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RMCE-ASAP: a gene targeting method for ES and somatic cells to accelerate phenotype analyses

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ABSTRACT

In recent years, tremendous insight has been gained on p53 regulation by targeting mutations at the *p53* locus using homologous recombination in ES cells to generate mutant mice. Although informative, this approach is inefficient, slow and expensive. To facilitate targeting at the *p53* locus, we developed an improved Recombinase-Mediated Cassette Exchange (RMCE) method. Our approach enables efficient targeting in ES cells to facilitate the production of mutant mice. But more importantly, the approach was Adapted for targeting in Somatic cells to Accelerate Phenotyping (RMCE-ASAP). We provide proof-of-concept for this at the *p53* locus, by showing efficient targeting in fibroblasts, and rapid phenotypic read-out of a recessive mutation after a single exchange. RMCE-ASAP combines inverted heterologous recombinase target sites, a positive/negative selection marker that preserves the germline capacity of ES cells, and the power of mouse genetics. These general principles should make RMCE-ASAP applicable to any locus.

INTRODUCTION

p53 is one of the most highly analyzed proteins for the past 25 years. Studies in cultured cells, often relying on the transfection of plasmids expressing various p53 mutants, have established models to explain how p53 is regulated. In recent years, some of these models were tested *in vivo* by targeting subtle mutations at the *p53* locus using homologous recombination in embryonic stem (ES) cells to generate mutant mice. The strength of this approach is that mutations are tested in a genomic setting and expressed from the endogenous promoter, ensuring physiological expression levels and correct spatio-temporal profiles. As significant differences between phenotypes from targeted p53 mutants *in vivo* and transfection data were observed [e.g. Refs (1–5)], more targeted mutations need to be generated and analyzed in multiple tissues to formulate more accurate models of p53 regulation.

However, using homologous recombination in ES cells to generate mutant mice is an inefficient, slow and expensive method because (i) homologous recombination typically occurs at low frequency in ES cells, requiring sophisticated selection schemes and screening of hundreds of clones to identify the desired mutant; (ii) large (15–20 kb) plasmids, often difficult to clone, are required to increase targeting efficiency and (iii) breeding mice to homozygosity and housing a mouse colony generate further delays and costs. Such limitations make the repeated targeting of a locus a technically daunting and economically impractical task.

Improvements in current technologies are needed to enable such analyses to be applied to the p53 or other genes. Developing methods to increase targeting efficiency in ES cells is clearly an important goal. In addition, efficient methods for gene targeting in fibroblasts could expedite phenotypic analyses. Indeed, siRNAs in fibroblasts often provide a faster read-out than equivalent gene knock-outs in animals (6). However, modeling most disease-associated mutations requires generating subtle mutations, not knock-outs or reduced expression alleles. Targeting point mutations in fibroblasts by homologous recombination is extremely inefficient, and targeting both alleles is required to reveal the phenotype of recessive autosomal mutations.

Here we report an approach that enables highly efficient targeting at the *p53* locus in both ES cells and fibroblasts. Recombinase-Mediated Cassette Exchange (RMCE) approaches were developed to improve targeting efficiency using a two-step process: the gene of interest is first replaced by a selection cassette flanked by recombinase target sites (e.g. loxP sites for Cre recombinase, to create a 'floxed' locus). Then, Cre-mediated recombination in the presence of a cassette containing a floxed mutant allele removes the resident sequence and inserts the mutant gene (7). Previously, technical difficulties have prevented RMCE from being applied routinely to generate mutant mice. For example,

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exchanges using cassettes with directly repeated loxP sites were inefficient because excisions dominated the intended exchanges (8). loxP sites with different sequences were generated to overcome this problem, but these sites also underwent intramolecular recombination, making RMCE efficient only if the replacement cassette contained a marker enabling selection of the desired recombinant (7,9–12). However, interference resulting from expression of the selection marker and the endogenous gene (13) necessitates strategies to remove the selectable gene. Together, previous studies indicate that an optimal RMCE requires (i) inverted heterologous loxP sites diverging by at least 2 nt to maximize the efficiency of exchange and (ii) an expression cassette enabling both positive selection to identify the initial recombinant and then negative selection to obtain a 'marker-free' mutant allele (14).

Most RMCE experiments have been performed at random sites in somatic cell lines. Only a few mutant mice generated by RMCE in ES cells have been reported, but the RMCE systematically introduced a selectable marker (15–17), or, when tested without an incoming marker, proved inefficient (12). A recent report disclosed an additional problem: the Hygromycin–Thymidine Kinase fusion gene used most frequently for positive/negative selection in RMCE, leads to mouse sterility, so that exchanges can only be performed in ES cells (16).

The RMCE strategy presented here relies on the integrated use of inverted heterologous loxP sites, a positive/negative selection marker that preserves the germline capacity of ES cells, and the power of mouse genetics to expedite phenotypic analyses. We show that our approach enables efficient targeting of marker-free mutations at the p53 locus in ES cells to generate mutant mice, but more importantly, it is Adapted for targeting in Somatic cells to Accelerate Phenotyping (ASAP). Because it relies on very general principles, RMCE-ASAP could be applied to any locus of interest.

MATERIALS AND METHODS

Targeting construct for a *p53* RMCE-ready locus

Details for plasmid construction are available upon request owing to space limitations mandating a brief outline. We started from a plasmid L3-1L containing heterologous loxP sites (L3 is the mutant loxP257 recently described (14), 1L is an inverted WT loxP). The WT loxP and loxP257 differ in their spacer sequences: the spacer sequence is 5′-ATGTATGC-3′ for WT loxP and 5′-AAGTCTCC-3′ for loxP257. The three mutations in the loxP257 spacer sequence prevent it to recombine with WT loxP, ensuring efficient RMCE in several cell lines: accurate RMCE with these loxP sites occurred with an average frequency of 81% at two loci in CHO cells and an average frequency of 69% at four loci in Hela cells (14). The L3-1L plasmid was first modified to include a ClaI and a FseI site between the LoxP sites, leading to plasmid L3-CF-1L. We next modified a puroΔTK plasmid (YTC37, a kind gift from A. Bradley) by using oligonucleotides to destroy a NotI site downstream of the puroΔTK gene and introduce a NotI site upstream, and a FseI site downstream of the gene (leading to plasmid CN-PuroΔTK-F). Next, a PmlI-MfeI 6.3 kb fragment from *Trp53* was subcloned in a modified pBluescript KSII+ (pBS, Stratagene), and the resulting plasmid was digested with SwaI to introduce an EagI site, leading to p53PmlEag, a plasmid containing exons 2–11 of p53. We then inserted a 5.5 kb ClaI-EagI fragment from p53PmlEag in plasmid CN-PuroΔTK-F digested by ClaI and NotI, and inserted the resulting fragment between the loxP sites of L3-CF-1L by ClaI and FseI digestion, leading to L3-p53PmlEagPuroΔTK--1L. We next engineered a plasmid containing the region for 3′-homology downstream of the p53 gene and the DTA gene in two steps: (i) we performed a three-way ligation between a modified pBS digested by HindIII and NotI, a HindIII-EcoRI fragment from *Trp53* for 3′homology and an EcoRI-NotI fragment containing the DTA gene, from plasmid pgkdtabpa (kind gift of P. Soriano), leading to plasmid 3′ + DTA and (ii) because the Bsu36I-EcoRI region downstream of p53 contains repetitive sequences (F. Toledo and G. M. Wahl, unpublished data), we later deleted this region, to obtain plasmid 3′ + DTA. The 5′ homology consists of a 3.4 kb-long BamHI-PmlI fragment from intron 1 of p53 cloned in a modified pBS (plasmid p5′). Finally, appropriate fragments from plasmids p5′, L3-p53PmlEagPuroΔTK-1L, and 3′ + DTA were assembled in a modified pSP72 plasmid (Promega). Plasmid Flox, the resulting targeting construct, was verified by restriction analysis, then sequenced using 30 primers chosen to precisely verify all p53 coding sequences, all exon–intron junctions and the sequences at and around the loxP sites.

Exchange constructs: making the $p53^{GFP}$ and $p53^{\Delta PGFP}$ plasmids

To make a p53-GFP fusion protein, we first subcloned a SacII-HindIII fragment of the p53 locus (corresponding to part of exon 10 to sequences downstream of the gene) into pBS, then mutated the HindIII site into a FseI site. We next mutated the C-terminal part of the p53 gene in two rounds of PCR mutagenesis, first with primers 5′-GGGCCTGACTCA-GACGGATCCCCTCTGCATCCCGTC-3′ and 5′-GACGGG-ATGCAGAGGGGATCCGTCTGAGTCAGGCCC-3′, which removed the stop codon and introduced a BamHI site, then with primers 5′-GACGGATCCCCTCTGAATTCCGTCCC-CATCACCA-3′ and 5′-TGGTGATGGGGACGGAATACA-GAGGGGATCCGTC-3′, which introduced an EcoRI site. We verified the sequence from the mutated plasmid, then digested it with BamHI and EcoRI, to insert in frame GFP sequences from a Bam HI-EcoRI fragment of plasmid phr-GFP-1 (Stratagene). We verified the sequence of this p53-GFP fusion fragment, then swapped it in the L3-p53PmlEagPuroΔTK-1L plasmid (see above) by HindIII and FseI digestion, resulting in the $p53^{GFP}$ exchange construct, the sequence which was verified before use. The $p53^{\Delta PGFP}$ exchange construct was engineered by combining sequences from the $p53^{GFP}$ exchange plasmid and sequences from the $p53^{\Delta P}$ targeting construct described recently (5). Its sequence was also verified before use.

Sequences and use of PCR primers

a: 5′-CCCCGGCCCTCACCCTCATCTTCG-3′, from the PuΔTK gene, assays targeting of Flox plasmid; b: 5′-AACA-AAACAAAACAGCAGCAACAA-3′, from sequences downstream of the p53 gene and outside Flox sequences, assays

targeting of Flox and RMCE with $p53^{GFP}$ or $p53^{\Delta PGFP}$ plasmids; c: 5′-TGAAGAGCAAGGGCGTGGTGAAGGA-3′, from GFP sequences, assays RMCE with $p53^{GFP}$ or $p53^{\Delta PGFP}$ plasmids; d: 5′-CAAAAAATGGAAGGAAATCAGGAACT-AA-3′, from p53 intron 3, and e: 5′-TCTAGACAGAGAAA-AAAGAGGCATT-3′, from p53 intron 4, assay RMCE with $p53^{\Delta PGFP}$ plasmid; f: 5′-ATGGGAGGCTGCCAGTCCTAA-CCC and g: 5′-GTGTTTCATTAGTTCCCCACCTTGAC-3′ amplify the WT p53 allele according to Taconic's procedures, h: 5′-TTTACGGAGCCCTGGCGCTCGATGT-3′ and i: 5′-GTGGGAGGGACAAAAGTTCGAGGCC-3′ amplify the Neo marker in the p53 KO allele according to Taconic's procedures.

Cell culture conditions

Primary MEFs, isolated from 13.5 day embryos, were cultured in DMEM with 15% FBS, 100 mM BME, 2 mM L-glutamine and antibiotics. 129/SvJae ES cells were grown in the same medium supplemented with 1000 U/ml ESGRO (Chemicon), on a layer of mitomycin C-treated SNLPuro-7/4 feeders (kind gift of A. Bradley). Selections were performed with 2 μg/ml puromycin, 0.2 μM FIAU or 2 μM ganciclovir.

Targeting/genotyping of the RMCE-ready locus

29/SvJae ES cells were electroporated with the Flox construct linearized with PmeI, and puromycin resistant clones were analyzed as described (Figure 2). Two clones were injected into blastocysts and transmitted through the germline.

Performing RMCE in ES cells

A total of 8×10^5 $p53^{RMCE/+}$ ES cells were grown without puromycin for 12 h, electroporated with 15 μg CMV-Cre plasmid (pOG231) and 200 μg of the exchange construct, and plated in T25 flasks at 10^5 cells per flask. FIAU was added to the medium 3–4 days after electroporation.

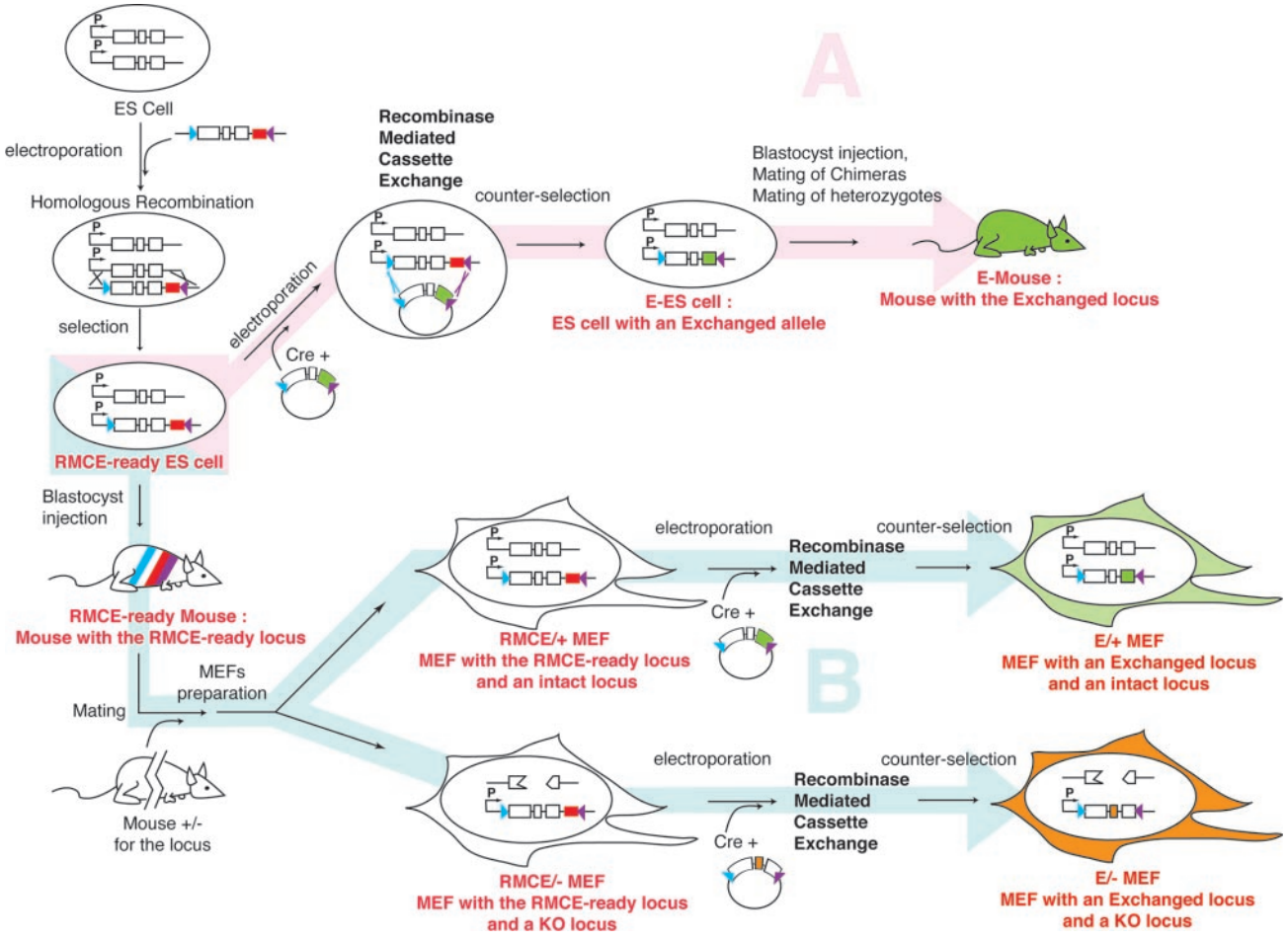

Figure 1. Rationale for a RMCE-ASAP. Using homologous recombination, the gene of interest (GOI, open boxes: exons), is targeted with a construct introducing upstream of coding regions one loxP (blue arrowhead) and downstream, a positive/negative selection cassette (red box) and a second inverted heterologous loxP (purple arrowhead) to create RMCE-ready ES cells. An exchange is performed in these cells by co-transfecting a Cre expression plasmid and a marker-free plasmid with a floxed mutant GOI (green box: mutated exon), to produce a mutant mouse (path A). Importantly RMCE-ASAP incorporates two major improvements over classical RMCE (path B): (i) the positive/negative selection cassette does not prevent germline transmission, so that RMCE-ready mice can be obtained; (ii) the selection cassette does not replace, but rather lies downstream of the GOI. This is a crucial requirement for accelerated phenotyping in somatic cells, as maintaining a functional GOI ensures that the RMCE-ready locus still behaves like a WT locus. Hence, after breeding the RMCE-ready mouse with mice heterozygotes for the GOI, somatic cells with an RMCE-ready locus and a WT or KO allele can be recovered [e.g. RMCE/+ and RMCE/− mouse embryonic fibroblasts (MEFs)]. Such cells, phenotypically similar to +/+ and +/− cells, can then be used for phenotypic analyses of dominant or recessive mutations after a single exchange.

Individual clones, picked 10 days after electroporation, were grown in 96-well plates and expanded to generate duplicate plates for freezing and DNA analysis by PCR and Southern.

Performing RMCE in MEFs

A total of 10^6 *p53*$^{RMCE/-}$ MEFs cells were grown without puromycin for 12 h, electroporated with 3 μg pOG231 and 30 μg exchange construct, and plated in a single 10 cm-dish, grown for 3 days then split in several dishes at 10^5 cells per dish. FIAU or ganciclovir was added to the medium 4 days after electroporation, for 3–4 days. Clones, picked 10 days after electroporation, were grown in 24-well plates and expanded for freezing and DNA analysis.

Western-blots

Cells, untreated or treated for 24 h with 0.5 μg/ml adriamycin, were lysed on the dish in a buffer consisting of 50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 1 mM sodium vanadate, 10 mM NaF and Complete Mini Protease Inhibitors (Roche Diagnostics) at 4°C for 30 min. Lysates were scraped, then spun at 6000× *g* at 4°C for 10 min. Protein concentration in the supernatant was determined using the Bio-Rad DC protein assay. Lysates were separated on single percentage SDS/PAGE gels, then electrophoretically transferred to poly(vinylidene difluoride), using standard procedures. Blots were incubated in 5% non-fat dried milk in TBST (0.02 M Tris, pH 7.6/0.35 M NaCl/0.1% Tween-20) for 1 h at room temperature before probing with primary antibodies against p53 (CM-5, Novacastra) and -actin (Sigma). Secondary antibodies used include peroxidase-conjugated goat anti-mouse IgG and anti-rabbit IgG (Pierce). Probed blots were incubated with Pierce Supersignal West Pico chemiluminescent substrate and exposed to X-ray films.

Flow cytometry

Log phase cells were irradiated at RT with a 60 Co γ-irradiator at doses of 6 or 12 Gy and incubated for 24 h. Cells were then pulse-labeled for 1 h with BrdU (10 μM), fixed in 70% ethanol, double-stained with FITC anti-BrdU and propidium iodide, then sorted by using a Becton Dickinson FACScan machine. Data were analyzed using Becton Dickinson Cellquest Pro.

RESULTS AND DISCUSSION

The rationale for RMCE-ASAP is detailed in Figure 1. The first step requires generating a floxed allele in ES cells that will serve as the substrate for subsequent exchanges (RMCE-ready ES cell, Figure 1). The targeting strategy is detailed in Figure 2. The frequency of targeting was 4% (12/300 puromycin-resistant clones, analyzed by Southern blot and long-range PCR, Figure 2).

We next tested the efficiency of RMCE in ES cells, using a replacement construct encoding p53 fused to GFP (p53^{GFP}) to enable tracking p53 in individual live cells. Importantly however, GFP fluorescence was not used to screen cells with targeted events, as we wanted to develop a general method to isolate marker-free recombinants. The exchange strategy is detailed in Figure 3A. We picked 65 ES cell clones

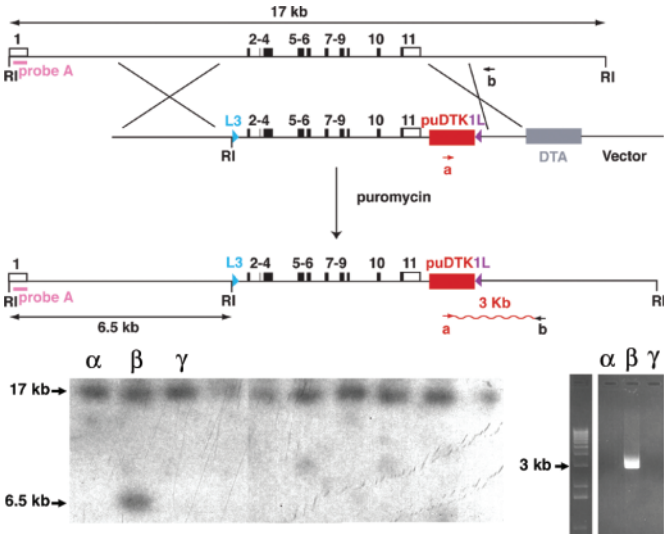

Figure 2. Generating ES cells with a *p53* RMCE-ready locus. The p53 gene is contained in a 17 kb-long EcoRI (RI) fragment (black boxes: coding sequences, white boxes: UTRs). The Flox targeting construct (below), the sequence which was verified before use (Materials and Methods), contains (i) a 3.4 kb-long 5′ homology region; (ii) 0.2 kb upstream of coding sequences, an EcoRI site and L3, a mutant loxP [loxP257, (14)]; (iii) p53 exons; (iv) 0.4 kb downstream, a puroΔTK fusion gene (puDTK) for positive/negative selection (21) and an inverted WT loxP (1L); (v) a 1.2 kb-long 3′ homology region and (vi) the diphteria α-toxin (DTA) gene for targeting enrichment. The recombinants resulting from the depicted crossing-overs are identified by a 6.5 kb band in Southern blot with probe A and a 3 kb band by PCR with primers a and b. A representative Southern, and PCR of one positive (β) and two negative clones, are shown.

resistant to 1-(-2-deoxy-2-fluoro-1-b-D-arabino-furanosyl)-5-iodouracil (FIAU) due to TK loss and analyzed their DNA by PCR and Southern blot. Strikingly, 54 proper recombinants were identified, indicating very high RMCE efficiency (83%). RMCE also proved to be precise, as no aberrant bands were detected in PCR and Southern blots (Figure 3A). *p53*$^{+/GFP}$ ES clones were analyzed by western blot with an antibody against p53, and found to express an additional band at the expected size (ca. 80 kDa). Surprisingly, the fusion of GFP to p53 apparently altered p53 stability: steady-state levels of p53^{GFP} were much higher than those of wild-type p53 (p53^{WT}) in unstressed cells, and did not vary significantly after DNA damage, so that the levels for both p53^{WT} and p53^{GFP} were similar after adriamycin treatment (Figure 3A).

Six independent *p53*$^{+/GFP}$ clones were injected into blastocysts and transferred to pseudo-pregnant females using standard procedures. Strikingly, no pregnancies were obtained. It has been shown that the p53 pathway is regulated very differently in ES and somatic cells: ES cells contain relatively high p53 levels and lack the p53-mediated DNA damage responses found in somatic cells (18). This, together with the observation that p53 levels decrease during mouse embryogenesis (19), suggested an explanation for the observed lack of pregnancies: we speculate that the high levels of p53^{GFP} in the ES cells injected into blastocysts might have prevented normal embryonic development once these cells began to differentiate and the p53 pathway became functional.

To test this possibility, we performed RMCE with a p53 fusion gene in which the p53 proline-rich domain (PRD)

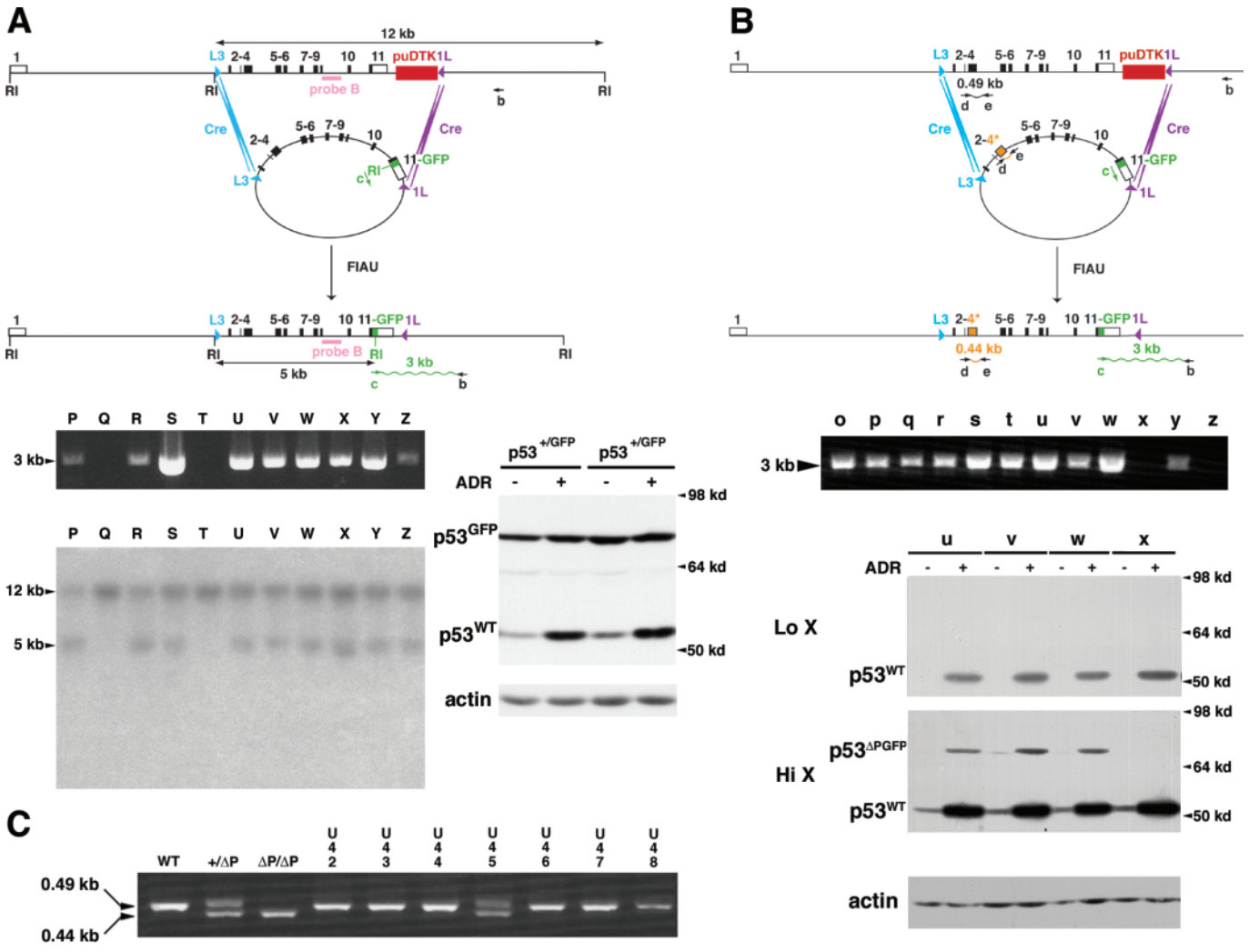

Figure 3. Performing RMCE in ES cells. **(A)** RMCE with a $p53^{GFP}$ plasmid. The exchange plasmid, the sequence which was verified before use, contains $p53^{GFP}$ coding sequences flanked by L3 and 1L sites. It was electroporated with a Cre expression plasmid. FIAU-resistant clones were analyzed by PCR with primers b and c and Southern blot with probe B. Both approaches led to identical results and identified 54/65 RMCE recombinants. Representative clones (P–Z) are shown (left), analyzed by PCR (top) and Southern (bottom): all clones but Q and T are positive with both assays. All positive clones produced a band of the expected size by PCR, indicating correct recombination at 1L, and displayed only the expected 12 and 5 kb bands by Southern, indicating correct recombination at L3. The absence of bands of aberrant size in Southerns also indicated that the exchange plasmid was neither rearranged nor inserted at ectopic sites. Thus RMCE was efficient and accurate. Recombinant clones were analyzed by western blot with an antibody to p53. In the representative western (right), cells from two independent $p53^{+/GFP}$ ES clones were left untreated or treated with adriamycin (ADR) at 0.5 μg/ml for 24 h, and protein extracts were prepared. $p53^{GFP}$ migrated at the expected size of 80 kDa and was expressed at unexpectedly high levels regardless of stress. **(B)** RMCE with a $p53^{\Delta PGFP}$ plasmid. The $p53^{\Delta PGFP}$ exchange construct (which sequence was verified before use) differed from the $p53^{GFP}$ construct only in that it contains a mutated exon 4 (4*) encoding a PRD deletion. RMCE was again very efficient, with 10/12 FIAU-resistant clones producing a 3 kb band by PCR with primers b and c. A western analysis of four FIAU-resistant clones is shown below (with low/high exposures: Lo X/Hi X). As expected, all except clone x expressed $p53^{\Delta PGFP}$. $p53^{\Delta PGFP}$ migrated at the expected size of 75 kDa and accumulated after stress, but at lower levels than $p53^{WT}$. **(C)** Germline transmission of the $p53^{\Delta PGFP}$ mutation. DNA of seven littermates (U42–U48), obtained from mating a $p53^{\Delta PGFP}$ chimera with a WT mouse, was analyzed by PCR with primers d and e (see B), with DNA from WT, $p53^{+/\Delta P}$ and $p53^{\Delta P/\Delta P}$ MEFs (5) as controls. U45, a $p53^{+/\Delta PGFP}$ mouse, demonstrated transmission of the mutation.

was deleted ($p53^{\Delta P}$), and the $p53^{\Delta P}$ was fused to GFP. We used this mutant because deleting the proline-rich domain decreases stability and compromises DNA-damage responses *in vivo* (5). According to our hypothesis, this hypomorphic mutant should not prevent embryonic development. RMCE with a $p53^{\Delta PGFP}$ replacement plasmid was again very efficient and western blots revealed an additional band of the predicted molecular weight only in $p53^{+/\Delta PGFP}$ ES cells (Figure 3B). As expected, the PRD deletion correlated with lower expression levels: $p53^{\Delta PGFP}$ was much less abundant than $p53^{WT}$ in all $p53^{+/\Delta PGFP}$ clones (Figure 3B). We next determined whether $p53^{+/\Delta PGFP}$ ES cells could generate chimeric mice and transmit the modified allele through the germline. Two $p53^{+/\Delta PGFP}$ ES cell clones were injected into blastocysts and highly chimeric (>80%) mice were obtained. Heterozygote pups were recovered from the mating the chimeras with WT mice (Figure 3C). These data demonstrate that marker-free RMCE is very efficient in ES cells and allows germline transmission of a targeted mutation (see Figure 1, path A).

We next determined whether the RMCE approach could be used to target mutations at the *p53* allele in somatic cells (Figure 1, path B). We first verified that the

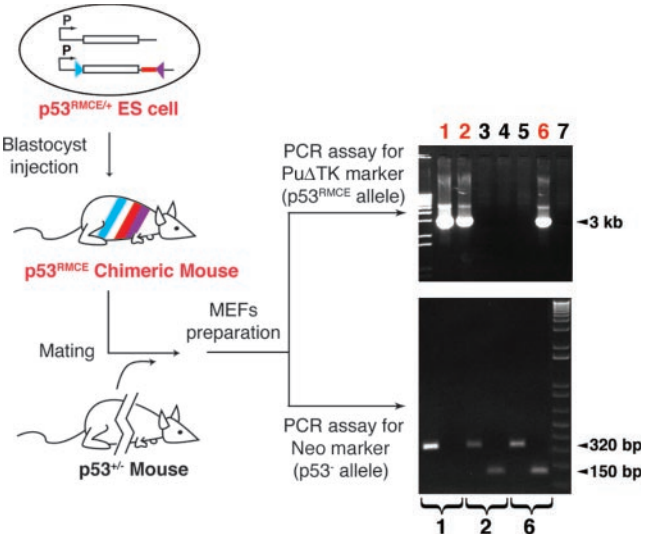

Figure 4. Germline transmission of the *p53* RMCE-ready locus. $p53^{RMCE/+}$ ES cells were injected into blastocysts to generate chimeric mice. Chimeras (>80%) were then mated with $p53^{+/-}$ mice (Taconic) and MEFs were prepared. MEFs were first genotyped by PCR with primers a and b (see Figure 2) to detect the PuroΔTK marker of the RMCE allele (top). This revealed germline transmission of the p53 RMCE-ready locus in MEFs 1, 2 and 6. Each of these three MEF clones was further analyzed (bottom) with primers f and g (left lanes) and h and i (right lanes), routinely used to genotype $p53^{+/-}$ mice (sequences in Materials and Methods). Primers f and g amplify a 320 bp product from a WT or RMCE allele, while primers h and i specifically amplify a 150 bp product from the Neo marker in the KO allele. MEF 1 are $p53^{RMCE/+}$ and MEFs 2 and 6 are $p53^{RMCE/-}$ cells.

RMCE-ready *p53* locus ($p53^{RMCE}$) could be transmitted through the germline by mating $p53^{RMCE/+}$ chimeras with $p53^{+/-}$ mice (20) (Figure 4). Importantly, this allowed us to generate $p53^{RMCE/-}$ MEFs, which were used to test RMCE at the *p53* locus in somatic cells. We first attempted RMCE in MEFs by electroporating $p53^{RMCE/-}$ MEFs with a Cre-expression plasmid and the $p53^{GFP}$ plasmid, followed by selection with FIAU or ganciclovir. Strikingly, no clones with an exchanged allele were identified (data not shown). RMCE with $p53^{GFP}$ in ES cells showed that $p53^{GFP}$ is expressed at high levels (Figure 3A), and as mentioned before, the p53 pathway that can be activated in MEFs is not readily activated in ES cells (18). The results above suggest that high levels of $p53^{GFP}$ could be tolerated by ES cells but toxic to MEFs, so that MEFs in which an RMCE had occurred failed to proliferate. To test this possibility, $p53^{RMCE/-}$ MEFs were electroporated with the $p53^{GFP}$ replacement construct with or without a Cre-expression plasmid, then analyzed by fluorescence microscopy 48 h after electroporation. The experiment was done without selection to enable observation of cells under conditions where a failure to proliferate would not derive from FIAU or ganciclovir toxicity but rather solely from the effects of $p53^{GFP}$. We observed a few fluorescent cells only when the Cre expression plasmid was co-electroporated, suggesting that such cells resulted from RMCE. Importantly, the rare fluorescent cells had a flat, 'fried-egg' appearance typical of senescent cells (Figure 5A), and when plates were observed 5 days later, the cells had detached. Altogether, the results suggest

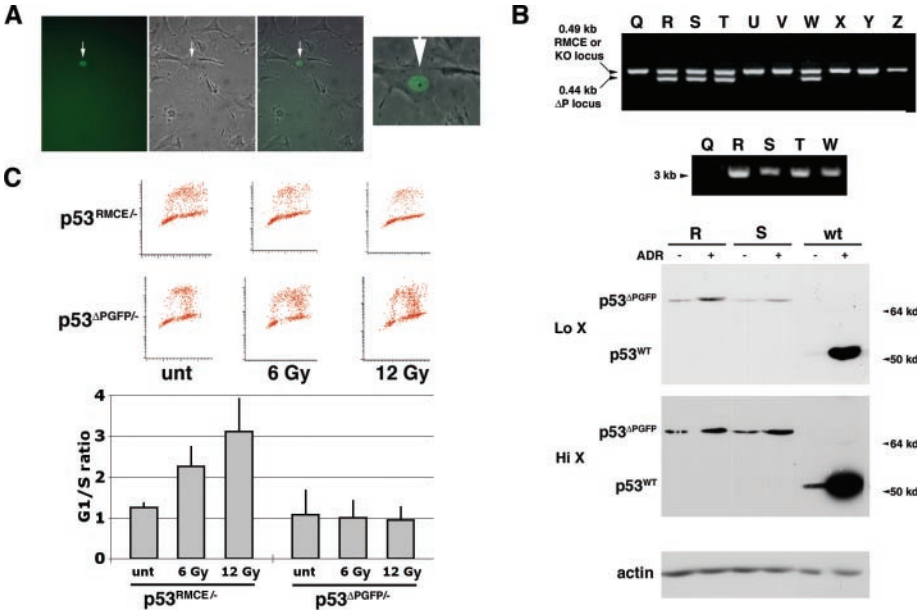

Figure 5. Performing RMCE in MEFs. **(A)** RMCE with the $p53^{GFP}$ plasmid leads to the transient observation of cells with intense nuclear fluorescence. $p53^{RMCE/-}$ MEFs, electroporated with a Cre expression plasmid and the $p53^{GFP}$ exchange plasmid, were analyzed 48 h later by fluorescence microscopy. A typical field (left to right: fluorescence, phase contrast, merged) with a fluorescent cell (arrow) is shown. The fluorescent cell is enlarged (extreme right). **(B)** RMCE with the $p53^{\Delta PGFP}$ plasmid. $p53^{RMCE/-}$ MEFs, electroporated with a Cre expression plasmid and the $p53^{\Delta PGFP}$ plasmid, were selected with ganciclovir. PCR with primers d and e (Figure 3B) indicated that 9/22 ganciclovir-resistant clones integrated the ΔP mutation [top row, a representative analysis of 10 clones (Q–Z) is shown]. PCR with primers b and c next verified that the detected PRD deletions resulted from RMCE at the p53 locus, not random integration (middle row, as expected clones R, S, T and W are positive, but not Q). Western analysis of positive clones (bottom row) showed that $p53^{\Delta PGFP}$ accumulated after ADR, but at lower levels than $p53^{WT}$. **(C)** Phenotypic assay of $p53^{\Delta PGFP}$: loss of cell cycle control. Asynchronous $p53^{RMCE/-}$ and $p53^{\Delta PGFP/-}$ MEFs left untreated, or irradiated with doses of 6 or 12 Gy, were analyzed (top shows a typical experiment; bottom plots results from ⩾4 independent experiments and ⩾3 independent MEFs). Note that the $p53^{RMCE}$ locus encodes a WT p53.

Table 1. Summary of targeting experiments

Cells	Electroporated plasmids	Selection drug	Targeting method	Targeting efficiency	Comments
WT ES	Flox	Puromycin	Homologous recombination	12/300 (4%)	Germline transmission of the RMCE locus
$p53^{RMCE/+}$ ES	$p53^{GFP}$ + Cre	FIAU	RMCE	54/65 (83%)	No pregnancy: $p53^{GFP}$ toxicity
$p53^{RMCE/+}$ ES	$p53^{\Delta PGFP}$ + Cre	FIAU	RMCE	10/12 (83%)	Germline transmission of the mutation
$p53^{RMCE/-}$ MEF	$p53^{GFP}$ + Cre	ganciclovir	RMCE	—	No clone: $p53^{GFP}$ toxicity
$p53^{RMCE/-}$ MEF	$p53^{GFP}$ + Cre	FIAU	RMCE	—	No clone: $p53^{GFP}$ toxicity
$p53^{RMCE/-}$ MEF	$p53^{GFP}$ + Cre	—	RMCE	Few fluorescent cells	Transiently observed (48h): $p53^{GFP}$ toxicity
$p53^{RMCE/-}$ MEF	$p53^{GFP}$	—	—	No fluorescent cells	
$p53^{RMCE/-}$ MEF	$p53^{\Delta PGFP}$ + Cre	Ganciclovir	RMCE	9/22 (41%)	Phenotypic read-out after a single exchange (loss of cell cycle control)

In ES cells, targeting $p53^{\Delta PGFP}$ or $p53^{GFP}$ by RMCE was ~20 times more efficient than targeting Flox by homologous recombination. In MEFs, due to $p53^{GFP}$ toxicity, only the targeting efficiency of RMCE with $p53^{\Delta PGFP}$ could be evaluated. Importantly, a phenotypic read-out of the $p53^{\Delta PGFP}$ mutation in MEFs, if it had been performed after targeting in fibroblasts by homologous recombination, would have required two rounds of inefficient targeting to target both *p53* alleles.

that RMCE can give rise to $p53^{GFP}$-expressing MEFs, but they die rapidly owing to $p53^{GFP}$ toxicity.

We also performed RMCE in $p53^{RMCE/-}$ MEFs with the $p53^{\Delta PGFP}$ construct. $p53^{\Delta PGFP}$-expressing MEFs were viable, recovered with an efficiency of ~40%, and, as expected from ES cell experiments, expressed a $p53^{\Delta PGFP}$ protein at much lower levels than $p53^{WT}$ (Figure 5B). Unlike WT MEFs, $p53^{\Delta P/\Delta P}$ MEFs are unable to arrest cycling after irradiation (5). Likewise, we found that irradiation doses that arrested $p53^{RMCE/-}$ MEFs (which express a wild-type p53 from the RMCE-ready locus, see Figure 2) did not arrest $p53^{\Delta PGFP/-}$ MEFs (Figure 5C). These data show that a single RMCE-ASAP reaction in heterozygous MEFs enables detection of a recessive phenotype. The results confirm that deleting the proline rich domain leads to less active p53 with impaired cell cycle control, and also indicate that a GFP C-terminal fusion can dramatically alter p53 regulation. A summary of our results is presented in Table 1.

These data report the development and implementation of an improved RMCE approach that enables efficient allele modification in ES cells to generate mice and in heterozygous MEFs to accelerate phenotypic analyses. The success of RMCE-ASAP relied on the integrated use of inverted heterologous loxP sites, a positive/negative selection marker that preserves the germline capacity of ES cells, and, for somatic cells, the existence of a knock-out allele of the gene of interest. These characteristics should make RMCE-ASAP a robust and general technology for analysis of mammalian genes under conditions that preserve normal control mechanisms in different tissues. In addition, RMCE-ASAP could be used to generate fibroblastic cell lines tailored for the repeated targeting of widely studied genes (p53, c-myc, NF-KB, etc.).

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