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Dual RMCE for efficient re-engineering of mouse mutant alleles

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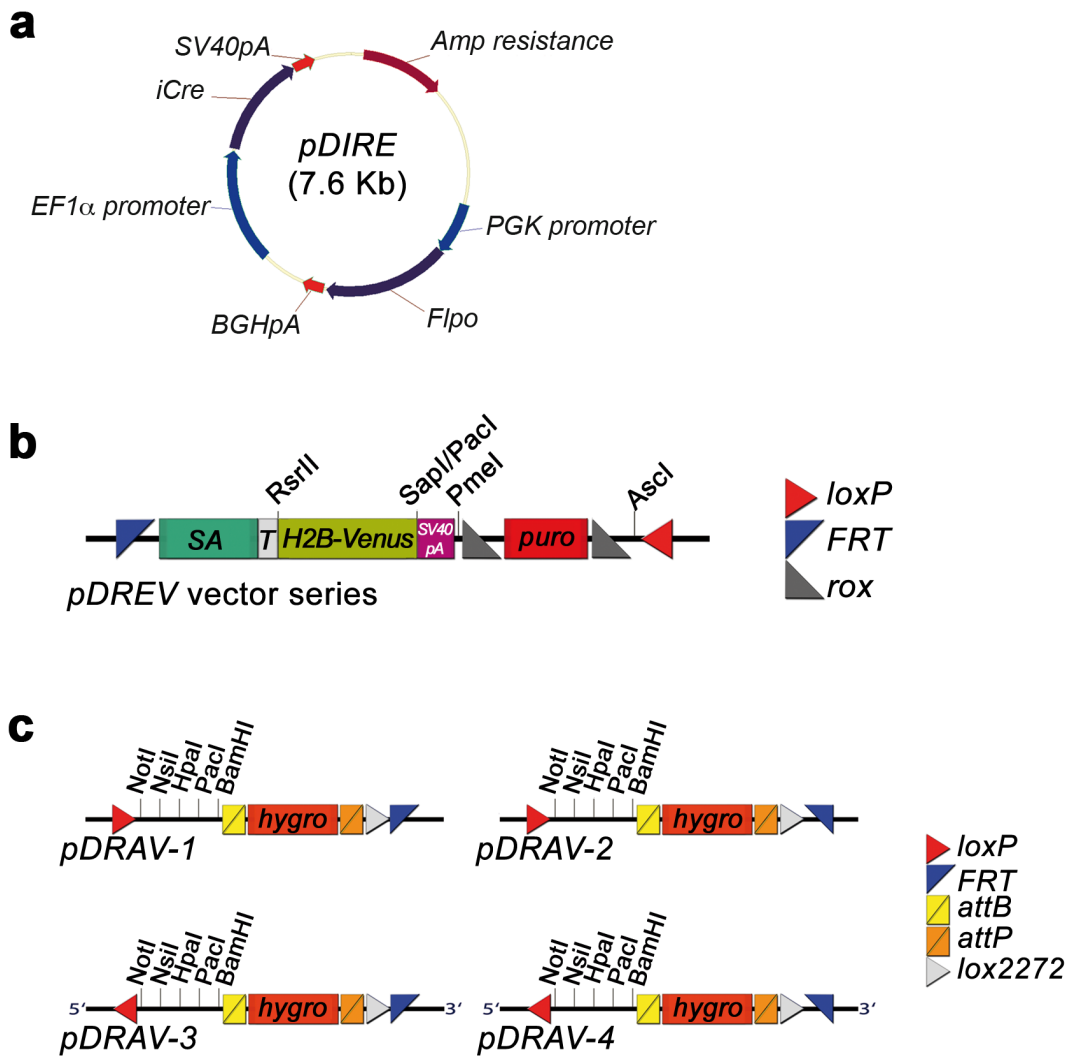
Note: Supplementary Data is available on the Nature Methods website.

Supplementary Figure 1. The dRMCE tool-kit.



(**a**) Map of the *pDIRE* (plasmid Dual Improved Recombinase Expression) vector. Simultaneous expression of both iCre and Flpo recombinases in mouse embryonic stem cells is achieved by the use of heterologous promoters (*PGK-Flpo*; *EF1α-iCre*). (**b**) The *pDREV* (plasmid Dual Recombinase EuComm Vector) series encode a 5' *FRT* site and 3' *loxP* site for re-engineering of the IKMC knockout first alleles. These plasmids encode the T2A self-cleaving peptide (T) and H2B-Venus fluorescent reporter fused in-frame to the splice acceptor of the mouse *En2* gene followed by the *SV40* polyadenylation site and *PGK-puromycin* selection cassette (flanked by *rox* sites). The *H2B-Venus* coding sequence can be substituted by any coding sequences of choice in a single cloning step. (**c**) The *pDRAV* (plasmid Dual Recombinase Acceptor Vector) series encodes the *loxP* and *FRT* sites in all possible orientations. The *PGK-hygromycin* selection cassette is flanked by φ31 target sites. A *lox2272* site has also been inserted to enable subsequent engineering. The polylinker in the *pDRAV* series provides the necessary versatility for rapid generation of custom-designed dRMCE replacement vectors. The complete sequences of the *pDIRE*, *pDREV* and *pDRAV* vectors are included as a **Supplementary Data File**.

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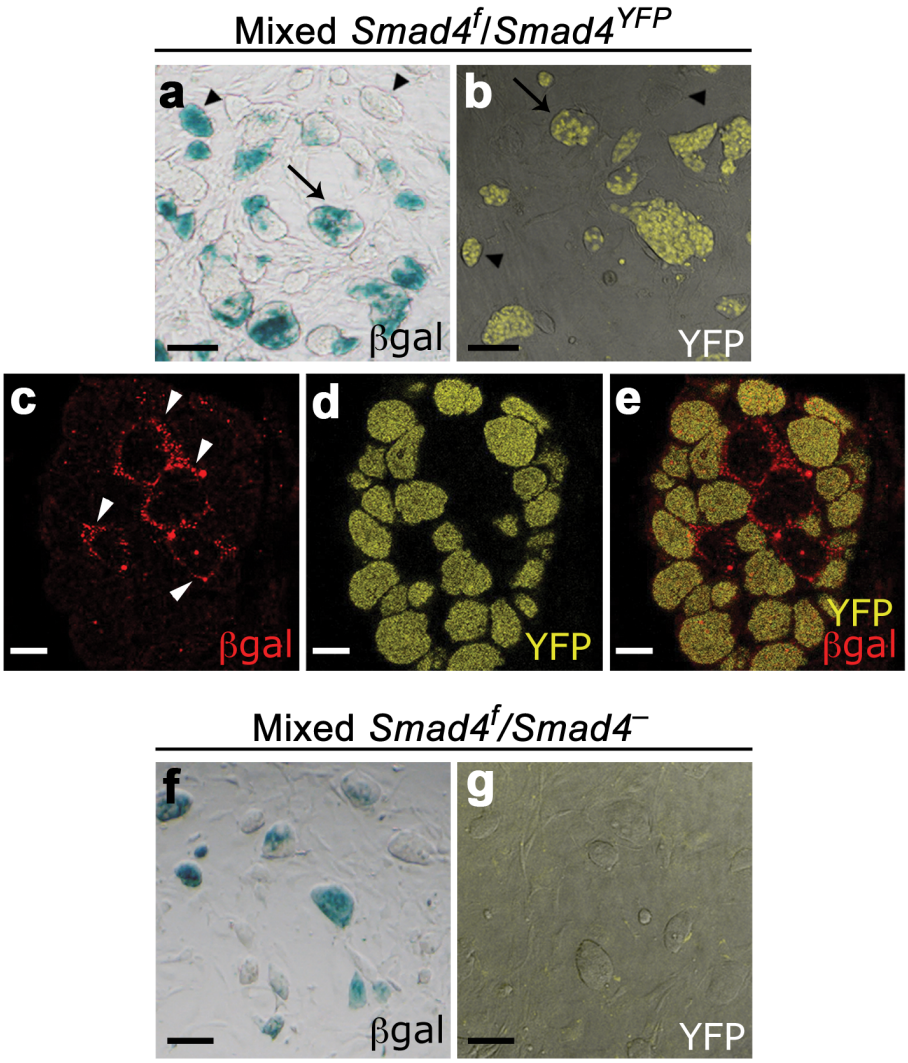
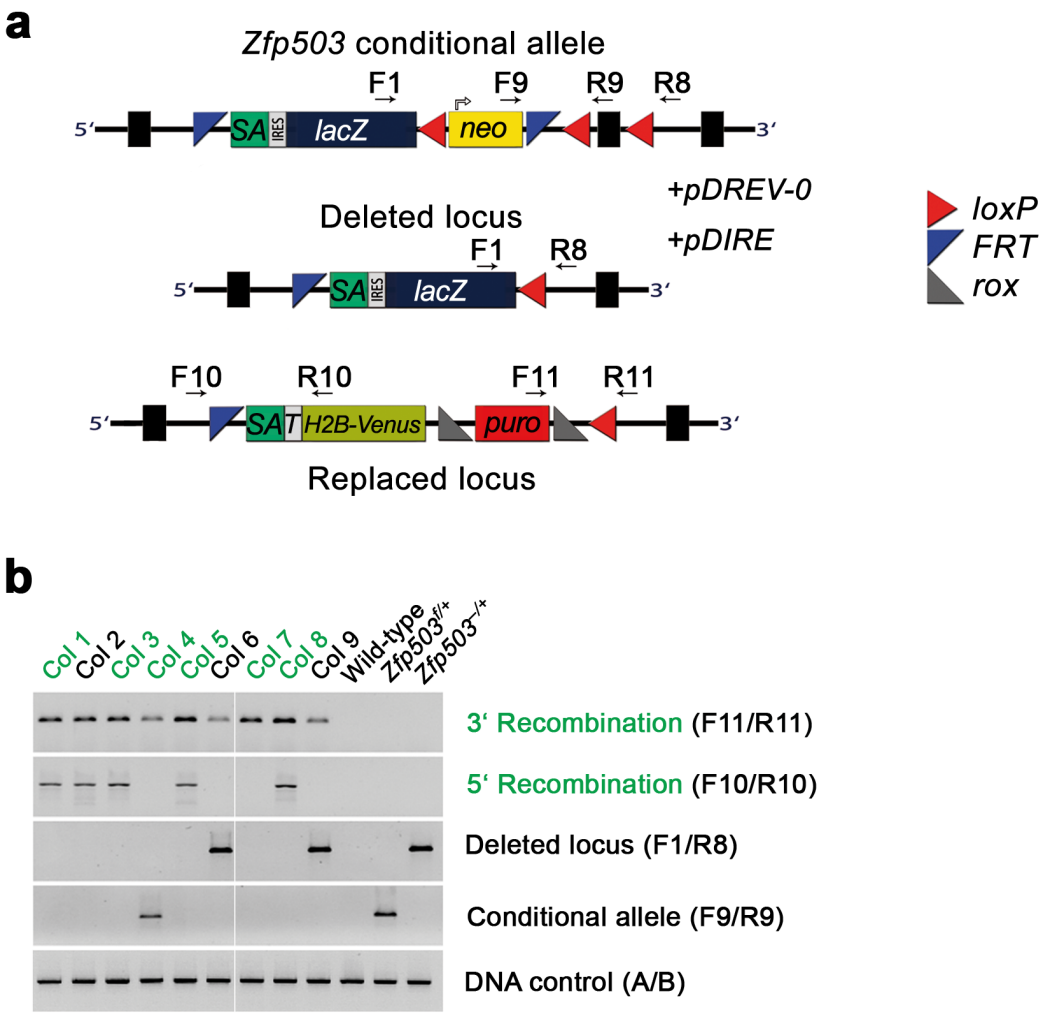
Supplementary Figure 2. Differential reporter activity distinguishes different types of mixed colonies.



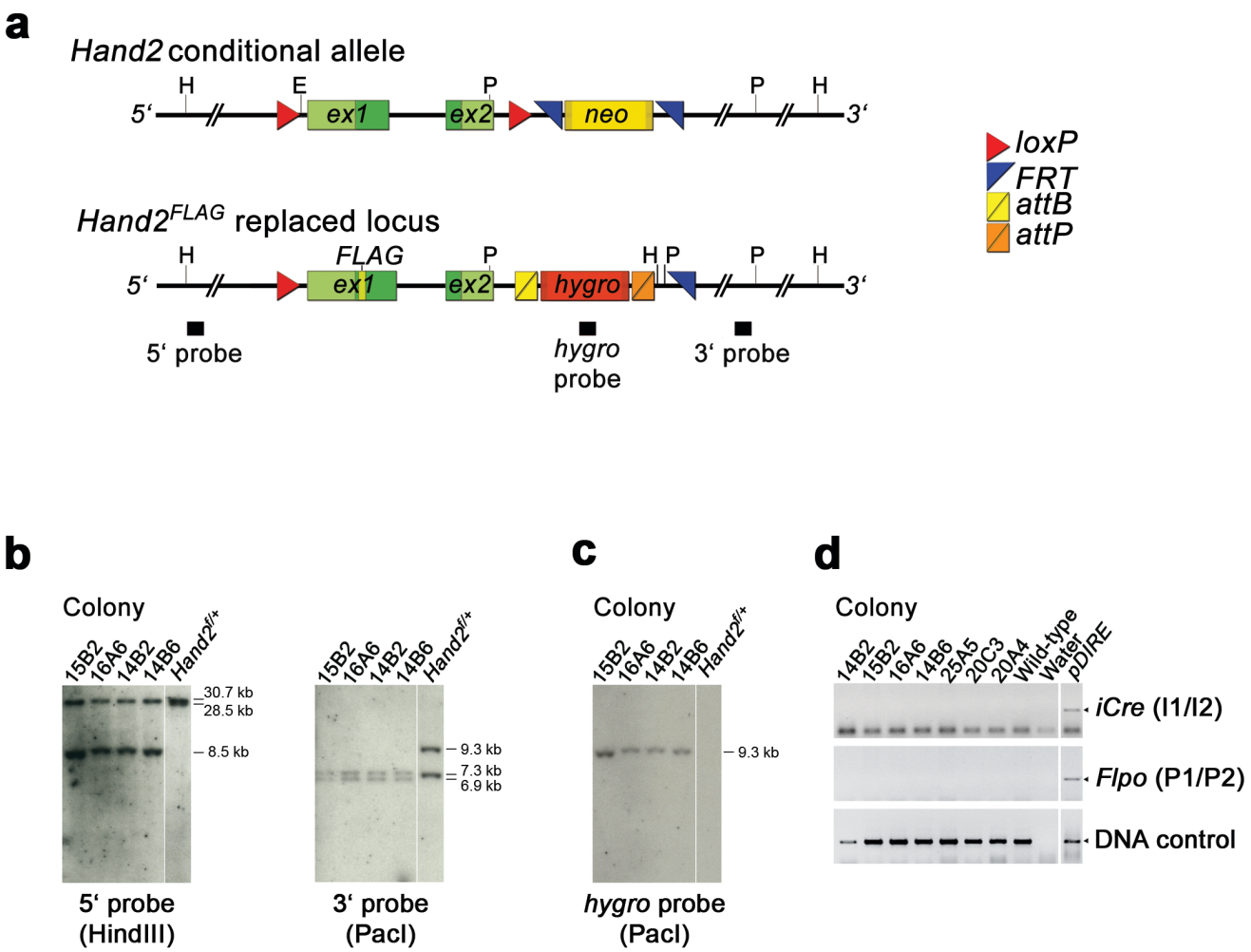
(**a**, **b**) Colony 2 is composed of both β-galactosidase (*Smad4*^{f}) and YFP-positive cells (*Smad4*^{YFP} allele) in agreement with PCR analysis (**Fig. 1c**). Some colonies are mixed (arrows), while others are either completely β-galactosidase or YFP-positive (arrowheads). (**c**, **d**, **e**) Co-immunolocalization of β-galactosidase (red) and YFP (yellow) in a mixed colony reveals that cells either express one or the other reporter (see panel **e**). β-galactosidase localizes to the cytoplasm (white arrowheads in panel **c**), while the YFP protein is nuclear. (**f**, **g**) In a mixed colony composed of heterozygous *Smad4*$^{–}$ and *Smad4*^{f} cells, some cells retain β-galactosidase while no YFP fluorescence is detected. Scale bars: 100 μm (panels **a**, **b**, **f** and **g**); 10 μm (panels **c**-**e**).

Supplementary Figure 3. dRMCE works efficiently with promoter-driven IKMC knockout-first alleles.



(**a**) Scheme of the dRMCE strategy for the *Zfp503* conditional allele (*Zfp503*^{f}), which encodes three *loxP* sites. The scheme illustrates the likely sequence of complete *cis*-deletion and subsequent *trans*-insertion that results in correct replacement and generation of the *Zfp503*^{YFP} allele. (**b**) PCR screening reveals the high frequency of clones with correct replacement (indicated in green) and some clones with only partial or no replacement. Detection of β-galactosidase activity or YFP fluorescence is not possible as *Zfp503* is not expressed by mouse embryonic stem cells. 3'Recombination, 5'Recombination: correct replacement at the 3' and 5' end, respectively.

Supplementary Figure 4. No random integration of the dRMCE plasmids in *Hand2*$^{FLAG/+}$ mouse embryonic stem cell clones.



(**a**) Schematic view of the *Hand2*^{f} and *Hand2*^{FLAG} alleles. The positions of restriction sites and the probes used for Southern blot analysis are indicated. H: HindIII, E: EcoRV, P: PacI. (**b**) Southern blot analysis confirms that replacement occurred correctly at the 5’ (8.5 kb band) and 3’ (6.9 kb band) ends and reveals the integrity of the *Hand2*^{FLAG} locus. (**c**) A single copy of the *hygromycin*-resistance cassette is present in all *Hand2*^{FLAG} clones. (**d**) PCR primers that amplify *iCre* and *Flpo* sequences fail to detect *pDIRE* sequences in *Hand2*^{FLAG} clones.

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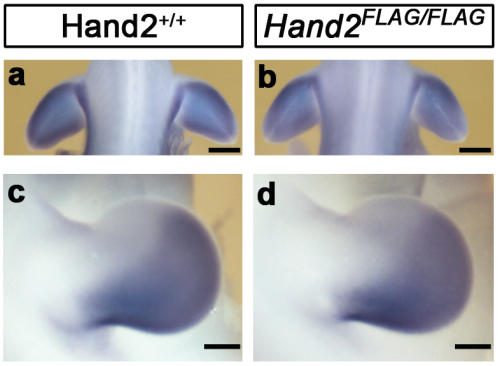
Supplementary Figure 5. *Hand2* expression in limb buds of mouse embryos.



(**a**, **b**) Forelimbs of wild-type (*Hand2*$^{+/+}$) and *Hand2*$^{FLAG/FLAG}$ mouse embryos at ~E10.5 (posterior view). (**c**, **d**) Forelimbs of mouse embryos of both genotypes at ~E11.5 (oriented with anterior to the top, posterior to the bottom). Scale bars: 200 μm (panels **a**, **b**); 500 μm (panels **c**, **d**).

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Supplementary Table 1. Frequencies of dRMCE-mediated correct replacement at the *Smad4*, *Zfp503*, *Hand2* and *Gli3* loci in mouse embryonic stem cells.

Gene Locus	Number of Colonies	Correct Replacement	Mixed Colonies	Negative
Smad4	48	33 (69%)	5	10
Zfp503	48	25 (52%)	0	23
Hand2	343	43 (13%)	11	289
Gli3	113	37 (33%)	0	76

Supplementary Table 2. Sequences of the PCR primers used in this study and primer pairs/amplicon sizes that were designed to specifically detect the different alleles of the *Smad4*, *Zfp503* and *Hand2* loci.

Primers of general use

In IKMC knockout-first alleles

Primer	Sequence
F1	AGCAGAGCGGGTAAACTGGC
R1	GCATCAGAGCAGCCGATTGTC
F9	CCAACCTGCCATCACGAGATT

In pDREV

Primer	Sequence
R3	TGGACGAAATGCCGGTGTCA
F4	GCAAAACCAAATTAAGGGCCA
R10	TGGACCTGCTTCAGAACCTTGTA
F11	CTCTTGATTCCCACTTTGTGGTTC

In pDRAV

Primer	Sequence
F6	ATGCGACGCAATCGTCCGATC
R7	CATCTGCACGAGACTAGTGAGACG

In pDIRE

Primer	Sequence
I1	GACTACCTCCTGTACCTGCAAGCCAG
I2	CTGCCAATGTGGATCAGCATTCTC
P1	CAGCCTGAGCTTCGACATCGTGAAC
P2	CTCAGGAACTCGTCCAGGTACACC

Locus specific primers

Primer	Sequence
F2	AACTAACTCTGTGTTCAGAGCCCCG
R2	GCTGCCCAAATCAATAGCCA
F3	GCAATCCAAACCAAGCATTGTC
F5	CCTCGGCAATTAGCAACGTGAACATC
R5	GTCCTCGCTCCTCAGGCTCTCTCG
R6	CCCTCCTCCACCACCACTGCTCAT
F7	CTGTGCCTGGTGCTTCGTTTTGTG
R8	TTGAACTGCGAACAGGGGAA
R9	TTCTGAGGAAGGCGACTTTGG
F10	CTTCCTGTGGGGTTTCTTTC
R11	GCACAAAACGAAACTCAAACGC
A	TCCAAGTCGATGGATATGCAACG (*Grem1*)
B	ATGAATCGCACCGCATACACTG (*Grem1*)

Locus specific amplicons

Smad4 locus

Primer pair	Allele	Size
F4/R2	*Smad4*^{YFP}(3')	456bp
F3/R3	*Smad4*^{YFP}(5')	1594bp
F2/R2	*Smad4*$^{-}$	565bp
	Smad4^{wt}	1265bp
F1/R1	*Smad4*^{f}	558bp

Zfp503 locus

Primer pair	Allele	Size
F11/R11	*Zfp503*^{YFP}(3')	396bp
F10/R10	*Zfp503*^{YFP}(5')	1449bp
F1/R8	*Zfp503*$^{-}$	987bp
F9/R9	*Zfp503*^{f}	599bp

Hand2 locus

Primer pair	Allele	Size
F6/R6	*Hand2*^{FLAG} (3')	965bp
F5/R5	*Hand2*^{FLAG} (5')	435bp
F5/R5	*Hand2*^{f} (5')	435bp (EcoRV: 340bp + 95bp)
F5/R6	*Hand2*$^{-}$	411bp
F7/R6	*Hand2*^{wt}	240bp
F7/R7	*Hand2*^{FLAG} (mice)	404bp