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When Cre-Mediated Recombination in Mice Does Not Result in Protein Loss

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ABSTRACT

Cre/loxP recombination enables cellular specificity and, in the case of inducible systems, temporal control of genomic deletions. Here we used a SM22α tamoxifen-inducible Cre line to inactivate β1 integrin in adult smooth muscle. Interestingly, analysis of two distinct β1 loxP transgenic mice revealed vastly different outcomes after β1 integrin deletion. Lethality occurred 4 weeks postinduction in one Cre/loxP line, while no apparent phenotype was seen in the other line. Genetic analysis revealed appropriate DNA excision in both cases; however, differences were found in the degree of protein loss with absolutely no change in protein levels in the model that lacked a phenotype. Seeking to understand protein persistence despite appropriate recombination, we first validated the flox allele using a constitutive Cre line and demonstrated its ability to mediate effective protein inactivation. We then examined the possibility of heterozygous cell selection, protein turnover, and deletion efficiency with no success for explaining the phenotype. Finally, we documented the presence of the Cre-recombination episomal product, which persisted in tissue samples with no protein loss. The product was only noted in cells with low proliferative capacity. These findings highlight the potential for protein expression from the products of Cre-recombinase excised genes, particularly when deletion occurs in low turnover populations.

INTEGRINS are a group of transmembrane, extracellular matrix receptors composed of heterodimeric pairs of α- and β-subunits that have been shown to function in cell survival, migration, and mechanotransduction (Schmidt *et al.* 1993; Wang *et al.* 1993; Chen *et al.* 1997; Hynes 2002; Stupack and Cheresh 2002; Martinez-Lemus *et al.* 2005; Abraham *et al.* 2008). To study the function of β1 integrin *in vivo*, knockout mice were generated by homologous recombination and found to be embryonic lethal prior to implantation (Fassler and Meyer 1995; Stephens *et al.* 1995). Due to this early lethality, Cre/loxP technology has been employed to dissect the contribution of β1 integrin in specific cell types (Raghavan *et al.* 2000; Pietri *et al.* 2004; Jones *et al.* 2006; Lei *et al.* 2008).

As with all targeted recombination technologies, the Cre/loxP system has advantages and some known limitations (Branda and Dymecki 2004). In the case of loxP sites, location is critical. LoxP sites can flank miRNAs resulting in their inadvertent deletion (Kuhnert *et al.* 2008) and insertion of a floxed *neo* cassette in the intron of a gene has been shown to unintentionally disrupt gene splicing (Wassarman *et al.* 1997; Meyers *et al.* 1998). Furthermore, unidentified alternative transcriptional start sites (Han and Zhang 2002) may circumvent attempts to inactivate a gene by loxP flanking a single exon. Given these caveats, it is important to use multiple controls to facilitate data interpretation and, whenever possible, validate the phenotype with an additional floxed allele having loxP sites located in different regions of the gene.

Four β1 integrin loxP constructs have been developed (Potocnik *et al.* 2000; Raghavan *et al.* 2000; Graus-Porta *et al.* 2001; Keller *et al.* 2001). Each of these transgenic alleles varies in their placement of loxP sites; however, they all have been shown to successfully eliminate β1 integrin protein when used with constitutive Cre-expressing systems (Li *et al.* 2005; Benninger *et al.* 2006; Jones *et al.* 2006; Lei *et al.* 2008). Our goal was to examine β1 integrin gene deletion in smooth muscle of adult mice. For this, we decided to use two distinct loxP transgenes designed to inactivate β1 integrin: an allele with loxP sites flanking exon 3 ($\beta 1^{e3}$) (Raghavan *et al.* 2000) and another allele with loxP sites spanning the region between exon 2 and the 3′-UTR ($\beta 1^{fl}$) (Potocnik *et al.* 2000). Both transgenic mice were crossed to an inducible SM-22α Cre-recombinase mouse (Kuhbandner *et al.* 2000) to temporally restrict deletion in adult smooth muscle cells.

To our surprise, the double transgenic lines provided different phenotypes despite clear verification of genomic

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recombination. Assessment of protein levels showed gradual protein loss in the β*1*^{e3}-Cre mouse, but no loss of protein was seen in the β*1*^{fl}-Cre mouse. This study communicates our path to reconcile the conundrum of having genomic recombination and yet no loss of protein, and in the process we provide evidence for episomal protein expression.

MATERIALS AND METHODS

Mice: β*1*$^{fl/fl}$ mice (Potocnik *et al.* 2000) were a gift from Reinhard Fassler (Max Planck Institute of Biochemistry, Germany). The β*1*$^{e3/e3}$ (Raghavan *et al.* 2000), *SM22αCre* (Holtwick *et al.* 2002), *R26R* (Soriano 1999), and the β*1*$^{+/-}$ (Stephens *et al.* 1995) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The *SM22aCre-ER*^{T2} mice (Kuhbandner *et al.* 2000) were generously provided by Robert Feil (Interfakultäres Institut für Biochemie, Universität Tübingen, Germany). *SM22*α protein binds actin and contributes to smooth muscle contractility (Je and Sohn 2007; Assinder *et al.* 2009) and is expressed specifically in adult smooth muscle cells of the vasculature and viscera. Genotyping primers used are listed in supporting information,Table S1.

Tamoxifen (MP Biomedical) was prepared at a concentration of 20 mg/ml in 10% ethanol and 90% sunflower seed oil (Sigma) according to previous reports (Monvoisin *et al.* 2006). Animals were allowed to mature (5–6 weeks old) and then received 1 mg of tamoxifen at 24-hr intervals for the specified number of injections. Mice receiving the 28-day injection regimen were aged an additional 2 weeks prior to evaluation.

Tissue to be immunostained was fixed in 2% PFA overnight. Samples were histologically sectioned and stained according to the manufacturer's protocol for phosphorylated histone 3 (Cell Signaling; 1:100). FITC-conjugated α-sm actin (Sigma, 1:100) was used for staining the bladder tissue.

β-Gal staining: To facilitate penetration of the reagents, the mucosa was dissociated from the bladder under a dissecting microscope. Embryos were stained whole mount. Tissue was then fixed at room temperature for 15 min in glutaraldhyde (0.2% glutaraldehyde, 5 mm EGTA pH 8, 2 mm MgCl, 1× PBS, pH 7.3). This was followed by three rinses of 30 min in detergent wash solution (2 mm MgCl, 0.01% DOC, 0.02% NP40, 1× PBS, pH 7.3). Tissues were stained overnight on an incubated shaker at 37° in detergent rinse with 5 mm ferrocyanide (II), 5 mm ferricyanate (III), 20 mm Tris, pH 7.3, and 1 mg/ml X-gal, pH 7.3. After staining overnight, samples were washed several times in 1× PBS at room temperature. Then they were fixed in 2% PFA overnight at 4°.

Western blot analysis: After the mucosa was removed, a portion of the bladder was placed in RIPA buffer (Lee *et al.* 2006). The Western blot antibodies used were anti-β1 integrin (Millipore, 1:750), anti-α-tubulin (Sigma, 1:5000) and GAPDH (Millipore, 1:5000).

Cell culture: Mouse embryonic fibroblasts (MEFs) were isolated from E13.5–E16.5 embryos according to Nagy *et al.* (2003). Mitomycin C (Calbiochem) was prepared at 200 mg/ml in HBSS and then filtered at 0.2 μm. Cells were incubated in 15 μg/ml mitomycin C in media for 3 hr at 37°. Cells were then rinsed two times with 1× HBSS and fresh media were added. Cells were allowed to recover overnight before replating. Induced quiescence was confirmed by a 6-hr incubation in 100 μm BrdU followed by fixation in 2% PFA for 30 min. Cells were then stained for BrdU incorporation using an anti-BrdU antibody (Thermo Scientific) and analyzed via confocal microscopy. Adeno-cre and adeno-gfp control viruses were applied to cells at an MOI of 30. DNA and protein were isolated from each treatment group, which included mock-infected controls.

Quantitative PCR primer design: Unique quantitative PCR (qPCR) primers were designed to facilitate a quantitative analysis of the rate of β1 loxP recombination and the rate of maintenance of the loxP-flanked DNA sequence (see Table S1 for primer sequences). The first primer set, "total-DNA," was designed to quantify the total number of chromosomal copies in each DNA sample from a template. This value was used to normalize all subsequent quantitative PCR values.

To determine the rate of recombination, a pair of primers, "unrecombined," was designed to anneal to the sequences on either side of a loxP site in the unrecombined β1 integrin locus. Following Cre-mediated excision of the loxP-flanked sequence, these primers anneal to distinctly separate fragments of DNA and will not generate an amplicon. Thus, the difference in the value from this primer set relative to the value from the total-DNA primer set indicates the degree to which the β1 integrin gene has been recombined and excised.

The total number of copies of β1-flanked DNA, both unrecombined (chromosomal) and recombined (episomal circles), were determined by the "flanked DNA" primer set. This primer set amplifies a segment just upstream of exon 3 that lies within the loxP-flanked region in both β*1*^{fl} and β*1*^{e3} cells. Comparison of the value from this primer set relative to the value from the total-genomic primer set indicates the degree of loss of loxP-flanked sequence.

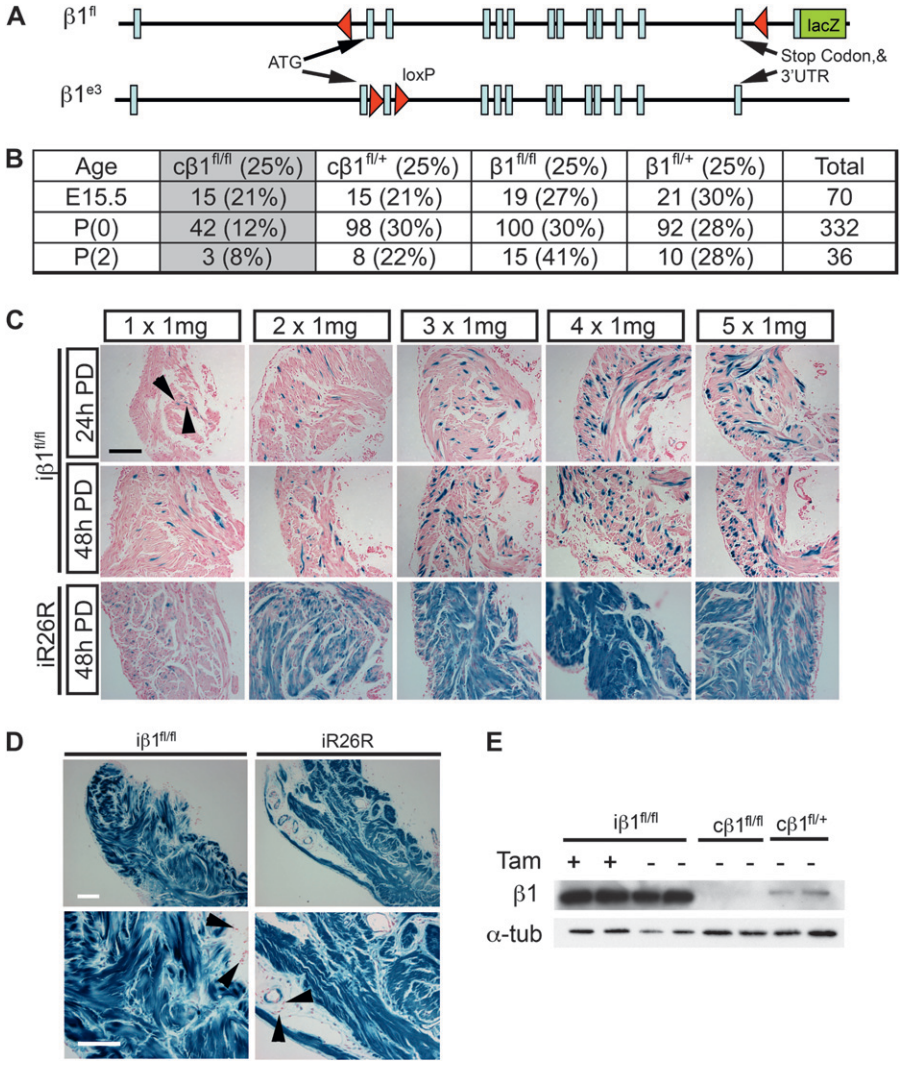
Validation experiments with these primers confirmed that each pair gave a unique, correctly sized product and that this was quantitative, relative to the amount of template present. The exception to this was the unrecombined-β*1*^{fl} primer set, which produced a marginal amount of an incorrectly sized product. To eliminate this from the analysis, a TaqMan probe was designed for this template to ensure that only the correct product was quantified.

Determination of recombination and floxed DNA persistence: DNA was extracted from bladder tissue, cardiac tissue, or MEFs using Phase Lock gel tubes (5 PRIME) according to the manufacturer's instructions. DNA was then resuspended in water and the concentration was normalized prior to PCR. Primer sequences are included in Table S1.

For qPCR, DNA was resuspended in TE (10 mm Tris, 1 mm EDTA) and subjected to qPCR with the following conditions: Each DNA sample was assayed in triplicate reactions in optical-grade, 96-well plates (Applied Biosystems, Foster City, CA). In the cases of total DNA, flanked DNA, and unrecombined-β*1*^{e3}, each 25-μl reaction contained 12.5 μl of Power SYBR Green Universal Master Mix (Applied Biosystems), 5 μl of purified DNA template, and primers (900 nm each final concentration, IDTDNA). Unrecombined β1^{fl} was performed in 25 μl total reaction containing 12.5 μl of TaqMan Universal Master Mix (Applied Biosystems), 5 μl of purified DNA template, primers (900 nm each final concentration), and a FAM-conjugated TaqMan probe (250 nm final concentration; Operon, Huntsville, AL). All PCR runs were performed on an ABI 7500 Real Time Thermocycler using SDS software v.1.3 (Applied Biosystems). Reaction conditions were as follows: 10 min at 94° followed by 40 cycles of 10 sec at 94° and 30 sec at 64°. The starting template copy number of each reaction was determined by comparison to a standard curve, which was generated by the serial dilution of DNA from uninfected β*1*^{fl} or β*1*^{e3} MEFs.

RESULTS

Induced β1 integrin deletion in vascular smooth muscle: Two floxed mice were used for deletion of β1 integrin in smooth muscle cells: β*1*^{fl} and β*1*^{e3} (Figure 1A;



Age	cβ1$^{fl/fl}$ (25%)	cβ1$^{fl/+}$ (25%)	β1$^{fl/fl}$ (25%)	β1$^{fl/+}$ (25%)	Total
E15.5	15 (21%)	15 (21%)	19 (27%)	21 (30%)	70
P(0)	42 (12%)	98 (30%)	100 (30%)	92 (28%)	332
P(2)	3 (8%)	8 (22%)	15 (41%)	10 (28%)	36

Figure 1.—Inducing β1 integrin deletion in smooth muscle cells. (A) Schematic representation of the location of the loxP sites in the β*1^{fl}* and β*1^{e3}* transgenic mice (Potocnik *et al.* 2000; Raghavan *et al.* 2000). (B) Distribution of genotypes and viability of genetic crosses between cβ*1$^{+/fl}$* mice and β*1$^{fl/fl}$* mice. (C) Histology of β-gal–stained bladders from *iβ1$^{fl/fl}$* and *iR26R* either 24 hr or 48 hr postdose (PD) at indicated doses. Arrows denoted β-gal positive cells. (D) Histology of β-gal–stained bladders from *iβ1$^{fl/fl}$* and *iR26R* mice exposed to 1 mg tamoxifen daily for 28 days. Arrows point to cells negative for β-gal. (E) Western blot from bladder lysates of 28 × 1 mg *iβ1$^{fl/fl}$*, oil-injected *iβ1$^{fl/fl}$*, with *cβ1$^{fl/fl}$* as a positive control and *cβ1$^{+/fl}$* as a negative control for protein loss and α-tubulin as a loading control.

Potocnik *et al.* 2000; Raghavan *et al.* 2000). The β1^{fl} allele contains loxP sites flanking the entire protein-coding region followed by a *lacZ* gene that functions as a reporter for the β1 promoter in cells with at least one recombined allele (Figure 1A; Potocnik *et al.* 2000). Expression of the *lacZ* reporter gene occurs upon excision of the floxed portion of the β1 integrin gene and subsequent splicing from exon 1 to a short segment of exon 2 placed in front of the *lacZ* gene. To examine the role of β1 integrin in smooth muscle *in vivo*, we crossed the β*1^{fl}* mouse to the SM22αCre (identified by "c" to indicate constitutive Cre) mouse, which constitutively expresses Cre-recombinase in smooth muscle and cardiomyocytes during development (Holtwick *et al.* 2002; Figure S2). We chose the SM22αCre because, in adult tissues, SM22α (transgelin) expression is restricted to smooth muscle cells (Duband *et al.* 1993; Kuhbandner *et al.* 2000). Deletion of β1 integrin using this Cre line resulted in lethality with ~50% of the progeny dying between E15.5 and P(0) (Figure 1B). The remaining progeny died prior to weaning. Due to their early lethality, the cβ*1$^{fl/fl}$* mice could not be used to study β1 integrin deletion in the adult. To bypass this lethality, the β*1^{fl}* allele was crossed to the inducible *SM22α Cre-ER^{T2}* (identified by "i" to indicate inducible Cre) transgenic mouse (Kuhbandner *et al.* 2000).

To identify an appropriate induction protocol for deletion of β1 integrin in adult smooth muscle, a regimen of five injections of 1 mg tamoxifen was tested (Figure 1C). Bladder smooth muscle was collected and stained for β-galactosidase (β-gal) at either 24 hr or 48 hr postfinal injection. Each tamoxifen injection was additive to total recombination, as indicated by β-gal positive cells (Figure 1C and Figure S1); however, a significant portion of cells remained negative for β-gal. To determine whether this poor degree of recombination was due to heterogeneous Cre-recombinase expression, the SM22αCre-ER^{T2} mouse was also crossed to the ROSA26R reporter (*R26R*) mouse (Soriano 1999). As seen previously (Kuhbandner *et al.* 2000), a sequence of five daily injections induced recombination in most bladder smooth muscle cells (Figure 1C), indicating that Cre was expressed and active in the

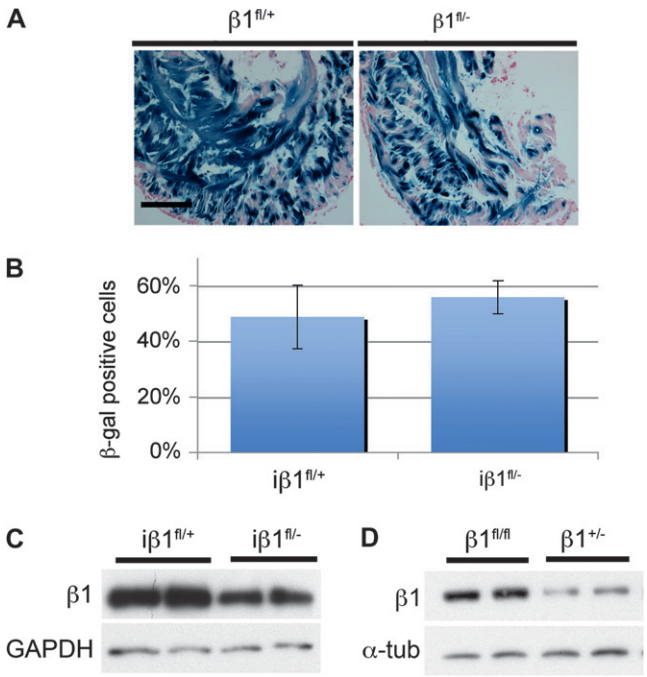


FIGURE 2.—Heterozygous and homozygous gene deletion. (A) Histology of β-gal–stained bladders from $i\beta 1^{+/fl}$ and $i\beta 1^{fl/-}$ mice that received 1 mg tamoxifen daily for 28 days. (B) Quantification of β-gal positive cells from $i\beta 1^{+/fl}$ and $i\beta 1^{fl/-}$ mice exposed to 1 mg tamoxifen daily for 28 days. (C) Western blot for β1 integrin on bladder lysates from $i\beta 1^{fl/-}$ and $i\beta 1^{+/fl}$ mice. (D) Western blot for β*1* integrin bladder lysates from $\beta 1^{+/-}$ and $\beta 1^{fl/fl}$ mice.

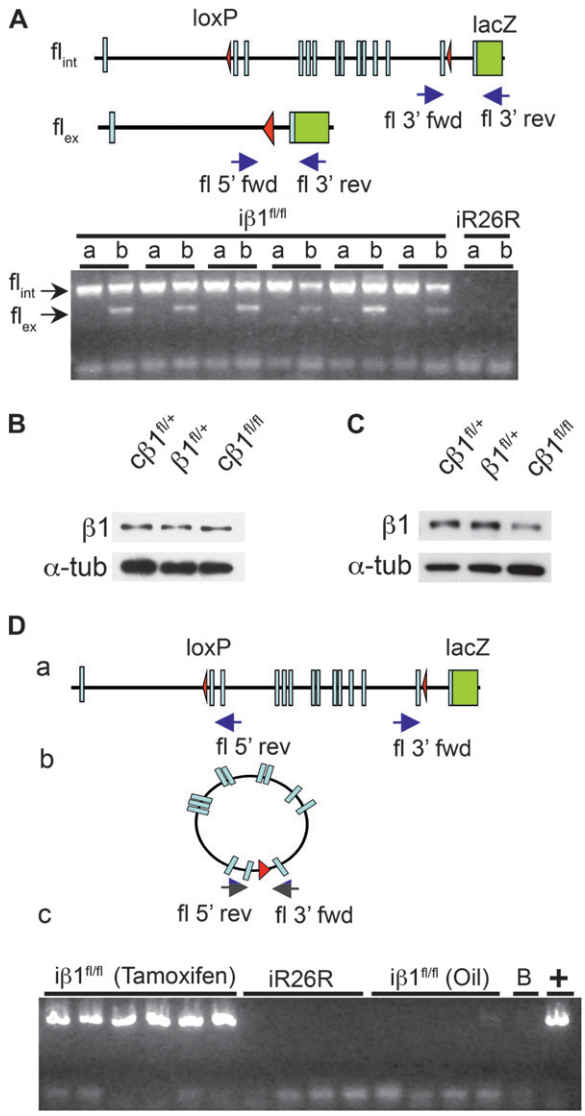


FIGURE 3.—Recombination and the generation of a $\beta 1^{fl}$ episome. (A) Schematic representation of excision product and primer locations (blue arrows). A representative PCR gel shows recombined (fl_{ex}) and intact (fl_{int}) alleles from DNA of abdominal muscle (a) and bladder (b) of $i\beta 1^{fl/fl}$ and *iR26R* mice. (B and C) Western blot analysis of bladder and heart protein lysates, respectively, from early postnatal $c\beta 1^{fl/fl}$, $\beta 1^{+/fl}$, and $c\beta 1^{+/fl}$ progeny. (D) Schematic representation of circular product and primer (blue arrows) with representative PCR results of DNA from bladder of $i\beta 1^{fl/fl}$ and *iR26R* and oil-injected $i\beta 1^{fl/fl}$ mice. B in panel c denotes a water blank and (+) is the $c\beta 1^{+/fl}$ bladder positive control.

large majority of these cells. To determine whether the low level of β-gal positive cells in the $i\beta 1^{fl/fl}$ mouse was associated with β1 integrin promoter activity in adult smooth muscle cells, bladders from the constitutive $c\beta 1^{fl/+}$ and the *cR26R* were harvested and stained for *lacZ* expression (Figure S1). In the constitutive mouse, most bladder smooth muscle cells were positive for β-gal, indicating that the β1 integrin promoter was indeed active in these cells. From this we concluded that the loxP sites flanking the $\beta 1^{fl}$ allele did not recombine as efficiently as the R26R construct, most likely due to the distance between loxP sites (ZHENG *et al.* 2000; COPPOOLSE *et al.* 2005; WANG *et al.* 2009) which is ~28 kb in $\beta 1^{fl}$ (POTOCNIK *et al.* 2000) *vs.* <2 kb in *R26R* (SORIANO 1999). Therefore, to improve excision, additional tamoxifen injections were used.

Extending the induction protocol to 28 days increased the number of β-gal positive cells (Figure 1D). β1 integrin deletion was further tested in bladder protein lysates by Western blot analysis (Figure 1E). Surprisingly, a Western blot for β1 integrin revealed no detectable change in protein levels in the $i\beta 1^{fl/fl}$ when compared to oil-injected controls despite the clear abundance of β-gal positive cells. This was in sharp contrast to protein isolated from the hearts of perinatal $c\beta 1^{fl/fl}$ and $c\beta 1^{fl/+}$ animals (Figure 1E).

Findings from the $c\beta 1^{fl/fl}$ mouse demonstrated that recombination of the $\beta 1^{fl}$ allele could indeed reduce β1 integrin protein levels. To determine whether the persistence of protein in the $i\beta 1^{fl/fl}$ mouse was due to a possible selection of heterozygous recombined cells (monoallelic recombination), we took advantage of the null ($\beta 1^{-}$) allele (STEPHENS *et al.* 1995) and generated $i\beta 1^{fl/-}$ and $i\beta 1^{fl/+}$ animals. Histological analysis of β-gal–stained bladders revealed a high proportion of recombination in both the $i\beta 1^{fl/-}$ and in the $i\beta 1^{fl/+}$ (Figure 2, A and B). Both $i\beta 1^{fl/-}$ and $i\beta 1^{fl/+}$ animals had approximately equivalent β-gal positive cells; therefore, heterozygous cells were not

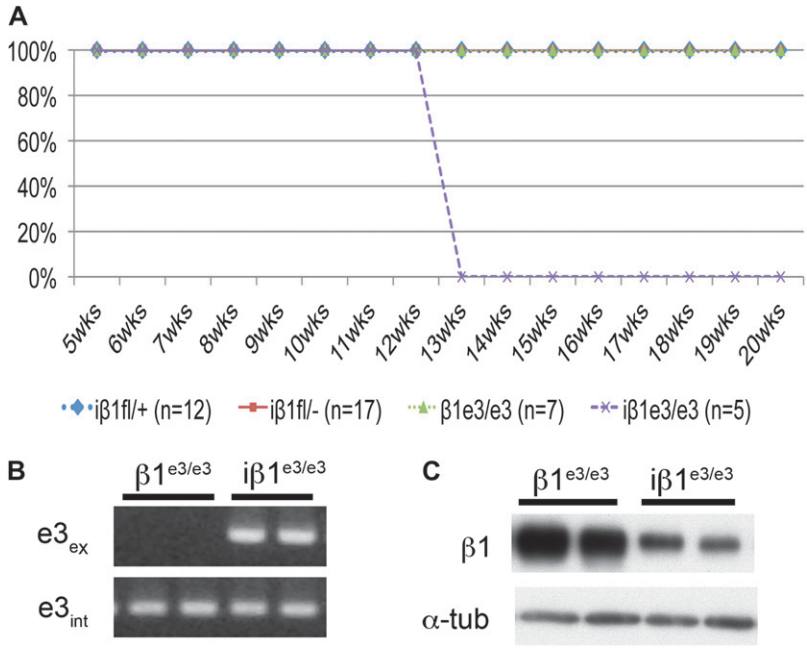


FIGURE 4.—Deletion of β1 integrin in $\beta 1^{e3}$ mice. (A) Survival curve for $i\beta 1^{fl/-}$, $i\beta 1^{+/fl}$, $i\beta 1^{e3/e3}$, and $\beta 1^{e3/e3}$ mice from the first injection through 3 months after final injection. (B) PCR of recombination ($e3_{ex}$) and intact allele ($e3_{int}$) in the $i\beta 1^{e3/e3}$ and $\beta 1^{e3/e3}$ mouse bladders. (C) Western blot analysis of bladder from $i\beta 1^{e3/e3}$ and $\beta 1^{e3/e3}$ adult mice exposed to 1 mg tamoxifen daily for 28 days.

being preferentially selected to survive in the $i\beta 1^{fl/-}$ bladders. Further, a Western blot showed reduced β1 integrin protein in $i\beta 1^{fl/-}$ bladders compared to the $i\beta 1^{fl/+}$ (Figure 2C), but approximately equivalent reduction was observed in $\beta 1^{+/-}$ and the $\beta 1^{fl/fl}$ (Figure 2D). These data demonstrate that persistence of the β1 integrin protein in the $i\beta 1^{fl/fl}$ bladders was not the result of selection of heterozygous cells and further showed that loss of a single β1 allele was detectable by Western blot.

Cre-induced recombination products: The persistence of β1 integrin protein in the $i\beta 1^{fl/fl}$ bladders could suggest that the flox sites in the gene were not being recombined. This is unlikely as β-gal staining indicated recombination of the β1 integrin locus. Nonetheless, we verified genetic excision by PCR analysis using primers that flanked the 5′ end in conjunction with the 3′ reverse primer (Figure 3A schematic). For the PCR, bladders from six $i\beta 1^{fl/fl}$ mice were used to examine recombination in smooth muscle (Figure 3A, b). We used abdominal muscle (Figure 3A, a), which is skeletal muscle as a negative control for recombination. The results confirmed the recombination (Figure 3A) and supported findings from the *lacZ* assay (Figure 1, C and D).

To reconcile the lack of protein loss after confirmed recombination, we examined the constitutive SM22αCre mice and assessed levels of β1 integrin protein in the heart and bladder (Figure 3, B and C). While the bladder of the knockout had similar β1 integrin protein levels as controls (Figure 3B), the heart, with higher cell proliferation rate at time of recombination, showed a significant decrease in β1 integrin protein over controls (Figure 3C and Figure S2). These results provide evidence that the $\beta 1^{fl}$ allele was appropriately targeted and excised during development, yet inactivation of the gene product was not accomplished in the adult bladder, despite clear evidence of recombination.

Recombination at loxP sites provides two outcomes: (a) elimination of chromosomal DNA flanking the loxP sites and (b) formation of an extrachromosomal circular product generated by the excised DNA (STERNBERG and HAMILTON 1981; HOESS *et al.* 1985; METZGER and FEIL 1999). Since excision in the inducible model was validated by PCR, we next sought the fate of the excised extrachromosomal DNA product. In the case of the $\beta 1^{fl}$ allele, the entire coding region, from the ATG translation initiation site in exon 2 to the stop codon and 3′-UTR, is flanked by loxP sites and forms a circle following recombination, which we refer to as an episome. Thus, we considered that the persistent β1 integrin protein in recombined cells might result from the activity of this extrachromosomal DNA. Next, we asked whether the circular excision product was present in recombined cells. PCR analysis using primers from opposite ends of the loxP flanked DNA showed that the episomal DNA was indeed present in the bladders of both the six $i\beta 1^{fl/fl}$ mice exposed to tamoxifen and in the adult constitutive $c\beta 1^{fl/+}$ bladders, but not in the four iR26R mice, which lack the allele, or in the four oil-injected $i\beta 1^{fl/fl}$ mice (Figure 3D).

Generation and retention of episomes from different β1 integrin flox alleles: The generation of an episomal product by Cre-recombinase has been examined at length (STERNBERG and HAMILTON 1981; HOESS *et al.* 1985; STERNBERG 1990; BIGGER *et al.* 2001). In fact, persistence of the episomal Cre-recombinase product was documented in plants (SRIVASTAVA and OW 2003) and was also noted to be retained after viral Cre infection in mice (DORIGO *et al.* 2004; GALLAHER *et al.* 2009). Yet, the potential expression from an intended

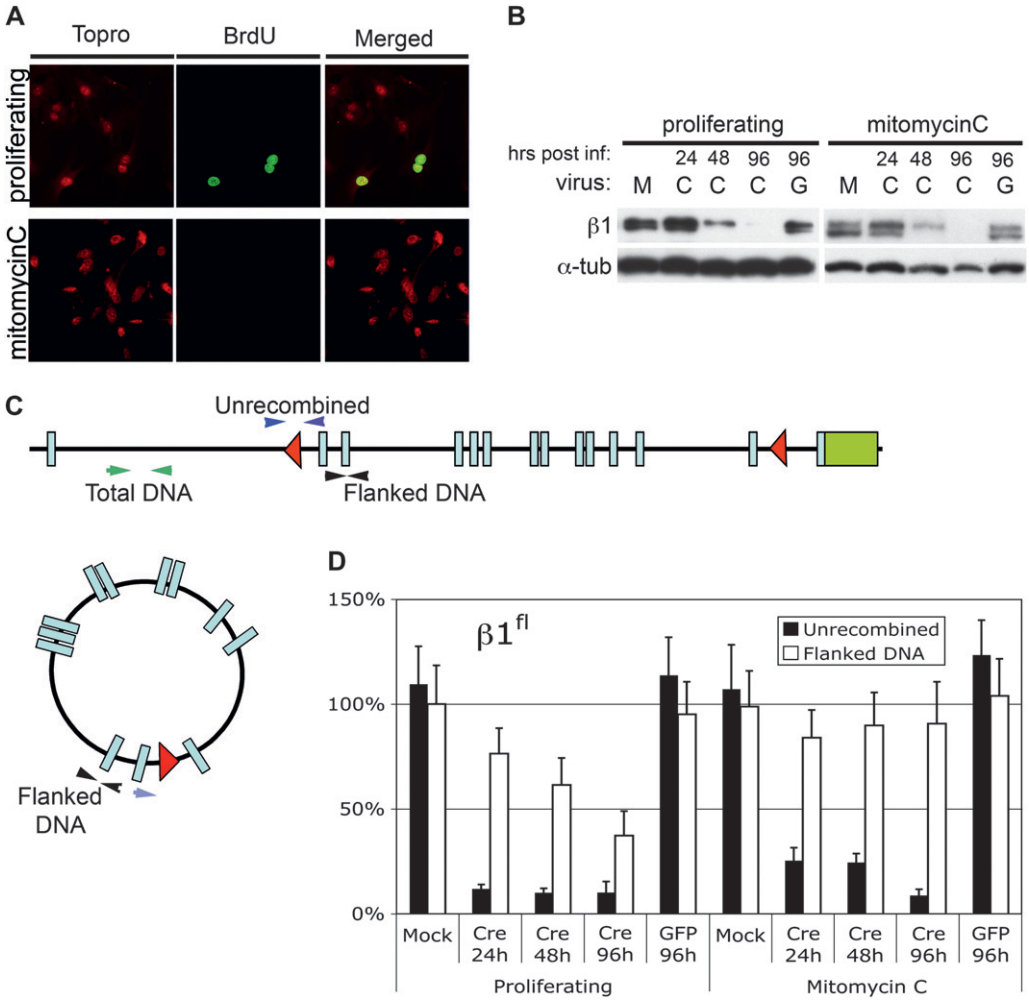


FIGURE 5.—Episome kinetics in β*1*$^{fl/fl}$ MEFs. (A) BrdU staining in untreated and mitomycin C treated β*1*$^{fl/fl}$ MEFs (BrdU-green, Topro-red). (B) Western blot showing a time course of β*1* integrin levels in mock (M), adeno-Cre (C), and adeno-GFP (G) infected cells up to 96 hr postinfection. (C) Schematic showing the location of the primers used in qPCR for detection of the flanked (episome) and unrecombined DNA. (D) Quantitative PCR of flanked and unrecombined DNA for the proliferating and mitomycin C-treated β*1*$^{fl/fl}$ MEFs.

gene deletion product has not been previously described in mammals. If the β*1*^{fl} episome was the reason for the persistence of β1 integrin protein in the recombined adult tissue, then utilization of a second loxP construct that disrupted the β1 integrin coding region should address protein persistence.

The β*1*$^{e3/e3}$ mouse has loxP sites that flank exon 3 and generate a frameshift and early termination mutation upon recombination (RAGHAVAN *et al.* 2000). We crossed the β*1*^{e3} mouse to the SM22αCre-ER^{T2} mouse. Upon treatment with tamoxifen, the *i*β*1*$^{e3/e3}$ mice die 4 weeks after the final injection with 100% penetration (Figure 4A), whereas tamoxifen-treated controls that included β*1*$^{e3/e3}$ and *i*β*1*$^{fl/+}$ as well as the iβ1$^{fl/-}$ mice remained viable throughout the experiment. PCR analysis confirmed genetic recombination (Figure 4B). Comparison of β1 integrin protein from bladders of *i*β*1*$^{e3/e3}$ and β*1*$^{e3/e3}$ mice showed the *i*β*1*$^{e3/e3}$ had a significant reduction in β1 integrin protein (Figure 4C). These results eliminated the possibility of an extended protein half-life. The findings further support the possibility that in the adult β*1*^{fl} mice, the persistence of protein was due to the excised β*1*^{fl} episome.

To evaluate episomal retention and expression in a more controlled environment, primary MEFs were isolated from β*1*$^{fl/fl}$ animals. Cells were expanded and proliferative quiescence was induced by mitomycin C treatment in half of the β*1*$^{fl/fl}$ MEFs. These lines were then subjected to either mock infection or infected with adeno-Cre virus or an adeno-gfp control virus. Mock-infected β*1*$^{fl/fl}$ quiescent and proliferating MEFs were harvested following similar treatments to that of the infected cells, but lacking virus. Adeno-cre–infected cells were harvested for protein and DNA at 24, 48, and 96 hr postinfection. Adeno-gfp control cells were harvested at 96 hr postinfection. BrdU was used to evaluate proliferation *vs.* quiescence (Figure 5A). Indeed, the quiescent MEFs were negative for BrdU. Western blots from the β*1*$^{fl/fl}$ MEFs indicate loss of protein at approximately the same rate as the β*1*$^{e3/e3}$ MEFs regardless of their proliferative status (Figure 5B and Figure S3). Quantitative PCR was performed to evaluate recombination and retention of the episome (Figure 5, C and D). The qPCR findings demonstrated high levels of recombination in all cells exposed to adeno-cre (Figure 5D). Further, β*1*$^{fl/fl}$ cells retained the episome in the quiescent state and lost it from proliferating cells. In contrast to

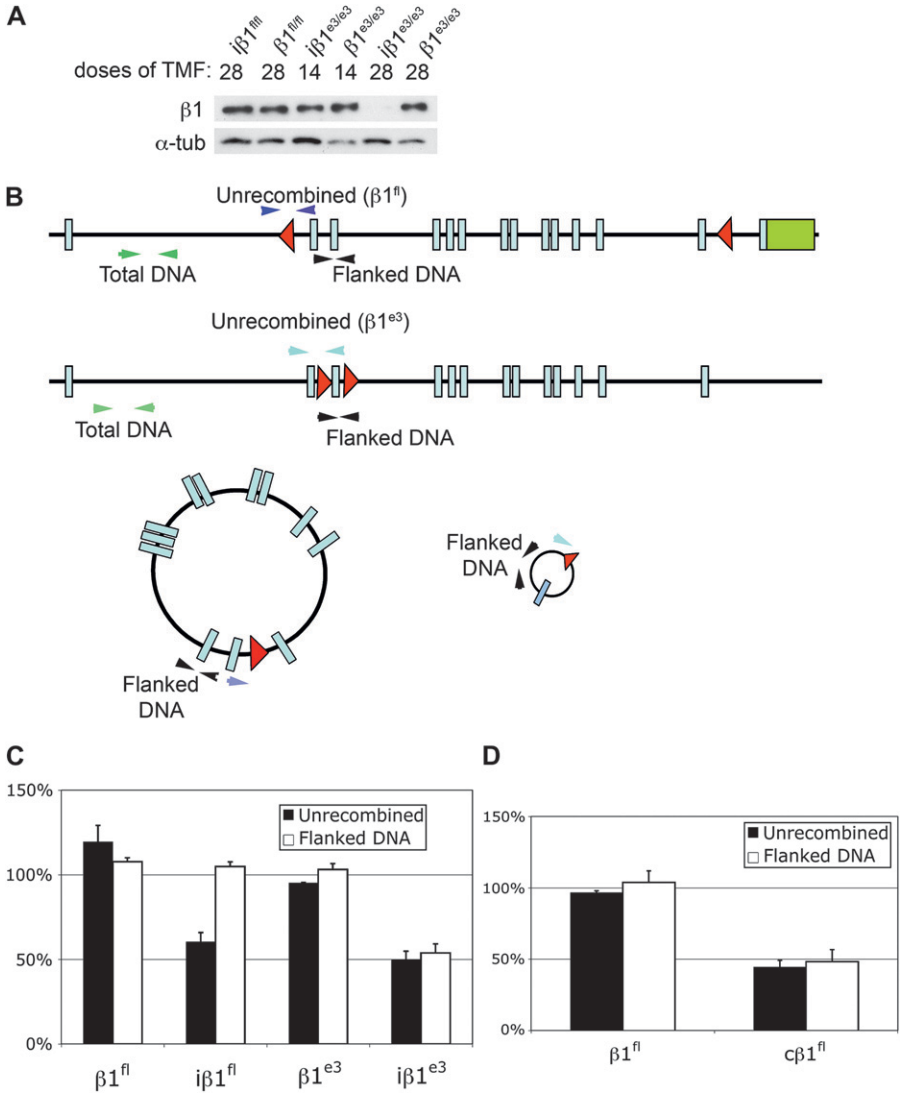


FIGURE 6.—Episome kinetics in β*1*$^{fl/fl}$ and β*1*$^{e3/e3}$ *in vivo*. (A) Western blot of *i*β*1*$^{fl/fl}$ and *i*β*1*$^{e3/e3}$ with β*1*$^{fl/fl}$ and β*1*$^{e3/e3}$ controls for tamoxifen dose indicated. The 14-dose bladders were collected 48 hr after the final injection while the 28-dose samples were collected 2 weeks after the last injection. (B) Schematic of the qPCR primers for detection of β*1*$^{fl/fl}$ and β*1*$^{e3/e3}$ flanked and unrecombined DNA. (C) Quantitative PCR for flanked and unrecombined DNA in *i*β*1*$^{fl/fl}$, β*1*$^{fl/fl}$, *i*β*1*$^{e3/e3}$, and β*1*$^{e3/e3}$ bladders of animals receiving 28 doses of tamoxifen. (D) Quantitative PCR of flanked and unrecombined DNA in the β*1*$^{fl/fl}$ and *c*β*1*$^{fl/fl}$ hearts.

the findings in the bladder, the data demonstrate that the β*1*$^{fl/fl}$-generated episome was not active in MEFs in culture (Figure 5B). Clearly the findings *in vitro* did not recapitulate either the rate of protein loss *in vivo* nor the expression from episomal recombined DNA, but demonstrated that the episome is retained in quiescent cells, while it is lost in proliferating cells.

To evaluate the episome and protein levels *in vivo*, protein and DNA was isolated from *i*β*1*$^{fl/fl}$, *i*β*1*$^{e3/e3}$, β*1*$^{fl/fl}$, and β*1*$^{e3/e3}$ mice after 28 doses of tamoxifen. Western blot analysis showed a precipitous loss of protein at 28 doses of tamoxifen in the *i*β*1*$^{e3/e3}$, while the *i*β*1*$^{fl/fl}$ mouse receiving 28 doses of tamoxifen showed no loss of protein (Figure 6A).

Quantitative PCR for recombination and presence of the episome in all four groups: *i*β*1*$^{fl/fl}$, β*1*$^{fl/fl}$, *i*β*1*$^{e3/e3}$, and β*1*$^{e3/e3}$, showed approximately equivalent total recombination in both Cre positive groups, but marked retention of episomal DNA with poor protein loss in the *i*β*1*$^{fl/fl}$ group only (Figure 6C). In contrast, DNA analysis from *c*β*1*$^{fl/fl}$ and β*1*$^{fl/fl}$ hearts showed that the episome was eliminated in the *c*β*1*$^{fl/fl}$ hearts, where protein also decreased significantly (Figures 1E, 3C, and 6D). A central difference between the *c*β*1*$^{fl/fl}$ hearts and the *i*β*1*$^{fl/fl}$ bladders is its proliferation status at times of recombination that was verified by the proliferative marker phosphorylated histone 3 (pHis3, Figure S2).

DISCUSSION

Here we have shown that recombination of the β*1*^{fl} and the β*1*^{e3} alleles in smooth muscle cells yield different outcomes. Our results indicate that this difference in phenotype can be attributed to the persistence of protein in the β*1*^{fl} allele. We addressed five hypotheses regarding the persistence of β1 integrin protein following recombination: (1) appropriate targeting, (2) heterozygous selection, (3) protein half-life, (4) recombination efficiency, and (5) episomal expression.

Testing the first three hypotheses was straightforward. Through PCR and DNA sequencing we confirmed that both loxP alleles were appropriately targeted within the β1 integrin gene. Further, work by us, as well as others, has

demonstrated that both alleles were capable of reducing β1 integrin protein levels *in vivo*, in the presence of Cre-recombinase when constitutive systems were used (Li *et al.* 2005; Benninger *et al.* 2006; Jones *et al.* 2006; Lei *et al.* 2008; Zovein et al. 2010). β1 integrin had been shown to be important in cell survival (Stupack and Cheresh 2002; Manohar *et al.* 2004; Pinkse *et al.* 2004). We showed by analysis of β-gal–stained bladders from $i\beta1^{fl/+}$ and $i\beta1^{fl/-}$ mice that heterozygous selection was unlikely. Finally, while the protein half-life of β1 integrin in cultured cells is 6–8 hr (De Strooper *et al.* 1991), there is no information as to β1 integrin turnover *in vivo*. Nonetheless, the $i\beta1^{e3/e3}$ findings confirmed that an extended protein half-life was not causing protein persistence. Thus, having eliminated the first three hypotheses, we turned our attention to recombination efficiency.

It has been shown that even small changes in the distance between loxP sites can affect recombination efficiency (Zheng *et al.* 2000; Coppoolse *et al.* 2005; Wang *et al.* 2009). The distance between loxP sites in the $\beta1^{fl}$ allele is ~28 kb (Potocnik *et al.* 2000), while the loxP sites for the $\beta1^{e3}$ allele (Raghavan *et al.* 2000) are <2 kb apart. The 28-day protocol was developed to compensate for the inefficiency of the $\beta1^{fl}$ construct and, indeed, we observed a significant increase in the number of β-gal positive cells when compared to the 5-day regimen. Surprisingly, there was no change in protein levels. Moreover, Western blot analysis of the $\beta1^{+/-}$ and $i\beta1^{fl/-}$ bladder tissue when compared to $\beta1^{fl/fl}$ showed that loss of a single allele was significant enough to reduce β1 integrin protein. This finding indicates that β1 integrin does not change promoter activity to compensate for lower total β1 integrin protein. Furthermore, it demonstrates that recombination efficiency of the $\beta1^{fl}$ allele is lower than the $\beta1^{e3}$, but this low efficiency is an unlikely explanation for the persistence of protein. At this point we began to consider the episomal hypothesis.

We found the episome generated by Cre-recombinase persisted *in vivo*, particularly with the $\beta1^{fl}$ transgene. Interestingly, unlike the $\beta1^{e3}$ mice, the $\beta1^{fl}$ recombination product included the ATG site at exon 2 through the final exon containing the stop codon. It is assumed that the lack of exon 1 and, therefore the promoter, would make this product transcriptionally irrelevant. However, recent analysis of the mouse and human genomes revealed that >50% of genes have alternative promoters and transcriptional start sites (Carninci *et al.* 2006). Of the genes with alternative transcriptional start sites, 97% generate identical protein products (Carninci *et al.* 2006). The persistence of the episome in the bladder of $i\beta1^{fl/fl}$ mice and the apparent loss of the episome and subsequent protein depletion in the heart of $c\beta1^{fl/fl}$ mice suggest potential expression *in vivo*. Consistent with this possibility Cre-recombinase–generated episomes have been shown to be competent for transcription in mammalian cells in culture, as well as *in vivo* (Bigger *et al.* 2001; Dorigo *et al.* 2004; Gallaher *et al.* 2009).

We attempted to recapitulate our *in vivo* data using MEFs *in vitro*. While we were able to generate quiescent $\beta1^{fl/fl}$ MEFs, which retained the episome upon adeno-cre–mediated recombination, β1 integrin protein loss was equivalent between proliferating and quiescent $\beta1^{fl/fl}$ MEFs. The discrepancy between the *in vivo* and *in vitro* experiments is difficult to interpret. It is possible that the promoter active in smooth muscle cells *in vivo* is not active in MEFs. Given the *in vivo* and *in vitro* qPCR data combined and the Western blots, it is difficult to resolve the difference in protein persistence by efficiency alone. We achieve nearly comparable recombination levels in the $i\beta1^{fl/fl}$ and $i\beta1^{e3/e3}$ bladders *in vivo*, yet changes in total protein do not equate and, more importantly, the biological outcome of the deletion in each model is distinct. While we find *in vitro* analyses are valuable, it may be a less optimal approach when exploring questions of gene regulation *in vivo*.

In summary, our data illustrate the value of analyzing a phenotype using multiple loxP alleles and reinforce the importance of verifying protein loss, not just genetic recombination in Cre/loxP experiments. Further, protein depletion needs to be validated *in vivo* for every novel Cre/loxP combination. Finally, this work illustrates a new approach to the Cre/loxP system *in vivo*. Our data together with previous work (Dorigo *et al.* 2004; Gallaher *et al.* 2009) indicate the potential for a new method for noninvasive gene expression that would be permanent in quiescent cells and transitory in proliferating cells. The technique may eventually enable novel and temporary gene therapies.

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GENETICS

Supporting Information
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When Cre-Mediated Recombination in Mice Does Not Result in Protein Loss

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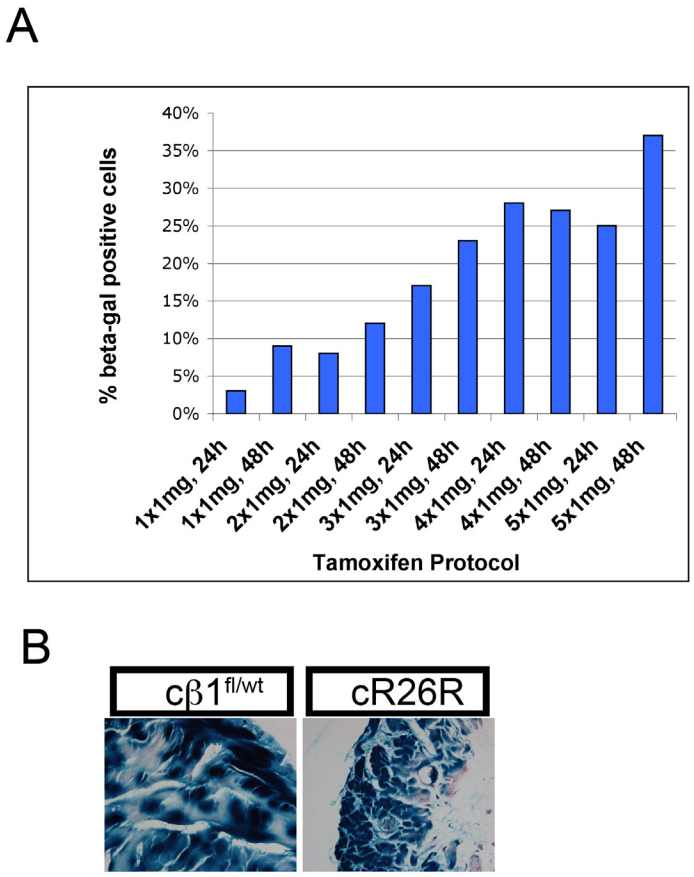

FIGURE S1.—β1fl bladder excision. A) Graph of β-gal positive cells in the bladder of *iβ1fl/fl* mice for the dose indicated. B) Histology of β-gal stained adult bladders from *cβ1fl/wt* and *cR26R* mice.

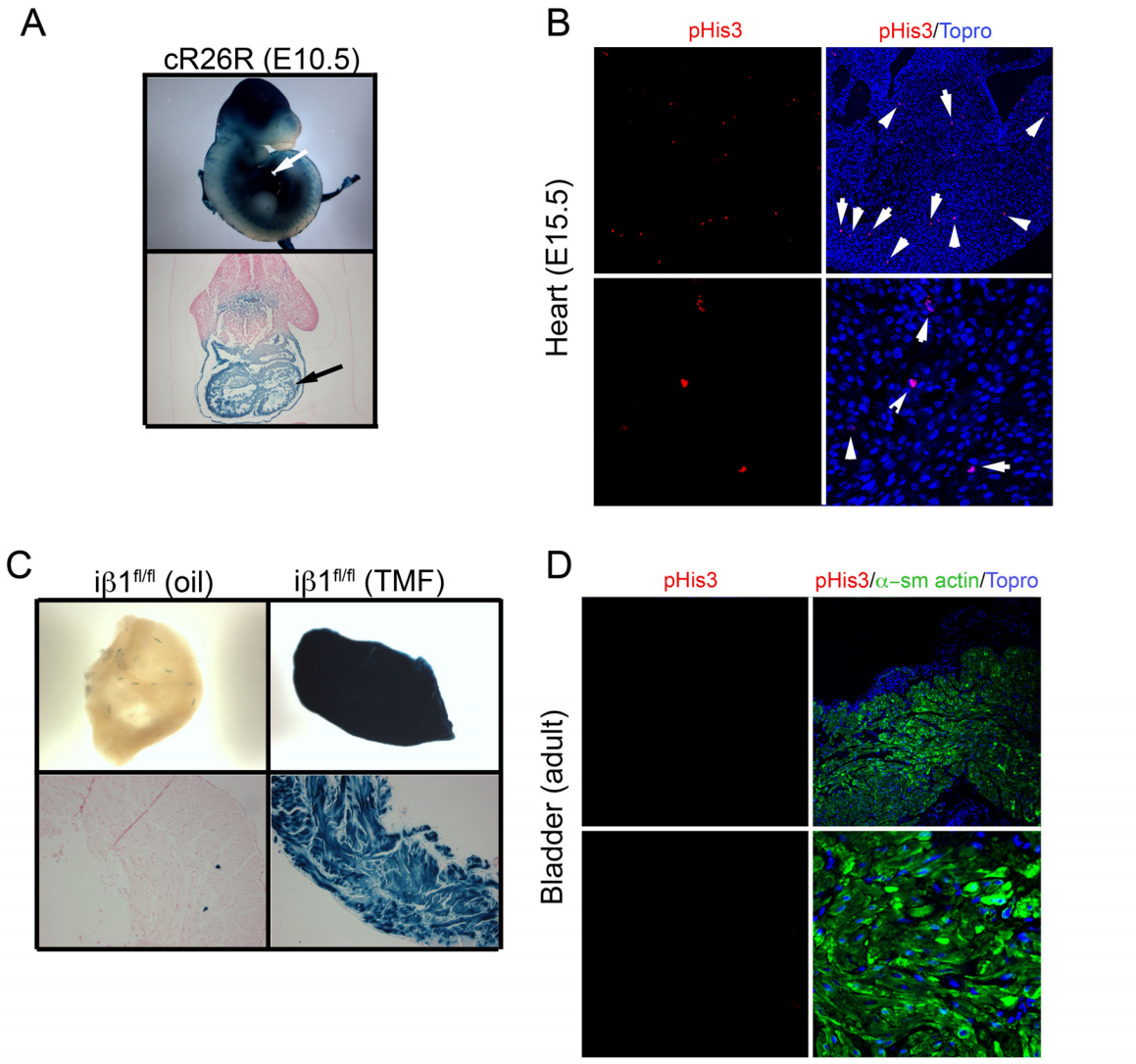

FIGURE S2.—Cre activity and proliferation in heart and bladder *in vivo*. A) Whole mount and histological section of cR26R heart (pHis3 – red, Topro-blue). Arrows indicate heart. B) Phosphorylated histone3 (pHis3) staining of wild type E15.5 heart. pHis3-red, Topro-blue. C) Whole mount and histological section of a small portion of bladder from 28-day oil (oil) and tamoxifen (TMF) injected $i\beta1^{fl/fl}$ mice. D) Phosphorylated histone (pHis3) staining of wild type adult mouse bladder. (pHis-red, α-sm actin-green, topro-blue).

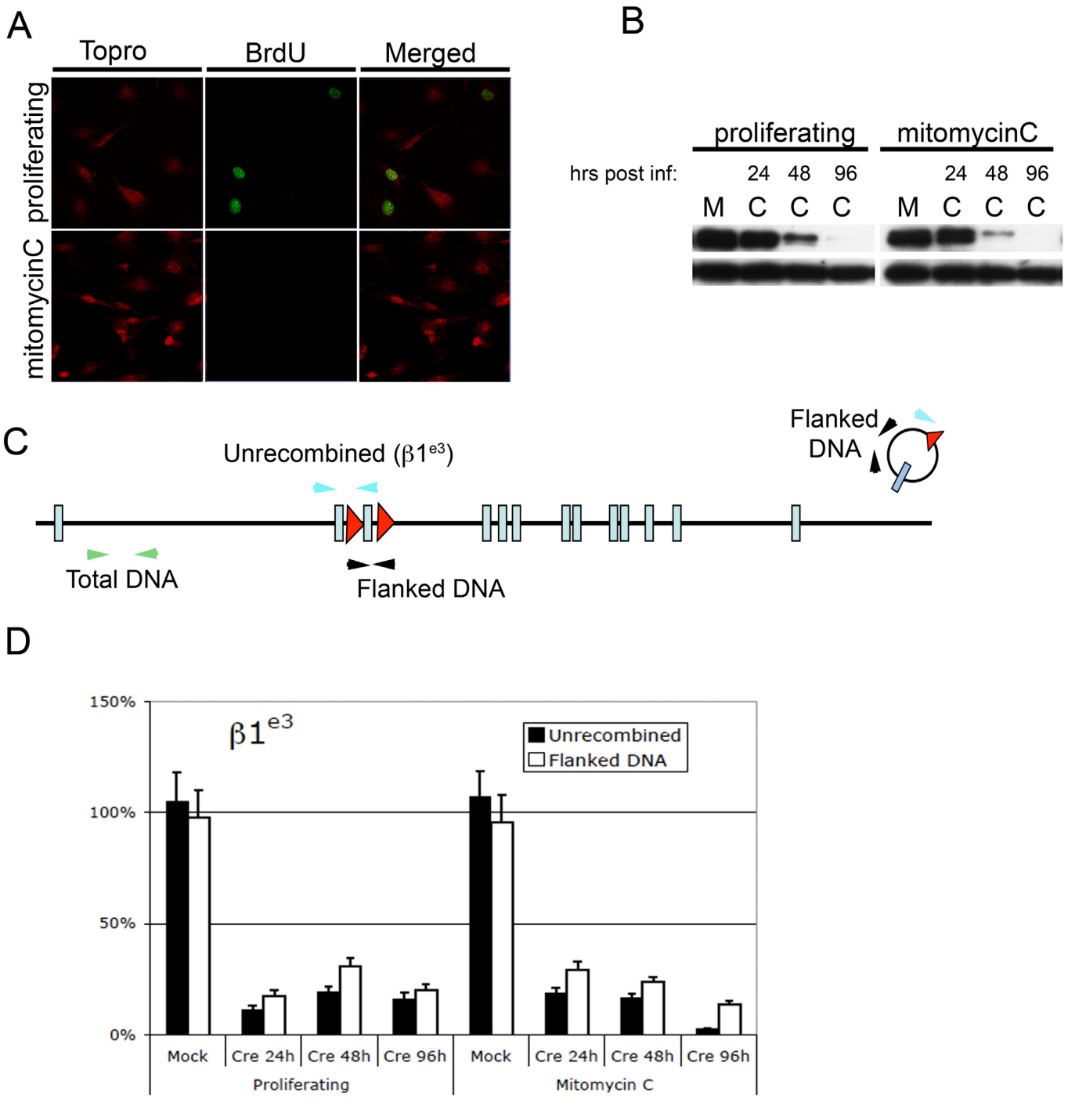

FIGURE S3.—A) BrdU staining of untreated and mitomycinC treated $\beta1^{e3/e3}$ MEFs (BrdU-red, Topro-blue). B) Western blot showing $\beta1$ integrin levels in mock (M) and adeno-Cre (C) treated cells up to 96h post infection. C) Schematic showing the location of the primers used in qPCR for detection of the flanked and unrecombined DNA. D) Quantitative PCR of flanked and unrecombined DNA for the proliferating and mitomycin C treated $\beta1^{e3/e3}$ MEFs.

TABLE S1

Genotyping and qPCR Primers

Flox primers	
fl 5' fwd	GTGAAGTAGGTGAAAGGTAAC
fl 5' rev	TTCTAGAGGGAAGGGCAA
fl 3' fwd	ACCATCAATTGTCACTATCAGTGCT
fl 3' rev (lacZ)	AAACCAGGCAAAGCGCCATT
E3 primers	
e3 5' fwd	CGCAGAACAATAGGTGCTGAAATTAC
e3 5' rev	CGTACACTGAGAACCACAAACGGC
e3 3' rev	CCACAACTTTCCCAGTTAGCTCTC
null primers	
null fwd	TCACCTCCTAACCCTGAGAT
null rev	GCAATCCATCTTGTTCAATG
null wt rev	CAATCCAGGAAACCAGTTGC
qPCR Primers	
Total Genomic fwd	GAGAGCCCCAGGAGACTGTGT
Total Genomic rev	CAAAGTTAGTATTCCTACAGCAGAAAGC
Flanked DNA fwd	GATTGCCGTTTGTGGTTCTCA
Flanked DNA rev	GCTATACCAGGCTACTCCAAAGAAA
Unrecombined e3 fwd	GTCAACAGTATGGTCTAATGGTGTGTAG
Unrecombined e3 rev	ACAAACGGCAATCATCTTGTCTT
Unrecombined flox fwd	TGTGAAGTAGGTGAAAGAAGGTAACG
Unrecombined flox rev	CGAGGAATTCCGATCATATTCAA
Unrecombined flox probe	CCGATAAGCTTGGCTGGACGTAAACTCC