Epigenetic regulation of gene structure and function with a cell-permeable Cre recombinase

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Studies of mammalian gene function are hampered by temporal limitations in which phenotypes occurring at one stage of development interfere with analysis at later stages. Moreover, phenotypes resulting from altered gene activity include both direct and indirect effects that may be difficult to distinguish. In the present study, recombinant fusion proteins bearing the 12 amino acid membrane translocation sequence (MTS) from the Kaposi fibroblast growth factor (FGF-4) were used to transduce enzymatically active Cre proteins directly into mammalian cells. High levels of recombination were observed in a variety of cultured cell types and in all tissues examined in mice following intraperitoneal administration. This represents the first use of protein transduction to induce the enzymatic conversion of a substrate in living cells and animals and provides a rapid and efficient means to manipulate mammalian gene structure and function.

The Cre recombinase from bacteriophage P1 has been widely used to induce DNA sequence-specific recombination in mammalian cells[1,2]. LoxP sites, which serve as targets of Cre-mediated recombination in the P1 genome, also function as recombination substrates in mammalian cells. Applications involving Cre-loxP recombination have included conditional mutagenesis[3–5], gene replacement[6] and chromosome engineering[7–12] in mice, and conditional gene expression[13–15]. However, the use of site-specific recombination in genetic studies is often hampered by difficulties expressing the recombinase enzyme in cells of the appropriate type and developmental stage. Moreover, even conditional mutants induced by tissue-specific Cre expression may interfere with tissue development, thus precluding later studies in terminally differentiated cells.

To address this issue, we have tested whether a protein domain with membrane-translocating activity could be used to deliver enzymatically active Cre recombinase into cultured cells and mice. The approach exploits the fact that leader sequences from Kaposi FGF-4 can promote the translocation of heterologous peptides across the plasma membrane and into the cytoplasm of cultured cells[16]. Protein transduction exploits biophysical properties common to mammalian cell membranes, and consequently, most if not all cell types were expected to acquire the enzyme. In addition to its potential utility in studies of mammalian gene function, a cell-permeable Cre could provide a convenient and stable marker of protein transduction in cells containing an appropriately modified reporter gene. This would permit a quantitative assessment of protein trafficking and uptake in both cells and animals.

Results and discussion

Recombinant cell-permeable Cre proteins. Purified recombinant fusion proteins bearing the 12-amino acid MTS from Kaposi FGF-4 (ref. 16) were used to deliver enzymatically active Cre proteins directly into mammalian cells. Of the four recombinant proteins tested, His_6-NLS-Cre-MTS (Fig. 1A), which also contains a nuclear localization sequence (NLS) from simian virus 40 (SV40) large-T antigen and an N-terminal His_6 affinity tag, displayed the best combination of yield, solubility, *in vitro* specific activity, nuclear localization, and activity in cells. Proteins with other affinity tags or lacking an NLS performed less well. For example, addition of the NLS to GST-Cre-MTS (tagged with glutathione-*S*-transferase) enhanced the biological activity of the protein in cells (see below) but also reduced the yield and solubility of the fusion protein. Replacement of the GST tag with the *Escherichia coli* maltose-binding protein (MBP) domain improved solubility but impaired enzymatic activity.

Localization of transduced protein. Uptake of recombinant Cre proteins into mammalian NIH3T3 cells was monitored by confocal fluorescence microscopy (Fig. 1B–E). All of the Cre fusion proteins were efficiently transduced, with 100% of treated cells showing intense staining. GST-Cre-MTS was localized predominantly in the cytoplasm (Fig. 1C) and MBP-NLS-Cre-MTS localized to both cytoplasm and nucleus (Fig. 1D), while His_6-NLS-Cre-MTS was predominantly nuclear (Fig. 2E). Treatment of cells with increasing concentrations of cell-permeable proteins resulted in increasing protein uptake as assessed by indirect immunofluorescence (data not shown).

Site-specific recombination in cultured cells. Cells containing individual genes modified with loxP sites were used to assess whether transduced Cre proteins could elicit recombination. Tex.loxp.EG is a T-lymphoctye line in which Cre-mediated recombination activates the expression of a green fluorescent protein (GFP) reporter gene. The cells were exposed to His_6-NLS-Cre-MTS, washed extensively, and cultured for 24 h, providing time for GFP expression. Treatment with 4 μM His_6-NLS-Cre-MTS for 2 h induced recombination in 50% of cells; this percentage increased to 69% following exposure to 10 μM Cre (Fig. 2A). Recombination was also observed in 50% of cells exposed for 30 min to 10 μM His_6-NLS-Cre-MTS, and this percentage increased to 75% after 2 h (Fig. 2B) and to 82% following three consecutive 2 h treatments with 10 μM enzyme (Fig. 2C). Southern blot analysis confirmed that expression of the GFP reporter accurately reflected the extent

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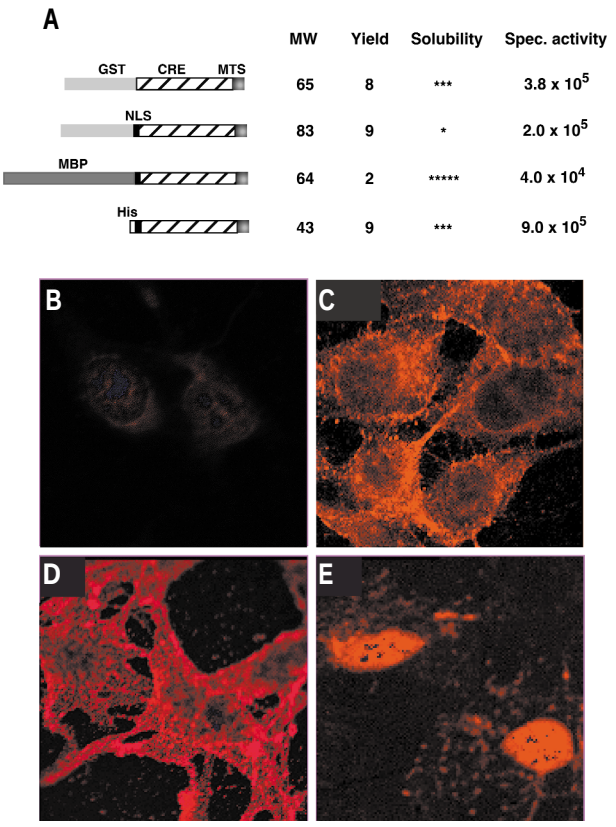

Figure. 1. Recombinant Cre Proteins and intracellular uptake. Cre proteins expressed in *E. coli* (A) contained a membrane translocation sequence (MTS); sequences for affinity purification (glutathione-*S*-transferase, GST; maltose-binding protein, MBP; and His); and SV40 T-antigen nuclear localization signal (NLS). The molecular weights (MW) of each protein, yield from *E. coli* cultures (mg/L), relative solubilities of the purified proteins, and specific activity *in vitro* (units per milligram of enzyme) are indicated. NIH3T3 cells were incubated with serum-free medium alone (B) or with serum-free medium containing 10 μM GST-CRE-MTS (C), MBP-NLS-Cre-MTS (D), or His_6-NLS-Cre-MTS (E) for 1 h, washed, and stained with anti-GST (C), anti-MBP (D), or anti-Cre (B, E) antibodies plus a rhodamine-labeled secondary antibody.

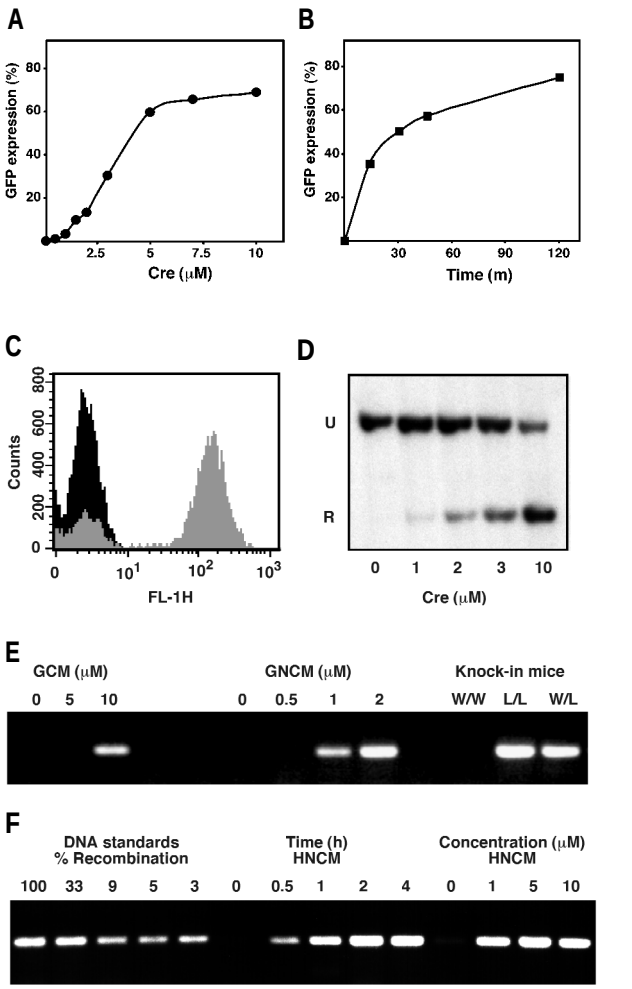

Figure 2. *In vivo* Cre-mediated recombination. Tex.loxp.EG is a cell line in which Cre-mediated recombination activates the expression of a green fluorescent protein (GFP) gene. Cells were exposed either to different concentrations of His_6-NLS-Cre-MTS for 2 h (A) or to 10 μM His_6-NLS-Cre-MTS for different amounts of time (B), and after one day the percentage of GFP-expressing cells was determined by flow cytometry, as illustrated in (C), which shows 82% recombination in cells exposed to three successive 2 h treatments with 10 μM His_6-NLS-Cre-MTS. Southern blot analysis (D) shows increased conversion of the loxP-containing gene (upper band, U) to the recombination product (lower band, R) following exposure to increasing concentrations of His_6-NLS-Cre-MTS. Cre-mediated recombination in S4R embryonic stem cells generates templates that can be detected by amplification of a 351-nucleotide PCR product. (E) S4R cells were exposed to the indicated concentrations of GST-Cre-MTS (GCM) or GST-NLS-Cre-MTS (GNCM). DNAs from wild-type (W/W) mice or mice containing either one (W/L) or two (L/L) deleted alleles were analyzed for comparison. (F) Cells were exposed either to 10 μM His_6-NLS-Cre-MTS (HNCM) for different amounts of time or to different concentrations of His_6-NLS-Cre-MTS for 4 h. Recombination standards were made by diluting DNA with a single deleted allele (100%) with different amounts of wild-type DNA.

of template recombination (Fig. 2D). S4R murine embryonic stem (ES) cells contain a single loxP-modified sulfonylurea receptor gene. Cre-mediated recombination generates a unique template that can be detected by PCR. GST-Cre-MTS induced detectable levels of recombination, but only at the highest concentration (10 μM) of protein tested (Fig. 2E). GST-NLS-Cre-MTS was ~10 times more active than GST-Cre-MTS *in vivo* (Fig. 2E), even though the protein was slightly less active *in vitro* (Fig. 1). His_6-NLS-Cre-MTS was also highly active. Exposure of cells to 5–10 μM His_6-NLS-Cre-MTS for 2 h was sufficient to induce recombination in 33–100% of templates (Fig. 2F) as compared to DNA standards.

ROSA26R (R26R; ref. 17) is a transgenic mouse line in which Cre-mediated recombination activates expression of a β-galactosidase reporter gene (Fig. 3A). Since the R26R promoter is expressed in all cell types, the locus provides a universal reporter of Cre-mediated recombination. As shown in Figure 3C, His_6-NLS-Cre-MTS induced recombination in ~50% of splenocytes explanted *ex vivo*, and cultured in the absence of lipopolysaccharide (LPS), a B-cell mitogen. By contrast, none of the splenocytes treated *ex vivo* with recombinant GST-MTS (containing only glutathione *S*-transferase and MTS domains) expressed detectable levels of β-galactosidase (Fig. 3B). Similar levels of recombination were observed in cells cultured in the presence of LPS (data not shown).

Transduction of Cre recombinase in mice. To examine systemic transduction of biologically active Cre recombinase in mice, ROSA26R mice were injected intraperitoneally on three consecutive days with 25 μg/g of His_6-NLS-Cre-MTS in 1 ml of RPMI medium or with PBS. After three days, organs were removed and analyzed for β-galactosidase expression. Cre-mediated recombination was observed in all tissues examined, including the brain, as evidenced by intense blue staining of intact organs (Fig. 4). Background staining in some organs from control mice (e.g., liver)

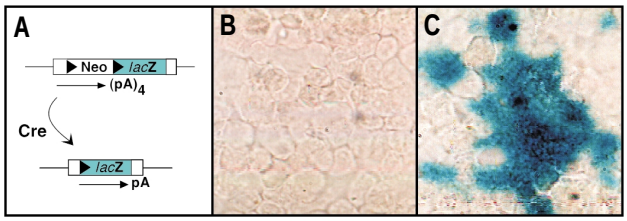

Figure 3. Cre-mediated recombination *ex vivo*. (A) ROSA26R is a transgenic mouse line in which Cre-mediated recombination activates the expression of a β-galactosidase reporter gene. Primary splenocytes from ROSA26R mice were treated *ex vivo* for 2 h with (B) recombinant GST-MTS (containing only GST and MTS domains) or (C) 10 μM His_6-NLS-Cre-MTS, and after two days in culture the cells were centrifuged onto glass slides and stained with X-Gal.

results from endogenous β-galactosidase activity. C57BL6 mice (i.e., mice lacking the ROSA26R reporter) injected on three consecutive days with 25 μg/g His_6-NLS-Cre-MTS also displayed only background levels of *lacZ* expression (data not shown). Sections through stained liver, brain, and kidney showed that *lacZ* expression was not confined to the surface of the organs or to the vascular system (Fig. 5A). β-galactosidase was also visualized by immunohistochemical staining of cryosectioned tissues in which β-galactosidase immune complexes are stained brown with a horseradish peroxidase–conjugated secondary antibody (Fig. 5B). β-galactosidase expression in Cre-injected animals was highest in regions surrounding blood vessels in brain and liver sections but was more evenly distributed in the kidney, consistent with systemic delivery of Cre through the bloodstream. Similar levels of recombination were obtained following intravenous injection (2.5 μg/g in 100 μl RPMI medium), and in mice injected intraperitoneally for five consecutive days (data not shown). All mice tolerated the recombinant protein with no apparent adverse effects.

The efficiency of recombination was assessed in mice injected intraperitoneally with 25 μg/g of His_6-NLS-Cre-MTS daily for one, three, or five days. After five days, splenocytes and thymocytes were analyzed for *lacZ* expression by flow cytometry, monitoring the conversion of 5-chlormethylfluorescein di-β-D-galactopyranoside (Molecular Probes, Eugene, OR) to a fluorescent product (Fig. 6). The percentage of thymocytes (Fig. 6A–C) and splenocytes (Fig. 6D–F) undergoing recombination following one (Fig. 6A, D), three (Fig. 6B, E), and five (Fig. 6C, F) treatments was approximately 14, 36, and 51% and 17, 34, and 37%, respectively. In a separate experiment, the efficiency of recombination in total splenocytes was measured at 48% and was somewhat lower in B cells (B220 positive) than in non-B cells (B220 negative), 43% and 62%, respectively (data not shown).

In summary, the present study demonstrates that cultured cells and a wide variety of nonproliferating, terminally differentiated cell types can be transduced with a cell-permeable DNA site-specific recombinase and are competent to undergo recombination soon after exposure to the enzyme. When used together with cells or mice containing loxP-modified genes, cell-permeable Cre permits rapid ablation or activation of gene expression both *in vivo* and *ex vivo*. The process circumvents the need to express Cre on cell type–specific promoters in order to induce recombination in specific cell types and is simpler than other methods for temporal control[5,18–22]. Moreover, recombination was achieved soon after exposure to the enzyme. This is particularly important in mice, where mutations induced by expressing Cre on a tissue-specific promoter may interfere with cell differentiation or survival and thus preclude studies of gene function in terminally differentiated cells. However, the percentage of cells that undergo recombination is less than can be achieved from the expression of Cre transgenes[1,2], and the widespread dissemination of the protein will complicate efforts to induce tissue-specific recombination in the intact animal.

Systemic protein transduction has been demonstrated in mice injected with a cell-permeable β-galactosidase reporter protein[23] and with a cell-permeable peptide that blocks nuclear factor κB (NF-κB) nuclear translocation[24]. The latter cell-permeable protein also protected animals from otherwise lethal doses of LPS. Likewise, systemic delivery of His_6-NLS-Cre-MTS was remarkably efficient, crossing even the blood–brain barrier. The striking efficiency of protein transduction is consistent with the suggestion that intercellular protein trafficking is a normal physiological process *in vivo*[25]. As cell-permeable Cre provides a stable record of protein trafficking and uptake in animal tissues, it will assist in the development of protein-based therapies for treating human diseases.

The present study represents the first use of protein transduction to induce the enzymatic conversion of a substrate in living cells. In addition to its utility in studies of mammalian gene function, cell-permeable Cre can be used as a reporter for quantitative studies of processes or conditions, such as nuclear transport or chromatin structure, that may affect the rates of recombination at specific loxP sites. Peptides from a variety of proteins including HIV Tat, FGF-4, Antennapedia, and VP22 have been used to deliver heterologous proteins and peptides into cells[26–28]. Protein transduction provides a new paradigm for the analysis of mammalian gene functions in cul-

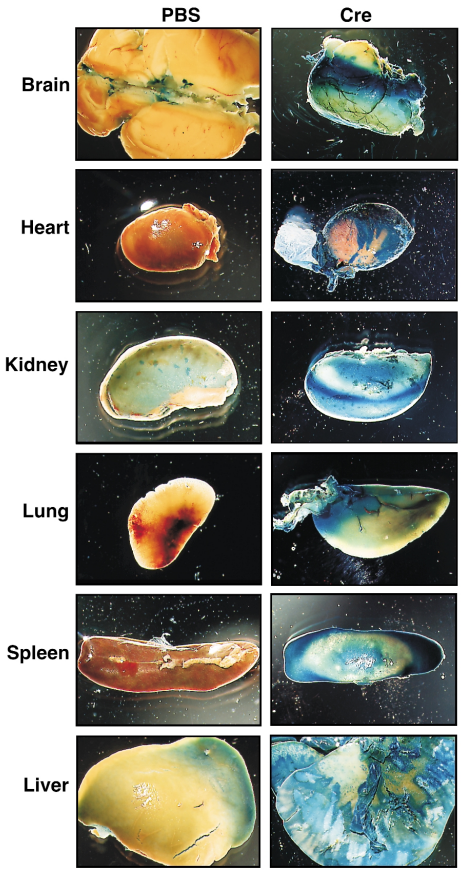

Figure 4. Cre-mediated recombination in mice. ROSA26R mice were injected intraperitoneally on three consecutive days with 25 μg/g of His_6-NLS-Cre-MTS or with PBS. Three days after the last injection, the mice were killed, and the organs from control (lefthand panels) and Cre-injected mice (righthand panels) were stained with X-Gal and examined by dark-field microscopy.

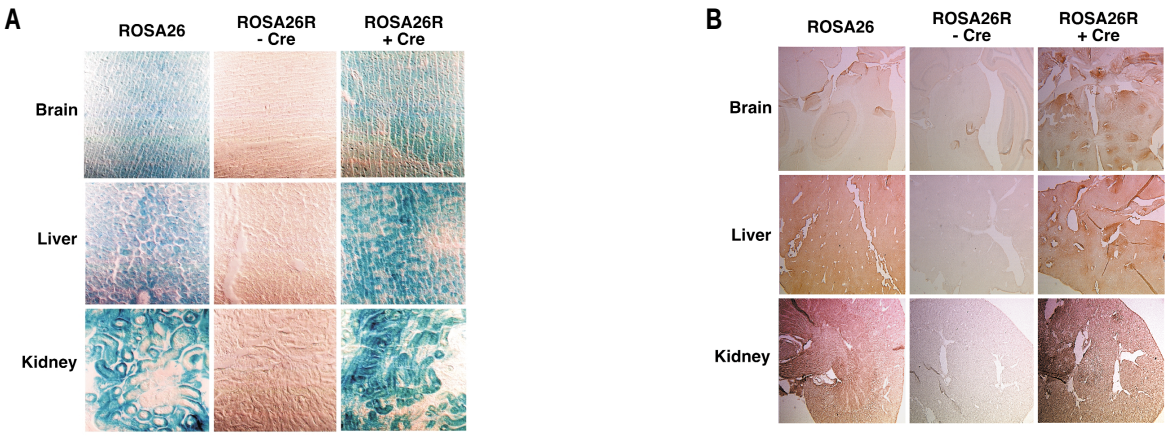

Figure 5. Histological examination of β-galactosidase expression. (A) X-Gal staining of *lacZ* expression. ROSA26R mice were injected with His_6-NLS-Cre-MTS protein (ROSA26R + Cre, righthand panels) or with a buffer control (ROSA26R – Cre, middle panels) as described in Figure 4. Three days after the last injection, the indicated organs were removed, stained with X-Gal, and sectioned at 20 μm. Tissues from ROSA26 mice, which constitutively express *lacZ* (lefthand panels), were analyzed as a positive control. (B) Immunostaining of *lacZ* expression**.** ROSA26R mice were injected with His_6-NLS-Cre-MTS protein (ROSA26R + Cre, righthand panels) or with a buffer control (ROSA26R – Cre, middle panels) on five consecutive days. Three days after the last injection, the indicated organs were removed, cryosectioned at 40 μm, and stained with rabbit anti-β-galactosidase antibody (1:500, Novagen) and horseradish peroxidase–conjugated goat anti-rabbit secondary antibody (Amersham). Tissues from ROSA26 mice, which constitutively express *lacZ* (lefthand panels), were analyzed as a positive control.

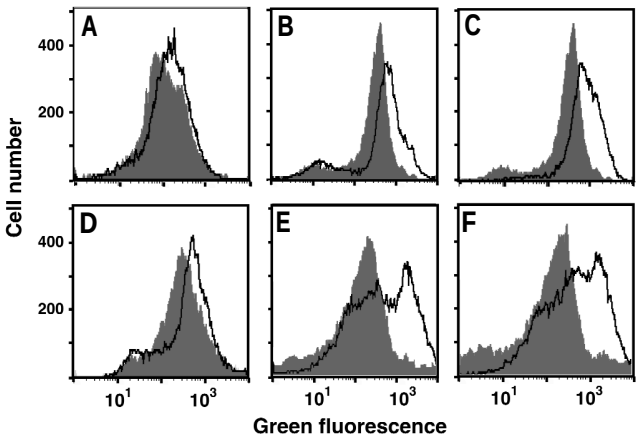

Figure 6. Time course of *lacZ* expression in splenocytes and thymocytes from Cre-treated mice. ROSA26R mice were injected intraperitoneally with His_6-NLS-Cre-MTS protein (black line) or with a buffer control (gray histograms) on one (A and D), three (B and E), or five (C and F) consecutive days, as described in Figure 5. After five days, β-galactosidase expression in thymocytes (A–C) or splenocytes (D–F) was measured by flow cytometry. The enhanced green fluorescence in cells from Cre-treated mice results from conversion of the β-galactosidase substrate, 5-chlormethylfluorescein di-β-D-galactopyranoside (Molecular Probes) to a fluorescent product.

tured cells and mice. The present study illustrates advantages of protein transduction over conventional gene-based approaches to quantify the direct effects of an enzyme on biochemical and biological processes in living cells under non-steady-state conditions.

Experimental protocol

Expression and analysis of recombinant Cre proteins. GST-Cre-MTS was constructed by amplifying Cre sequences from bp 484 to 1,513 (GenBank accession no. X03453) using primers A and B. The PCR product was cleaved with *Bgl*II and cloned into the *Bam*HI site of pMTS2 (ref. 29). A NLS sequence was added to GST-Cre-MTS by PCR amplification of Cre-MTS sequences using primer C that contained the 5-amino acid NLS sequence of SV40 large-T antigen together with primer B. The PCR product was cleaved with *Bgl*II and cloned into the *Bam*HI site of pMTS2. The resulting plasmids were used to express GST-Cre-MTS and GST-NLS-Cre MTS under the control of the *lac*I promoter in *E. coli* strain BL21. High levels of the fusion protein were expressed 2.5 h after the addition of 0.6 mM isopropyl-β-D-thiogalactoside (IPTG), and the recombinant proteins were purified by glutathione affinity chromatography, as directed by the supplier of the affinity matrix (Amersham/Pharmacia, Piscataway, NJ). To construct MBP-NLS-Cre-MTS, NLS-Cre-MTS sequences were PCR-amplified from the GST-NLS-Cre-MTS plasmid by using primers D and E and cloned in-frame into the *Bam*HI site of the MBP expression vector, pMAL-c2 (New England Biolabs, Beverly, MA). MBP-NLS-Cre-MTS was purified[30] from *E. coli* TB1 cells grown to A_{600} of 0.5 and induced for 5 h with 0.3 mM IPTG. To construct His_6-NLS-Cre-MTS, NLS-Cre-MTS sequences were PCR-amplified from the GST-NLS-Cre-MTS plasmid by using primers F and G and cloned into the *Nde*I site of the His_6 expression vector, pET-28a(+) (Novagen, Madison, WI). His_6-NLS-Cre-MTS was purified, as directed by the supplier of the affinity matrix (Qiagen, Valencia, CA), from *E. coli* BL21 cells grown to an A_{600} of 0.8–1.0 and induced for 5 h with 0.7 mM IPTG. After affinity purification as directed by the supplier (Qiagen), all of the recombinant proteins were concentrated by ultrafiltration and dialyzed for 4 h against cell culture medium (DMEM or RPMI).

Primer A: 5′-CCGGAGATCTTAATGTCCAATTTACTGACCGTA-3′
Primer B: 5′-GCCGGAGATCTCATCGCCATCTTCCAGCAGGCG-3′
Primer C: 5′-CCGCCGGAGATCTTAATGCCCAAGAAGAAGAG-GAAGCTGTCCAATTTACTGACCGTACAC-3′
Primer D: 5′-CCGCCGAGATCTCCCAAGAAGAAGAGGAAGGTGTC-CAATTTACTGACCGTACAC-3′
Primer E: 5′-CCGCCGAGATCTTTAGGGTGCGGCAAGAA-GAACAGGGAGAAGAACGGCTGC-3′
Primer F: 5′-CCGCCGCATATGCCCAAGAAGAAGAGGAAGGTGTC-CAATTTACTGACCGTACAC-3′
Primer G: 5′-CCGCCGCATATGTTAGGGTGCGGCAAGAA-GAACAGGGAGAAGAACGGCTGC-3′

Assays of Cre enzyme activity measured the release of a circular plasmid inserted into a λ-phage (Novagen) by transformation of *E. coli*. The specific activity of His_6-NLS-Cre-MTS purified through one cycle of affinity chromatography was 9.0×10^4 U/mg. One unit (U) of enzyme produces 10^4 colonies (equivalent to 2×10^6 circular molecules) in a 30 min reaction containing 200 ng DNA substrate in 50 mM Tris-HCl pH 7.5, 33 mM NaCl, and 10 mM $MgCl_2$ in a total volume of 15 μl.

Cell culture and protein transduction. Tex.loxp.EG cells were derived by infecting Tex cells (a murine thymoma line derived from p53-deficient mice) with the pBABE.lox.stp.EGFP retrovirus. pBABE.lox.stp.EGFP contains the

STOP cassette from pBS302 (ref. 13) positioned upstream of the enhanced GFP gene (EGFP; Clontech Laboratories, Palo Alto, CA) and cloned into the pBABE vector[31]. Ecotropic retroviral stocks were prepared in BOSC 23 packaging line[32]. Cells were incubated with serum-free medium alone (either DMEM or RPMI), or with serum-free medium containing Cre fusion proteins. FBS inhibits transduction of recombinant Cre proteins. Cre uptake was monitored by using anti-Cre antibodies (rabbit polyclonal; Novagen), together with a rhodamine-labeled secondary antibody (goat anti-rabbit IgG; Kirkegaard and Perry, Gaithersburg, MD).

Primers (5′-CAATTCCTCAACTGAGGCTCTTAA-3′ and 5′-GCTTGAAGTTCCTATCCGAAGTTCC-3′) complementary to the targeted S4R locus were used to amplify a 351-nucleotide fragment generated by Cre-mediated recombination. PCR reactions (100 ng genomic DNA, 0.2 μM each primer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1× GeneAmp Gold PCR buffer (Perkin-Elmer, Foster City, CA), and 2.5 U AmpliTaq Gold (Perkin-Elmer)) involved 40 cycles of denaturation (94°C), primer annealing (60°C), and primer extension (72°C) for 1 min each.

Primary splenocytes, predominantly B and T lymphocytes, were cultured for 24 h in RPMI medium and treated for 2 h with RPMI or with serum-free RPMI containing 10 μM His_6-NLS-Cre-MTS. FBS reduces the efficiency of protein transduction. The cells were washed and cultured for 3 h in serum-free RPMI and then cultured in normal medium (RPMI plus 10% FBS) or in medium containing 10 μg/ml LPS, a mitogen that activates proliferation of primary B cells. After 48 h, the cells were centrifuged onto glass slides and were stained with X-Gal. Mice were killed by CO_2 inhalation, and the organs were fixed and stained (24 h in 0.1% X-Gal solution) for β-galactosidase expression[33].

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