL-KD2:ER* mice were constructed by targeting *loxP-Stop-loxP ROCK2:ER* or *KD2:ER* transgenes under the transcriptional control of a *CAG* promoter by gene-targeting each to the *Hprt* locus using an approach we have previously described (Samuel *et al.*, 2009; Schachtner *et al.*, 2012). The *CAG loxP-Stop-loxP* transgene was made by first replacing the SA region of the plasmid pBigT (gift of Frank Costantini; Addgene plasmid 21270) (Srinivas *et al.*, 2001) with the chimeric synthetic promoter *CAG* (Niwa *et al.*, 1991) from the plasmid pTurbo-CRE (a gift from Prof. Tim Ley, The Genome Center at Washington University, St. Louis, USA). The *3-phosphoglycerate kinase* (*PGK*) promoter and EM7 promoter sequence from pL452 were then inserted into pBigT.*CAG* by recombineering in *E. coli* (Liu and Bambara, 2003). Short 5′ and 3′ homology arms from the *Hprt* targeting plasmid pSKB1 (Bronson *et al.*, 1996) were placed flanking the expression cassette to generate pHprt.*CAG.loxP-STOP-loxP*. ROCK2:ER or KD2:ER fusion protein cDNAs were then cloned downstream of the *loxP-Stop-loxP* (*LSL*) transcriptional termination cassette. The transgene was then recombineered into pSKB1 (Bronson *et al.*, 1996) to generate the final targeting pHprt-*CAG-LSL-ROCK2:ER* or pHprt-*CAG-LSL-KD2:ER* vectors.

The targeting vector was linearized and transferred into *Hprt*-deficient HM1 embryonic stem (ES) cells (Magin *et al.*, 1992) by electroporation, which were then cultured on DR4 mouse embryonic fibroblast feeder layers (Tucker *et al.*, 1997). Recombinants were selected in HAT supplement containing medium (Sigma) and appropriate targeting of the *Hprt* locus on 5′ and 3′ sides was confirmed by PCR using genomic DNA template prepared from HAT-resistant colonies. Genotyping

used the Expand™ Long Template PCR system (Roche) according to the manufacturer's instructions. Primer pairs for genotyping the targeted ES cells were 5′ GTTGCTGAGGCAAAAATAGTGTAAT and CCATTTACCGTAAGTTATGTAACGC and 3′ CTACCTAGTGAGCCTGCAAACTG and ATGTAAGTGCTAGGAATTGAACCTG.

Following identification of correctly targeted clones, mouse lines were derived by injection into C57BL/6J blastocysts according to standard protocols (Nagy et al., 2003). Germline transgene transmission was identified by inspection of progeny coat colour and confirmed by PCR.

All procedures were performed under appropriate licenses and according to the UK Home Office guidelines or under approval granted by the SA Pathology/CALHN Animal Ethics Committee and according to Australian Code for the Care and Use of Animals for Scientific Purposes, 8th edition (2013). Mouse lines described herein will be made available to the research community.

Polymerase chain reaction (PCR)

2.5 µl of genomic DNA was used as a template in a 25 µl reaction volume containing 2.5 mM $MgCl_2$, 0.5 µM reverse and forward primers for each transgene, 0.25 mM dNTPs and 1 unit Taq DNA polymerase (1 U/µl) in a 200 µl PCR tube. PCR was performed for each transgene in a thermal cycler. Electrophoresis was performed on the PCR products using 1% agarose gels that were stained with ethidium bromide. *ROCK2* primers: Forward CGACCACTACCAGCAGAACA; Reverse GACGAACCAACTGCACTTCA. *K14-CRE:PR* primers: Forward CGGTCGATGCAACGAGTGAT; Reverse CCACCGTCAGTACGTGAGAT.

Keratinocyte isolation and culture

Primary keratinocytes were extracted from tail skin as described previously (Samuel *et al.*, 2011). Cells were maintained at 37°C, with 5% CO_2 and 3% O_2, in keratinocyte growth medium (KGM-2; Lonza) and 50 μM Ca^{2+} on collagen-1 coated dishes (10 μg/mL; Advanced BioMatrix 5005).

Small molecules

4-Hydroxytamoxifen (Sigma H7904), RU486 (Sigma M8046), β-Naphthoflavone (Sigma N3633), tamoxifen (Sigma T5648), tamoxifen citrate salt (Sigma T9262).

Immunoblotting

Standard protocols were used for Western blot analysis. Whole cell lysates were prepared in cell lysis buffer (1% SDS (w/v), 50 mM Tris pH 7.5) and protein concentration was determined by bicinchoninic assay (Sigma). ROCK1/2 (Millipore 07-1458) or GFP (Abcam ab6556) primary antibodies were used at 1:500 dilution. Alexa-Fluor 680-conjugated (Thermo Fisher Scientific) secondary antibody was detected by infrared imaging (Li-Cor Odyssey).

Immunohistochemistry and immunofluorescence analysis

Formalin-fixed, paraffin-embedded sections were rehydrated and immersed in 10 mM citric acid buffer at pH 6.0, boiled for 20 min, cooled, and sequentially blocked with 3% H_2O_2 and 10% normal goat serum in PBS. Sections were then incubated with primary antibody, followed by incubation with Envision + System-HRP labeled Polymer (Dako). Primary antibodies were routinely used at 1:50 or 1:100 dilutions. Staining was visualized with Liquid DAB + Substrate (Dako). Immunofluorescence

analysis was carried out as for immunohistochemistry, eliminating the peroxide blocking step. HRP-labeled secondary antibodies were replaced by Alexa Fluor 488 (green) or 594 (red)-conjugated secondary antibodies (Thermo Fisher Scientific) and slides were mounted in VECTASHIELD mounting medium (Vector Labs). Primary antibodies used: ROCK2 (Santa Cruz SC-5561), GFP (Clontech 632377), pMYPT1 (Thr696; Millipore ABS45), cytokeratin 14 (Leica Biosystems). Photomicrographs were obtained with a Zeiss microscope and Axiovision image capture software. Immunofluorescence images were acquired using an LSM 700 confocal system (Zeiss) or using a Leica SCN 400f scanner and Leica Slidepath Digital Hub software, respectively.

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Figure Legends

Figure 1. Targeting a conditionally-active *loxP-Stop-loxP ROCK2:ER* transgene to the *Hprt* locus. (**A**) The conditionally-active ROCK2:ER fusion protein is inactive in the absence of ligand. Upon stimulation with estrogen analogues including 4-hydroxytamoxifen (4HT), the specific activity of the kinase domain of ROCK2 increases leading to phosphorylation of substrates including MYPT1, MLC and LIM kinases 1 and 2 (LIMK1/2), which results in the generation of actomyosin contractile force. EGFP = enhanced green fluorescent protein. ER = modified hormone binding domain of the estrogen receptor. (**B**) The *Hprt* locus in mouse HM1 embryonic stem cells lacks a promoter and first two exons, allowing for targeting by the homology arms of the pHprt-*CAG-LSL-ROCK2:ER* plasmid that includes a promoter and first exon of human *HPRT* (yellow) and second exon of mouse *Hprt*. Successful targeting and homologous recombination reconstitutes an active *Hprt* locus that allows for positive selection with HAT medium. (**C**) By crossing with mice expressing CRE recombinase from tissue-selective promoters, CRE-mediated recombination between loxP sites removes the transcription termination cassette (STOP) to allow ROCK2:ER transgene expression.

Figure 2. Validation of *LSL-ROCK2:ER* expression in mouse skin. (**A**) *LSL-ROCK2:ER* mice were crossed to a line expressing a CRE fusion with the hormone-

binding domain of the progesterone receptor (PR) from a *K14* promoter. Treatment with the progesterone ligand RU486 enables recombination and consequent ROCK2:ER expression. Stimulation with 4-hydroxytamoxifen then stimulates ROCK2:ER catalytic activity. (**B**) PCR analysis of the *ROCK2:ER* and *CRE* transgenes in mouse ear skin without or with RU486 treatment. Unrecombined *LSL-ROCK2:ER* results in the production of a 575 bp PCR band in all samples, while a 600 bp *CRE:PR* is seen only in the 4 transgene expressing mice. Following RU486 treatment, recombination resulted in the production of a 500 bp PCR band, indicating dependence on both *CRE* expression and RU486 treatment. (**C, D**) Validation of *LSL-ROCK2:ER* recombination and consequent ROCK2:ER expression in mouse skin following topical RU486 treatment by EGFP or ROCK2 immunohistochemical staining. Scale bar = 100 μm. (**E**) Treatment with RU486 does not lead to increased MYPT1 phosphorylation (pMYPT1) unless combined with topical 4HT treatment to conditionally activate ROCK2:ER. (**F**) Validation of LSL-ROCK2:ER expression in epidermal keratinocytes following crossing with K14-CRE expressing mice. Primary keratinoctyes were isolated from the tails of mice with *LSL-ROCK2:ER* alone, *LSL-ROCK2:ER* plus *K14-CRE* or *K14-ROCK2:ER* as indicated. A ROCK2:ER protein band was only detected by blotting with ROCK2 or GFP antibodies in keratinocytes from *LSL-ROCK2:ER* plus *K14-CRE* or positive control *K14-ROCK2:ER* mice. Consistent with previous observations, treatment with 4HT increased ROCK2:ER protein levels relative to untreated cells.

Figure 3. Validation of *LSL-ROCK2:ER* expression in mouse mammary epithelium. (**A**) *LSL-ROCK2:ER* and *LSL-KD2:ER* mice were crossed separately onto a strain expressing a CRE recombinase under the control of the *MMTV* promoter. Mice will

express the ROCK2:ER or KD2:ER fusion protein constitutively, but transgene-derived ROCK will not be activated until systemic treatment with tamoxifen, which is metabolized to 4HT and activates ROCK2:ER but not kinase-dead KD2:ER. (**B**) Immunofluorescence analysis of mammary tissues derived from *LSL-KD2:ER, LSL-ROCK2:ER, MMTV-CRE; LSL-KD2:ER* or *MMTV-CRE; LSL-ROCK2:ER* mice showing expression of the EGFP:KD2:ER or EGFP:ROCK2:ER fusion proteins in CRE-expressing mice. Scale bars = 50 μm (**C**) Immunofluorescence analysis (cytokeratin 14 [white in monochromatic panels and red in Merge] to mark basal mammary epithelium and pMYPT1 [white in monochromatic panels and green in Merge]) of mammary tissue derived from *LSL-KD2:ER, LSL-ROCK2:ER, MMTV-CRE; LSL-KD2:ER* and *MMTV-CRE; LSL-ROCK2:ER* mice that have been treated systemically with tamoxifen, showing elevated phosphorylation of MYPT1 in CRE-expressing *LSL-ROCK2:ER* mammary epithelium but not *LSL-KD2:ER* expressing tissues. Scale bars = 50 μm.

Figure 4. Validation of *LSL-ROCK2:ER* expression in mouse intestinal epithelium. (**A**) *LSL-ROCK2:ER* and *LSL-KD2:ER* mice were crossed to a line expressing a CRE recombinase under the control of the cytochrome P450 *Cyp1A1* (Ah) promoter. Mice will express the ROCK2:ER or KD2:ER fusion protein upon activation of the *Cyp1A1* promoter by systemic treatment with β-Naphthoflavone, but transgene-derived ROCK will not be activated until systemic treatment with tamoxifen, which is metabolized to 4HT and activates ROCK2:ER. (**B**) Immunofluorescence analysis (pMYPT1 [green]) of intestinal epithelium derived from *Ah-CRE; LSL-KD2:ER* and *Ah-CRE; LSL-ROCK2:ER* mice that have been treated systemically with tamoxifen,

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showing elevated phosphorylation of MYPT1 in CRE-expressing *LSL-ROCK2:ER* intestinal epithelium but not *LSL-KD2:ER* tissues.

Figure 5. Consequence of conditional activation of ROCK2:ER in whole mice. (**A**) *LSL-ROCK2:ER* and *LSL-KD2:ER* mice were crossed to a line expressing a CRE:ER fusion recombinase under the control of an artificial chicken *actin gene* (*CAG*) promoter. Mice express the ROCK2:ER or KD2:ER fusion protein in a tamoxifen-induced manner in most tissues. Systemic treatment with tamoxifen, which is metabolized to 4HT, activates ROCK2:ER but not KD2:ER fusion protein. (**B**) Survival curves for *CAG-CRE:ER; LSL-ROCK2:ER* and *CAG-CRE:ER; LSL-KD2:ER* mice that were untreated or treated with tamoxifen as indicated. Survival p-value determined by Mantel-Cox test (*CAG-CRE:ER; LSL-ROCK2:ER* untreated n = 3; *CAG-CRE:ER; LSL-ROCK2:ER;* tamoxifen treated n = 6: *CAG-CRE:ER; LSL-KD2:ER* untreated n = 3). (**C**) Brain sections from tamoxifen treated *CAG-CRE:ER; LSL-KD2:ER* (left) and *CAG-CRE:ER; LSL-ROCK2:ER* (middle) mice. Scale bars = 1 mm. Red and blue rectangle insets indicate sites of cerebral hemorrhagic lesions in the *CAG-CRE:ER; LSL-ROCK2:ER* image are shown at higher magnification at right with corresponding borders. Scale bars = 100 μm.

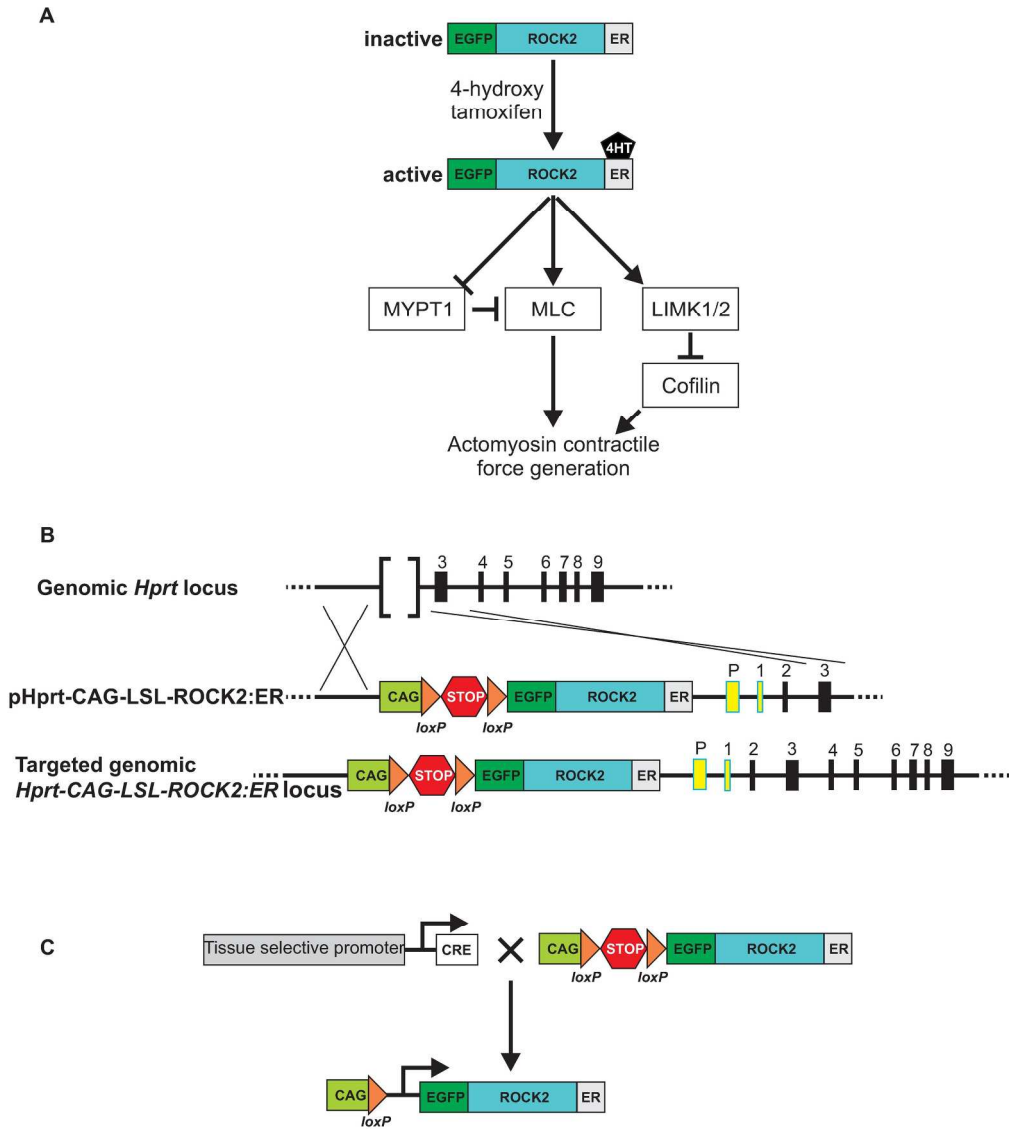

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Figure 1
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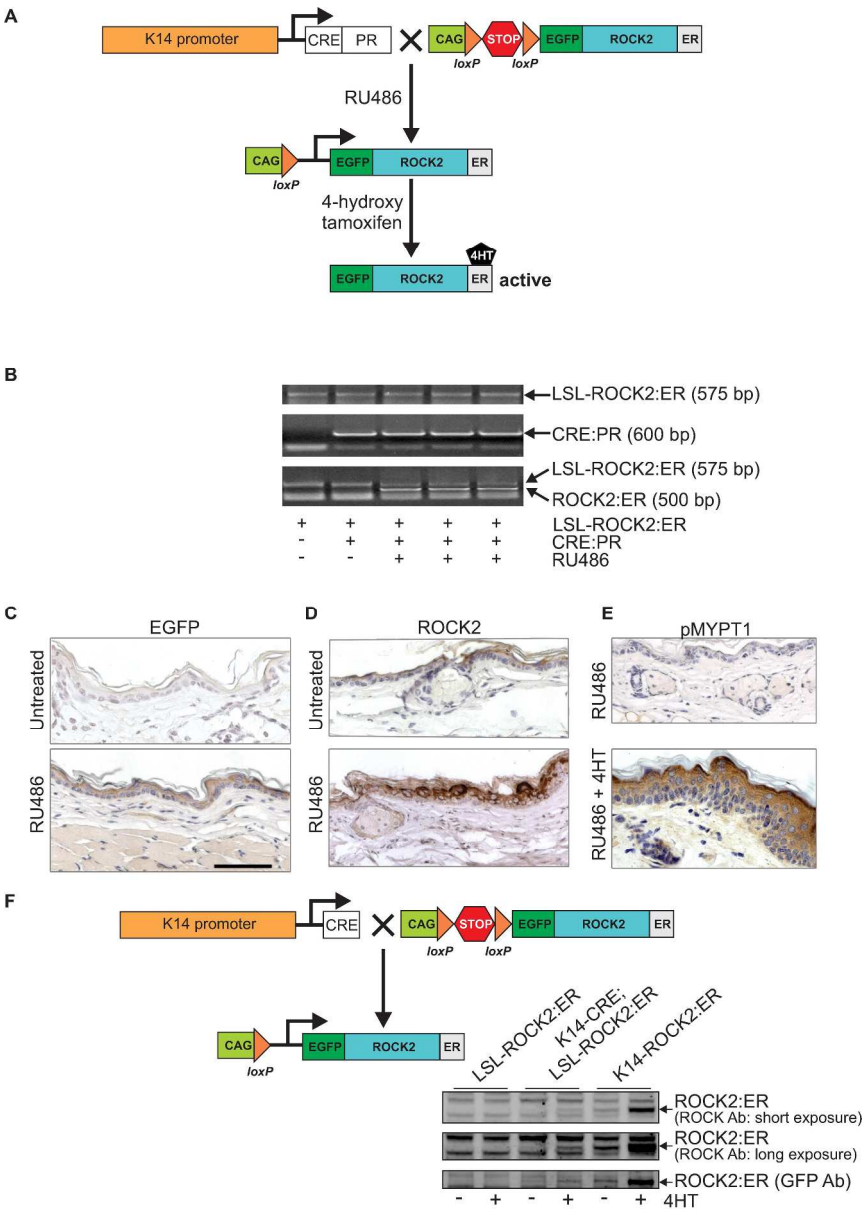

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Figure 2
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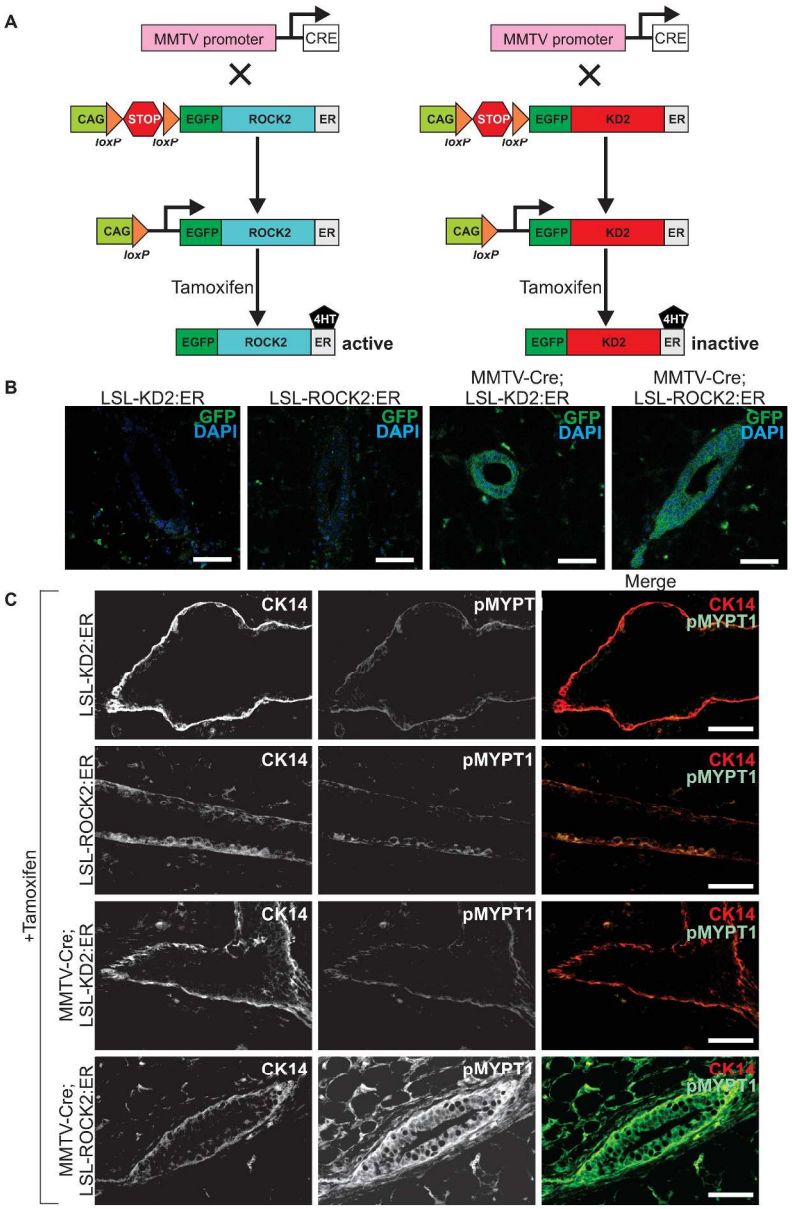

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Figure 3
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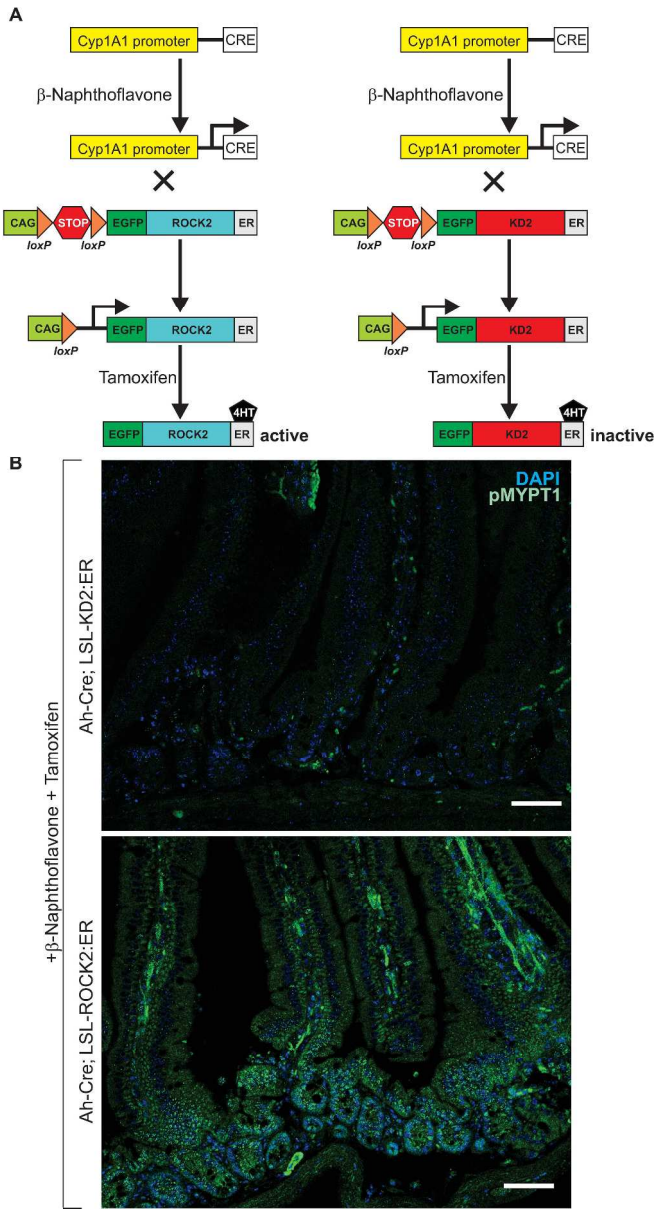

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Figure 4
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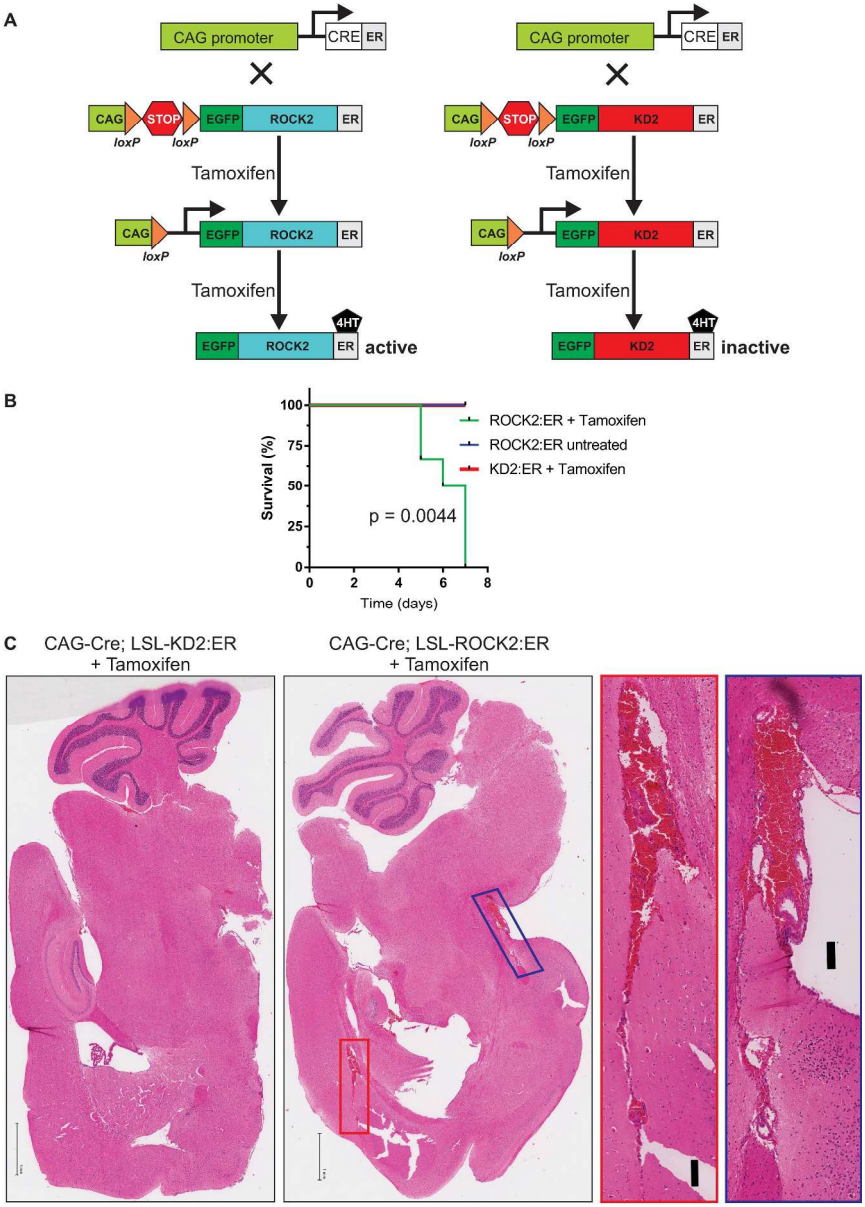

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Figure 5