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RUSH & CRUSH: a rapid and conditional RNA interference method in mice

Juliana R. Brown^{1,2,4,*}, Bernd Zetsche^{1,2,*}, and Laurie Jackson-Grusby^{1,2,3,4,*}

¹Pathology Department and Kirby Center for Neuroscience, Children's Hospital Boston, 300 Longwood Ave, Boston, MA 02115

²Harvard Stem Cell Institute, Boston, MA 02115

³Biological and Biomedical Sciences Program, Harvard Medical School, Boston, MA 02115

⁴Pathology Department, Harvard Medical School, Boston, MA 02115

Abstract

RNA interference (RNAi) is a powerful approach to phenocopy mutations in many organisms. Gold standard conventional knock-out mouse technology is labor- and time-intensive, however off-target effects may confound transgenic RNAi approaches. Here we describe a rapid method for conditional and reversible gene silencing in RNAi transgenic mouse models and embryonic stem (ES) cells. RUSH and CRUSH RNAi vectors were designed for reversible or conditional knockdown, respectively, demonstrated using targeted replacement in an engineered ROSA26^{lacZ} ES cell line and wildtype V6.5 ES cells. RUSH was validated by reversible knockdown of Dnmt1 in vitro. Conditional mouse model production using CRUSH was expedited by deriving ES cell lines from Cre transgenic mouse strains (nestin, cTnnT, and Isl1) and generating all-ES G₀ transgenic founders by tetraploid complementation. A control CRUSH^{GFP} RNAi mouse strain showed quantitative knockdown of GFP fluorescence as observed in compound CRUSH^{GFP}, Ds-Red Cre-reporter transgenic mice, and confirmed by western blotting. The capability to turn RUSH and CRUSH alleles off or on using Cre recombinase enables this method to rapidly address questions of tissue-specificity and cell autonomy of gene function in development.

Keywords

RNAi; ES cells; transgenesis; mouse models

Introduction

Gene silencing through RNA interference (RNAi) is a widely implemented “reverse genetics” approach for defining loss or reduction-of-function phenotypes in diverse

Correspondence: Laurie Jackson-Grusby, Children's Hospital Boston, 300 Longwood Avenue, Boston, MA 02115, Phone: 617-919-2104, FAX: 617-730-0148, laurie.jackson-grusby@childrens.harvard.edu.

*These authors contributed equally

Author Contributions

L.J.-G., J.R.B. and B.Z. designed the experiments; all authors performed experiments and interpreted the data; J.R.B and L.J.G wrote the manuscript; all authors reviewed the manuscript.

eukaryotic organisms (Hannon and Rossi, 2004). In mice, the gold-standard approach for disease modeling and examining gene function *in vivo* is knock-out technology (Rajewsky *et al.*, 1996). However, despite a considerable global effort to create a genome-wide mouse model resource, the EUCOMM and KOMP repositories of knockout mice are incomplete and *de novo* knockout model generation and validation remains laborious and time intensive (Ryder *et al.* 2013). RNAi offers a more rapid approach to control endogenous gene expression through inducible or reversible construct design (Dickins *et al.*, 2007; Premssirut *et al.*, 2011; Seibler and Schwenk, 2010; Vidigal *et al.*, 2010). Use of RNAi transgenesis in mice, however, has yielded more limited successes as compared to gene targeting approaches. Several reports have identified technical issues associated with high levels of shRNA expression, including increased risk of off-target gene silencing (Jackson and Linsley, 2010), interference with endogenous micro-RNA functions (Grimm *et al.*, 2006; Khan *et al.*, 2009), and physiological complications of cellular toxicity and animal morbidity (Ehlert *et al.*, 2010; Liu and Berkhout, 2011; McBride *et al.*, 2008). Here we demonstrate a robust and efficient method, termed RUSH & CRUSH, for generation of conditional single-copy transgenic RNAi mouse strains requiring less work and providing substantial time savings compared with established methods. We created an engineered ES cell line to facilitate this approach, and we addressed potential confounding effects of RNAi transgenes using two proof-of-method mouse strains.

Results

We designed a pair of vectors, RUSH (ROSA26 U6 short hairpin) and CRUSH (Conditional RUSH), which incorporate a green fluorescent protein (GFP) gene-trap at the ROSA26 locus (Soriano, 1999) coupled to Pol III driven Cre-lox regulated shRNA cassettes (Ventura *et al.*, 2004), enabling knockdown with Cre-rescue or conditional knockdown, respectively (Fig. 1a). Knock-in at a defined locus controls for position effects and the GFP gene trap facilitates screening for targeted integration in mouse embryonic stem (ES) cells (outlined in Supplementary Fig. 1). The reversible RUSH vector uses Cre-mediated recombination to eliminate the shRNA, causing target gene reactivation for testing reversion of knockdown phenotypes by “conditional-rescue.” By contrast, the inducible CRUSH vector shRNA is silent until Cre-mediated deletion of a stop sequence (Fig. 1a).

To enable doxycycline-inducible rescue of target gene expression, we derived custom ES cells from the tet-operator controlled LC1 Cre transgenic strain (Schönig *et al.*, 2002), and engineered the Z•LCR ES cell line (Fig 1b). This line incorporates a Rosa26-lacZ reporter allele, a bidirectional tet-operator that expresses both Cre and luciferase upon tetracycline induction and a reverse tetracycline transactivator (rtTA) knocked into the second Rosa26 allele. Upon targeted replacement of the ROSA26-lacZ allele by the RUSH vector, lacZ expression is abolished, which allowed detection of residual lacZ expression using parallel cultures of picked ES cell colonies to screen for mosaic targeted clones (Fig. 1c). In combination with fluorescence-guided selection of ES colonies mediated by the GFP gene-trap cassette, histochemical staining for loss of β -galactosidase activity allows identification of homologously recombined clones with 100% fidelity, 33-fold more efficiently than random selection (Fig. 1d).

To validate RNAi efficacy and rescue, RUSH vectors were engineered to express shRNAs for GFP or for Dnmt1 and targeted in Z•LCR ES cells. Analysis of ROSA26-GFP knockdown was determined using flow cytometry by comparing RUSH^{GFP}, Cre transfected RUSH^{GFP}, and parental Z•LCR ES cells. The results reveal an approximately 15-fold reduction in GFP intensity between RUSH^{GFP} and the “rescued” cells that deleted the shRNA (Fig. 1e). Knockdown of endogenous Dnmt1 in RUSH^{Dnmt1} cells was confirmed by western blotting (Fig. 1f). Cre-mediated deletion of the Dnmt1 shRNA was induced by treatment of the ES lines with 1 µg/ml doxycycline for 2, 6, 12 or 24 hours to activate Cre expression, and quantitative reactivation of Dnmt1 RNA and protein expression was observed within 12 hours (Fig. 1f and data not shown).

We sought to test this approach *in vivo* by constructing a control RNAi mouse strain exhibiting conditional expression of the validated GFP shRNA upon Cre-recombination. To this end, we constructed CRUSH^{GFP} (Fig. 2a), engineered targeted clones in V6.5 ES cells, and generated sibling knockdown clones by transient transfection with Cre. Flow cytometry revealed a 95% knockdown of GFP (Figs. 2b, 2d). Using these CRUSH^{GFP} V6.5 clones in tetraploid complementation (Eggan *et al.*, 2001; Kunath *et al.*, 2003; Nagy *et al.*, 1990) yielded all-ES derived transgenic neonates readily identified by widespread GFP expression (Supplementary Fig. 1). We tested this allele in combination with two Cre deleter lines. When CRUSH^{GFP} mice were crossed to Isl1-Cre transgenics (Yang *et al.*, 2006), in which Cre is expressed in early second heart field progenitors (Laugwitz *et al.*, 2008), we observed strong GFP knockdown in hearts of double-transgenic animals (Fig. 2c). CRUSH^{GFP} mice crossed to the neural stem cell deleter Nestin-Cre transgenic strain (Tronche *et al.*, 1999) revealed 71% knockdown of GFP fluorescence compared to CRUSH^{GFP} alone as analyzed by flow cytometry, but negligible reduction (0.2%) in control spleen tissue (n=6 for each, Fig. 2d). We also observed reduced GFP by E12.5 embryonic brain by fluorescence microscopy in compound CRUSH^{GFP}; Nestin-Cre transgenics (Fig. 2e) commensurate with Nestin-Cre activity *in vivo* (data not shown).

We used a quantitative neurosphere clonal plating assay to examine toxicity of the GFP shRNA in single copy as compared with high copy lentivirus-mediated (Ventura *et al.*, 2004) GFP shRNA expression on self-renewal of neural stem-like cells. No significant consequence of GFP RNAi was measurable in a neurosphere formation assay of neural stem cell self-renewal in CRUSH^{GFP};Nestin-Cre cells compared to littermate control cultures (ANOVA, Fig. 2f, right panel). By contrast, lentiviral shGFP expression significantly reduced the frequency of neurosphere formation (Student's t-test, $p < 0.0001$) compared to cells infected with an empty vector control (Fig. 2f, left panel, see Methods). These data suggest that single-copy shRNA expression can mediate efficient gene knockdown while exhibiting reduced cellular toxicity in neural stem cells.

To functionally parse knockdown from non-recombined cells *in vivo*, we engineered a contrasting ROSA26-DsRed2 Cre reporter allele (R26DsRedR Fig. 3a) and established a dual-color RNAi “sensor” mouse strain in which Cre expression causes the induction of both DsRed fluorescence and the GFP shRNA. Mice carrying the R26DsRed^R; CRUSH^{GFP} and Nestin-Cre alleles showed efficient GFP knockdown in eyes and clonogenic neural stem cells concomitant with activated DsRed2 expression (Fig. 3b); GFP knockdown and

efficiency of self-renewal remained stable through serial passaging of the neurospheres (data not shown). Analyses of dissected lens tissue confirmed potent GFP knockdown by fluorescence microscopy (Fig. 3c) and western blotting (Fig. 3d). Together, these data validate the Cre-dependent tissue-specificity of the approach.

A schematic of the RUSH & CRUSH process illustrates the rapid timeline for creation of global and tissue-specific knockdown mouse models using these improved alleles (Fig. 3e). Introducing constructs into custom-derived Cre deleter ES cell lines and generating founders using tetraploid complementation eliminated two mouse breeding cycles as compared with the standard conditional targeting method. High efficiencies of founder (G_0) mouse generation were observed using both the established V6.5 and newly-isolated Cre-transgenic (Cre^{tp}) ES lines by this method (Fig. 3f). To illustrate this, we generated conditional *Bmi1* knockdown mice using a *CRUSH^{Bmi1}* vector transfected into ES cells derived from nestin-Cre mice. Targeted ES cells transfected with Cre to delete the stop cassette *in vitro* yielded *CRUSH^{Bmi1}* clones with $18 \pm 4.8\%$ residual *Bmi1* mRNA expression (Fig. 3g). Compound transgenic nestin-Cre;*CRUSH^{Bmi1}* mice were generated by tetraploid complementation. Founders crossed with R26DsRed^R (Fig. 3h) recapitulated the deficit observed in *Bmi1* knockout mice (Molofsky *et al.*, 2003) in clonogenic neural stem cell self-renewal (Fig. 3i). Together, these data demonstrate *in vitro* validation of an shRNA in ES cells and rapid generation of conditional mouse lines for *in vivo* analysis.

Discussion

The approach to mouse RNAi transgenesis we describe incorporates single-copy shRNA expression, Cre-lox based conditional knockdown, and reversion rescue to fulfill the principles of an effective RNAi experimental system (Hannon and Rossi, 2004). Premsirat *et al.* have described a parallel system for doxycycline-inducible shRNA transgenes that rely upon tet-transcription factors for tissue-specific induction (Premsrirut *et al.*, 2011). Our complementary system permits a choice of Cre-off (reversion rescue) and Cre-on (conditional) constructs with high efficiency of recovering targeted ES cell colonies, and the use of Cre deleter ES cell lines and tetraploid complementation enables the streamlined creation of transgenic RNAi mouse lines. The independent genomic targeting sites and induction strategies described by Premsirat *et al.* and in this study offer versatility in transgenic RNAi experimental design for use of tet-inducible or Cre-lox regulated approaches respectively.

Despite numerous reported vector systems and strategies for rapidly generating transgenic RNAi mice (Dickins *et al.*, 2007; Hitz *et al.*, 2007; Premsrirut *et al.*, 2011; Seibler and Schwenk, 2010; Vidigal *et al.*, 2010; Yu and McMahon, 2006), none of these approaches have been widely adopted to date. Our results, along with a recent validation study of the EUComm and KOMP pipelines, revealed significant inefficiencies are caused by mosaic ES lines produced during gene targeting (Ryder *et al.*, 2013, Fig. 1c). We detected ES mosaicism initially from inconsistent RNA knockdown between sibling “isogenic” clones, which we suspect contributes substantially to the lack of reliability of transgenic RNAi more generally. Our method circumvents this problem in two ways. First, we have incorporated a counter screening for replacement of *lacZ* in our using a customized mouse ES cell line.

Second, we assess the uniformity of clonal GFP expression during the expansion of targeted ES lines, which is generalizable to other ES cell lines.

We envisage improved reliability of transgenic RNAi using the technical nuances we describe here will advance numerous applications in mouse physiology and development.. Moreover, the uniquely complementary RUSH and CRUSH alleles will facilitate analysis of cell autonomous gene function. An appropriate Cre deleter crossed separately with RUSH and CRUSH strains would generate reciprocal knockdown patterns, namely, target knockdown in all tissues except the lineage of interest (“conditional rescue”), or conditional gene knockdown within the lineage of interest, respectively. Production of global and conditional knockdown embryos or mice also provides a rapid means to create cohorts for validating hits from genome-wide based screens in the physiologic context of a transgenic mouse. Lastly, the CRUSH^{GFP} mouse strain we describe is also a useful control to substantiate “on-target” phenotypes observed in other transgenic knockdown strains.

Methods

Construction of RUSH & CRUSH and ROSA26-DsRed^R vectors

RUSH and CRUSH targeting vectors were constructed by modification of pRosa26-1, a ROSA26 genomic targeting plasmid,(Soriano, 1999) by deleting the HpaI site, converting the XhoI site to AscI, and cloning a splice acceptor-GFP-polyA into the XbaI site. Cre-lox regulated U6 cassettes derived from pSICO^R and pSICO lentiviral vectors(Ventura *et al.*, 2004) were modified by replacing the lentiviral GFP gene with drug selection markers (pgk-neo or pgk-puro), and cloned into the XbaI site 3' of GFP. Unique HpaI and XhoI sites were maintained for single step short hairpin oligonucleotide cloning in the design of the RUSH & CRUSH vectors, which was preserved from the pSICO system vectors. The ROSA26-DsRed Cre reporter allele was constructed by replacing the GFP in the genetrap cassette with DsRed2-N1 (Clontech), and insertion of a loxP flanked neomycin resistance stop cassette(Soriano, 1999) between the splice acceptor and DsRed. Plasmid vectors will be available from Addgene.

Construction of shRNA plasmids

Targets were selected using the program pSicoOligomaker1.5 (Ventura *et al.*, 2004) to identify 19-mer sequences on the basis of thermodynamic profiles, and screened using BLAST(Altschul and Gish, 1996) for seed-complement frequency filtering. Double-stranded shRNA oligo inserts were custom synthesized (Invitrogen), (see Supplementary Fig. 1), we treated with T4 phosphonucleotide kinase (NEB), annealed by slow cooling from 100°C to 4°C. Annealed oligos were diluted to 500nM, and 1µl of diluted oligos was ligated to 75ng of HpaI and XhoI digested vector. Oligo insertion was confirmed by bacterial colony PCR screening. RUSH primers, RUSH-for 5' CACAAAAGGAACTCACCTAA 3' and RUSH-rev 5' CTAAAGCGCATGCTCCAGAC 3', yielded 231bp and 177bp for positive and negative products, respectively; CRUSH primers, CRUSH-for 5' CATCAGAAGCTGGTCTGACTCT 3' and CRUSH-rev 5' GGGCCACAACCTCCTCATAAA 3' yielded 429bp and 374bp products from insert positive

and vector alone respectively. DNA from positive clones was purified (Qiagen) and sequence analyzed using RUSH-for or CRUSH-rev primers.

ES cell culture and line derivation

ES cells were cultured on irradiated mouse embryonic fibroblasts (MEFs) in DMEM (Invitrogen), 20 mM HEPES, 14 mM sodium bicarbonate (Sigma), pH adjusted to 7.3, supplemented with 15% fetal calf serum (Millipore), 0.1 mM non-essential amino acids (Invitrogen), 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma), 1000 U/ml leukaemia inhibitory factor (Millipore).

For derivations of new cre transgenic ES lines, Nestin-cre(Tronche *et al.*, 1999) or LC-1; ROSA26 lox-stop-lox-lacZ reporter(Schönig *et al.*, 2002) female mice were injected with 5 IU Pregnant Mare Serum Gonadotropin (Calbiochem) and 46-48h later 5IU of human chorionic gonadotropin (Calbiochem) to induce superovulation and mated to C57BL/6 males. Morulae were flushed at 2.5 dpc and cultured for 2 days in potassium simplex optimized media (KSOM-AA)(Ho *et al.*, 1995). After removal of zona pellucida in Acid Tyrode's solution (Sigma), individual blastocysts were transferred onto freshly plated MEFs in 96-well plates and cultured in 100 μ l ES media supplemented with 50 μ M Mek1 inhibitor (PD98059, Cell Signaling). Five days after plating, attached blastocysts were dissociated with trypsin, and replated on MEFs in single wells of a 96 well plate. Wells containing ES colonies were expanded and passaged to lines. The Z•LCR ES cell line was generated by targeted insertion of dox-inducible rtTA2^s-m2, pgk-puro at ROSA26(Wutz *et al.*, 2002) using early passage LC-1; ROSA26-lox-stop-lox-lacZ ES cells. Doxycycline (1 μ g/ml) treatment for 2 days activated lacZ expression assessed by Xgal staining. For recombination rescue experiments, RUSH targeted Z•LCR cells were grown in ES media containing 1 μ g/ml doxycycline for 2-24 hours then exchanged with regular ES media for 2 days prior to protein and RNA extraction.

ES cell transfection and screening

RUSH and CRUSH vectors were linearized with AscI and purified by phenol-chloroform extraction prior to transfection. 1-2x10⁶ cells were electroporated with 20 μ g of vector at 25 μ F and 0.40kV (Biorad Gene Pulser). 0.5-1.0 x10⁶ cells were plated on 10cm plates with neomycin-puromycin resistant MEFs. G418 (200 μ g/ml GIBCO) or puromycin (2 μ g/ml, Sigma) was added 24 hours post-transfection for 6 days. V6.5 and Nestin-cre ES cell transfections were screened for ROSA26 targeting by southern blot(Soriano, 1999) or nested-PCR using primers: TGT1-ROSA: CGCCTAAAGAAGAGGCTGTG, TGT1-SA GAAAGACCGCGAAGAGTTTG; TGT2-ROSA TCATCAAGGAAACCCTGGAC, TGT2-SA CAGAGAGCCTCGGCTAGGTA. Z•LCR ES cell transfected colonies were picked into duplicate 96-well plates. One plate was scored for lacZ replacement using Xgal staining: fixation for 5 minutes in 2% PFA, 0.2% glutaraldehyde, three washes with PBS, and staining for 1-2 hour at 37°C. X-gal negative clones were expanded from a duplicate plate. Xgal stain solution: PBS containing 5 mM K₃Fe(III)(CN)₆ and 5 mM K₄Fe(II)(CN)₆•3H₂O, 2 mM MgCl₂, 0.2% NP40, 0.1% Sodium deoxycholate, 1 mg/ml Xgal.

Residual expression analyses

Q-RT-PCR—Target gene knockdown was measured using quantitative real time RT-PCR (Q-PCR). Dissociated ES cells were plated on gelatinized plates for 30 min to remove MEFs. Total ES cell RNA was isolated using the RNeasy Kit (Qiagen) with on-column DNase digestion (Qiagen). cDNA was synthesized from 1µg RNA (iScript, BioRad). Target gene expression was analyzed in triplicate and normalized to GAPDH with relative expression levels calculated using the $\Delta\Delta C_t$ method (Grace *et al.*, 2002). Q-PCRs were performed with the ABI StepOnePlus Real Time PCR System. Statistical analysis was performed using Prism 5.0 (GraphPad).

Western blot—Cells and homogenized tissues were lysed in RIPA buffer (Upstate) supplemented with protease inhibitor cocktail (Roche). Total protein concentrations were determined using the DC-Protein Assay (Bio-Rad). Western analysis was performed with 25µg of total protein per sample, with the following antibodies: rabbit anti-Dnmt1 (1:1000, custom antibody), mouse anti-GFP (1:2500, Rockland), mouse anti-β-actin (1:5000, Abcam), anti-mouse-HRP (1:10,000, Jackson ImmunoResearch), anti-rabbit-HRP (1:10,000, Jackson ImmunoResearch).

Flow Cytometry—ES cells were dissociated and single cell preparations of brain and spleen were achieved by meshing tissues through 70µm and 30µm nylon mesh filters (BD Falcon) respectively in PBS. Tissues were isolated from 9-week old CRUSH^{GFP} mice, CRUSH^{GFP}; Nestin-Cre mice, and C57Bl/6 mice. Viable cells were gated based on cell size and propidium iodide (Sigma) staining. Quantification of mean fluorescence intensity (MFI) was examined using FlowJo (Three Star Inc, OR). Percentage of GFP knockdown was calculated using the formula: $(\text{CRUSH}^{\text{GFP}} \text{ MFI} - \text{CRUSH}^{\text{GFP}}; \text{Nestin-Cre MFI}) / (\text{CRUSH}^{\text{GFP}} \text{ MFI} - \text{C57Bl/6 MFI}) \times 100\%$.

Generation of transgenic mice

All experiments were performed as approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital. ES cell-derived mice were generated by tetraploid complementation (Eggan *et al.*, 2001). Briefly, CD-1 females were hormone primed as above, embryos were isolated at 2-cell stage, and electrofused in 0.3M mannitol, 0.3% BSA using a 50 millisecond pulse at 140V delivered from a BTX ECM 2001 Electrofusion Generator (Genetronics, Inc., San. Diego, CA). Fused embryos were cultured for 2 days in KSOM-AA. Each tetraploid blastocyst was microinjected with 5-10 transgenic ES cells, and a total of 18-24 blastocysts, including 6-7 diploid carrier blastocysts, were transferred to pseudopregnant CD-1 foster mice to induce pregnancy. All-ES mice were interbred or backcrossed to C57Bl/6 under pathogen-free conditions.

CNS Tissue Dissection, Neurosphere Culture

Tissues isolated by microdissection were imaged by fluorescence microscopy on an Olympus SZX16 microscope with matching magnifications and exposures. Non-adherent neural stem cell neurosphere cultures were prepared from neonatal cerebella at postnatal day 7. (Lee *et al.*, 2005) Cerebella were individually isolated, dissociated into single cells with Accutase (Millipore) and resuspended in 10ml neurosphere media containing DMEM/F12,

B27 serum-free supplement, 10µg/ml basic FGF, and 10µg/ml EGF (all media components from Invitrogen). Primary cultures were cultured in suspension for 7 days in T75 flasks. Following dissociation with Accutase and cell counting, secondary platings of neurosphere cells were carried out at clonal density (1500 cells/ml) in replicate wells of a 6-well plate for neurosphere quantification and imaging. (Molofsky *et al.*, 2003) Triple transgenic reporter mice (CRUSH^{GFP}, DsRed^R, Nestin-Cre) were used to validate clonal densities based on uniform fluorescence classes (DsRed⁺;GFP^{kd} or DsRed⁻;GFP⁺). Individual GFP^{kd} or GFP⁺ clones were picked, dissociated and replated to confirm clonality and stability of gene silencing, as compared to their parental clones.

Lentiviral infections

For lentiviral transfection, viral supernatant was produced by transient calcium chloride transfection of human embryonic kidney cells (HEK293) cells with lentiviral packaging (Pax) and envelope (VSV) vectors and pSicoR-puro-shRNA vectors. Viral supernatant was collected 48 hours after transfection, supplemented with polybrene (8ug/ml) and passed through a 0.45µm filter. Dissociated neurospheres isolated from Ptc1^{+/-};trp53^{-/-} mice were resuspended with the filtered viral supernatant and centrifuged for 45 minutes at 2500rpm. Puromycin selection (2ug/ml) was applied 1d post-infection for 48 hours and on d3 post-infection, the lentivirally-infected neurospheres were plated in self-renewal assays.

Statistical analysis

All statistical tests were performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Efficient knockdown using single-copy knock-in RNAi

a, Diagram of RUSH and CRUSH ROSA26 locus targeting constructs. ROSA26 controls expression of eGFP. Locations of southern probe (back box) and PCR primers (arrows) are indicated. The U6 promoter short hairpin cloning cassette in CRUSH is regulated by a stop cassette flanked by TATA-lox sites (Ventura *et al.*, 2004). The constitutive U6-shRNA cassette in RUSH can be excised by Cre. **b**, Schematic of the ROSA26 loci in Z•LCR ES cells before and after transfection with RUSH and after doxycycline (Dox) induced shRNA deletion through rtTA activated expression of Cre from the LC-1 transgene. Correct

targeting of RUSH to ROSA26 is visualized by ablation of β -gal activity and expression of GFP. **c**, Frequency of ES clones bearing homologous insertion at ROSA26 derived from selection for GFP⁺ and lacZ⁻ (green) is 33-fold enriched compared to random picking (blue). **d**, Time course of Dox administration leading to Dnmt1 reactivation was measured by western blot using β -actin as an internal control.

Figure 2. Quantitative GFP knockdown in CRUSH^{GFP} ES cells and mice

a, Schematic of CRUSH^{GFP} vector targeted to Rosa26. Seed sequence of shRNA is underlined. **b**, FACSscan histogram of GFP fluorescence of CRUSH^{GFP}-targeted V6.5 ES cells before transfection with Cre (solid green line) and after Cre activation of shRNA expression (dotted green line). Non-transgenic V6.5 cells (grey) were used as a background control. **c**, GFP knockdown in CRUSH^{GFP}; Isl1-Cre double transgenic heart (left) compared to wild-type (right) and CRUSH^{GFP} single-transgenic heart (center); representative individuals shown. **d**, GFP knockdown measured by FACSscan analysis of CRUSH^{GFP}

targeted ES cells, and brain and spleen of adult nestin-Cre; CRUSH^{GFP} mice (n=6). **e.**, GFP knockdown in embryonic brain at E12.5 visualized in bright field and GFP channels. **f.** Left panel: neurosphere formation frequency is reduced in medulloblastoma tumor stem cells lentivirally transfected with shGFP (green) versus empty vector (white) (n=6 for each; unpaired t test with Welch's correction). Right panel: secondary neurosphere formation frequency is unchanged in p7 CRUSH^{GFP} (green; n=3) and CRUSH^{GFP};Nestin-Cre (striped; n=3) compared to wildtype age-matched controls (white; n=4) (ANOVA with Dunnett's multiple comparison test).

Figure 3. Validation of rapid RNAi transgenesis using RUSH & CRUSH

a, Schematic of ROSA26-DsRed Cre-reporter strain created and breeding strategy for generating compound transgenics to analyze tissue specific GFP knockdown *in vivo*. **b**, Dual-color RNAi reporter mice display tissue-specific GFP knockdown with contrasting Ds-Red activation shown in the central nervous system. Merged bright field, green and red channel images of eyes (left) and cultured neural stem cells (right) from dual-color sensor mice carrying R26DsRedR; CRUSH^{GFP}; without (top) and with (bottom) Nestin-Cre. Secondary low-density cultures of neurospheres demonstrate self-renewal and stable

maintenance of GFP knockdown through serial passaging. **c**, Fluorescence microscopy of dissected lens tissues from compound transgenic animals demonstrates robust cre-dependent GFP knockdown and contrasting DsRed activation. **d**, Western analysis of GFP protein using lens tissues from nestin-cre, CRUSH^{GFP}, and CRUSH^{GFP}; nestin-cre P21 littermates, compared to β -actin. **e**, Schematic for creating expedited RNAi transgenics using RUSH & CRUSH. Global knockdown in ES cells using RUSH is carried out for selection of effective shRNA alleles and generation of global knockdown mice. ES cell derivation from Cre transgenic strains and founder (G₀) derivation using tetraploid complementation each save one mouse generation in creating conditional models. **f**, All ES-derived mice were created from ROSA26 targeted derivatives of wildtype 129:C57Bl/6 F1 ES cells (V6.5, grey bar) or from newly derived Cre transgenic ES cells and their ROSA26 targeted derivatives (Cre^{tp}, green bar). Comparably high efficiencies in transgenic founder generation, shown as a percentage of injected embryos, were observed. **g**, qRT-PCR comparison of residual Bmi1 expression in CRUSH^{Bmi1} ES clones with or without Cre activation, **h**, Representative neurospheres from CRUSH^{Bmi1} mice with or without nestin-Cre, **i**, Quantified self-renewal in secondary low-density neurosphere cultures demonstrate deficit in Bmi1 knockdown animals (n=3 mice per genotype).