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8 **Knock-In of a 25-Kilobase Pair BAC-Derived Donor Molecule**
9 **by Traditional and CRISPR/Cas9-Stimulated Homologous Recombination**

10 **Short title: 25-Kilobase Pair Traditional and CRISPR/Cas9-Stimulated Knock-Ins**
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1 **ABSTRACT**

2 Here, we describe an expansion of the DNA size limitations associated with CRISPR knock-in technology,
3 more specifically, the physical extent to which mouse genomic DNA can be replaced with donor (in this case,
4 human) DNA at an orthologous locus. Driving our efforts was the desire to create a whole animal model that
5 would replace 17 kbp of the mouse *Bcl2l11* gene with the corresponding 25-kbp segment of human *BCL2L11*,
6 including a conditionally removable segment (2.9-kbp) of intron 2, a cryptic human exon immediately 3' of this,
7 and a native human exon some 20 kbp downstream. Using two methods, we first carried out the replacement
8 by employing a combination of bacterial artificial chromosome recombineering, classic ES cell targeting, dual
9 selection, and recombinase-driven cassette removal (traditional approach). Using a unique second method, we
10 employed the same vector (devoid of its selectable marker cassettes), microinjecting it along with CRISPR
11 RNA guides and *Cas9* into mouse zygotes (CRISPR approach). In both instances we were able to achieve
12 humanization of *Bcl2l11* to the extent designed, remove all selection cassettes, and demonstrate the
13 functionality of the conditionally removable, *loxP*-flanked, 2.9-kbp intronic segment.

1 **AUTHOR SUMMARY**

2 Clustered regularly interspaced short palindromic repeat (CRISPR) technology can be used to place DNA
3 sequences (designed in the laboratory) into the genomes of living organisms. Here, we describe a new method,
4 whereby we have replaced an exceptionally large segment of the mouse *Bcl2l11* gene with the corresponding
5 segment of human *BCL2L11* gene. The method represents an expansion of the DNA size limitations typically
6 associated with the introduction of DNA sequences through traditional CRISPR methods.

7

1 INTRODUCTION

2 The discovery of clustered regularly interspaced short palindromic repeat (CRISPR) systems, the
3 elucidation of their function, and their exploitation as genome engineering tools are revolutionizing genetic
4 engineering (1-5). Discovered as a form of adaptive immunity in bacteria and archaea, CRISPR systems
5 consist of a series of DNA spacer elements derived from invading plasmids or viruses. Interdigitated among
6 the spacers is a series of direct repeats. Depending on the particular system, these series are transcribed and
7 processed into single spacer/repeat units called crRNAs (CRISPR RNAs). In turn, these crRNAs may interact
8 with other short RNAs (e.g., tracrRNA) and one or more CRISPR-associated (Cas) proteins (e.g., Cas9 of
9 *Streptococcus pyogenes*), culminating in the assembly of an RNA-guided endonuclease directed at degrading
10 DNA from the offending plasmid or virus.

11
12 As genome engineering tools, the CRISPR-Cas endonucleases serve as instruments for generating DNA
13 double-strand breaks (DSBs) with locus-of-interest specificity, at high frequency, and across a wide variety of
14 strains and organisms (6). When faced with DSBs, cells of the organism being perturbed respond with one or
15 both of two DNA repair pathways known as the non-homologous end joining (NHEJ) pathway and the
16 homology-directed repair (HDR) pathway (7, 8). DNA DSBs repaired by the more rapid and error-prone NHEJ
17 pathway are characterized by the deletion or insertion of a small number of nucleotides. As one might expect,
18 these insertion/deletion events (INDELS) within the open reading frame of a protein of interest may lead to the
19 deletion of one or more endogenous amino acids, the insertion of one or more non-native amino acids,
20 premature termination, or frameshift mutations. In each of these instances the modified mutant locus will
21 commonly encode a hypomorphic or null allele of the original gene of interest.

22
23 In contrast, DSBs repaired in the presence of a homologous template (e.g., sister chromatid, donor
24 molecule) may be repaired by HDR (9). For genetic engineers, this provides the opportunity to introduce
25 precise DNA modifications, created at the laboratory bench, into the organism under investigation, at the site of
26 the DSB.

1 For traditional gene-targeting, of the sort in use in the mouse for the past thirty years (10-14), the traditional
2 paradigm based on a large body of literature, has been to create plasmid vectors with two homology arms of a
3 few to several kilobase pairs in length to act as donor molecules (15, 16). These arms are situated within the
4 plasmid so as to flank investigator-altered sequences that will be incorporated into the genome after
5 introduction of the plasmid vector into embryonic stem (ES) cells and HDR. Positive and negative selection
6 cassettes are frequently employed to aid in selecting the rare ES cell clones containing properly integrated
7 sequences. This technique is sufficient for modifying genomic sequence on a scale from one nucleotide to
8 several thousand base pairs. The method may fall short, however, when attempting to alter entire mouse
9 genes that often extend over 10s or 100s of thousands of base pairs.

11 In these instances, other genetic engineering technologies are employed including such methods as
12 random transgenesis (17, 18), targeted transgenesis (19, 20), and recombinase-mediated cassette exchange
13 (RMCE) (21, 22). Each of these methods has its drawbacks as well. For example, random transgenic methods
14 deviate from genome modification at the cognate endogenous locus, sufficing to allow transgenes to integrate
15 randomly (where they are subject to variegated expression). During targeted transgenesis, transgenes may be
16 directed specifically to standardized safe harbor sites to limit this position-effect variegation but even here the
17 transgenes are unlinked to their endogenous cognate genes. Like the related RMCE method, targeted
18 transgenesis may involve the use of antibiotic selection cassettes flanked by recombinase-binding sites. In
19 addition to the added complexity, deleting these selection cassettes requires breeding to specific recombinase-
20 expressing mice thereby prolonging strain development (23-26).

22 With the advent of CRISPR technologies many new avenues have opened. For example, by dramatically
23 increasing the frequency of DSBs at specified sites, gene-targeting need no longer be married to the culture of
24 ES cells or the use and removal of selection cassettes. In fact, in mice, most experiments begin with the
25 microinjection of Cas9 and CRISPR RNA guides, (and when needed, donor molecules) into single-celled
26 zygotes (27). Furthermore, in species where ES cell technology is lacking, CRISPR technology is a viable
27 alternative, a fact that has opened gene-editing experimentation to a wide variety of strains and a broad range
28 of species from bacteria to humans (6). However, here again, DNA modifications have generally been limited

1 to physical extents on the order of a few to a few thousand base pairs. Moreover, systematic studies of the
2 effect of homology arm length on CRISPR-associated HDR are lacking.

3
4 Despite the associated uncertainties, as described in the report that follows, we sought to expand the limits
5 of CRISPR knock-in technology. Specifically, we attempted to increase the physical extent to which mouse
6 genomic DNA could be replaced with donor (in this case, human) DNA at an orthologous locus. Driving our
7 efforts was the desire to create a whole animal model that would replace 17 kbp of the mouse *Bcl2l11* gene
8 with the corresponding segment of human *BCL2L11*, including a conditionally removable segment (2.9-kbp) of
9 intron 2, a cryptic human exon immediately 3' of this, and a native human exon some 20 kbp downstream (28).
10 Using two approaches, we first carried out the replacement by employing a combination of bacterial artificial
11 chromosome (BAC) recombineering, classic ES cell targeting, dual selection, and recombinase-driven cassette
12 removal (hereafter referred to as our traditional approach) (29). In the second approach, we used the same
13 vector (devoid of its selectable marker cassettes), microinjecting it along with CRISPR RNA guides and *Cas9*
14 into mouse zygotes (hereafter referred to as our CRISPR approach). In both instances we were able to
15 achieve humanization of *Bcl2l11* to the extent designed, remove all selection cassettes, and demonstrate the
16 functionality of the conditionally removable, *loxP*-flanked, 2.9-kbp intronic segment.

17
18 Our latter result may well represent the largest segment of mouse DNA to be replaced by an orthologous
19 human DNA using a CRISPR-directed approach with zygotic injection, to date. The study offers a proof-of-
20 principal demonstration that a minimum of at least 25 kbp of genomic DNA can be effectively humanized in
21 mouse, and provides a foundation for further technical optimization in mouse and specialization for use in other
22 species.

24 **METHODS**

25 Husbandry

26 All mice were obtained from The Jackson Laboratory (Bar Harbor, ME), housed on a bedding of white pine
27 shavings, and fed NIH-31 5K52 (6% fat) diet and acidified water (pH 2.5 to 3.0), *ad libitum*. All experiments
28 were performed with the approval of The Jackson Laboratory Institutional Animal Care and Use Committee

1 (IACUC) and in compliance with the Guide for the Care and Use of Laboratory Animals (8th edition) and all
2 applicable laws and regulations.

3 4 Preparation of the Targeting Vectors/Donor Molecules

5 We designed a targeting vector/donor molecule with three objectives in mind — 1), to humanize a central
6 segment of the *BCL2L11/Bcl2l11* gene; 2), to place selectable markers immediately 5' and 3' of the humanized
7 segment; and 3), to flank a 2,903-bp region within one of the humanized introns with *loxP* sites in order to
8 model a disease-associated deletion observed in 12% of the East Asian population (28).

9
10 Specifically, we constructed a targeting vector/donor molecule containing a 27,282-bp central segment of
11 the human *BCL2L11* gene flanked by 12,773- and 26,632-bp homology arms consisting of the proximal and
12 distal regions of the mouse *Bcl2l11* gene, respectively. This construct was designed such that it could be used
13 both for homologous recombination in embryonic stem (ES) cells, as well as for a CRISPR/Cas9 knock-in
14 approach (See Fig 1).

15
16 Initially, BAC DNAs were purified from BAC clones containing the corresponding *BCL2L11* and *Bcl2l11*
17 genes (human: library RP11, clone 695-B-23; mouse: library RP23, clone 331-K-22) (30, 31). Purified DNAs
18 were then electroporated into the recombinogenic *E. coli* strain, SW102 (32).

19
20 Segments from the mouse and human BACs were amplified using the oligonucleotides described in Table
21 1, restriction-digested at sites incorporated into the oligonucleotides, gel-purified, and assembled into small
22 plasmid vectors as follows:

1 **Table 1. Oligonucleotides.**

Abbreviation	Synonym	Orientation	Species	Genome Build	Chromosomal Coordinates	Product Size	Homology Length	Overall Length	Sequence	Enzyme
A	oTLD38	+	Mouse	mm10	Chr 2 : 128116776 128116795	424	20	31	5' -dCGCATACTAGTTCCATCCGGTCAATTTCTCTC-3'	SpeI
B	oTLD39	-	Mouse	mm10	Chr 2 : 128117158 128117177		20	31	5' -dCGCATAAAGCTTTTTGCTTGGTCCAGATTCC-3'	HinDIII
C	oTLD29new	+	Mouse	mm10	Chr 2 : 128129327 128129348	246	22	35	5' -dCGCATGCGCGCGCATAGTTAATAACCACAGGCA-3'	NotI
D	oTLD30	-	Mouse	mm10	Chr 2 : 128129528 128129548		21	32	5' -dCGCATAAAGCTTAAGTGTAGCCCAAGAA-3'	HinDIII
E	oTLD27	+	Human	hg38	Chr 2 : 111124898 111124917	742	20	31	5' -dCGCATAAGCTTTATGCTCAGAGGGTTTGGGA-3'	HinDIII
F	oTLD28	-	Human	hg38	Chr 2 : 111125598 111125617		20	31	5' -dCGCATGGATCCTGATTTACCTCACTGAAGCC-3'	BamHI
G	oTLD20	+	Human	hg38	Chr 2 : 111125618 111125641	765	24	35	5' -dCGCATGAATTCGGCAGGCCTTGGCCATGTTATAG-3'	EcoRI
H	oTLD21new	-	Human	hg38	Chr 2 : 111126335 111126358		24	37	5' -dCGCATGGCGCCCTACTTTACTTACAGGTATAACC-3'	AscI
I	oTLD31	+	Mouse	mm10	Chr 2 : 128129549 128129573	843	25	38	5' -dCGCATGGCGCGCGGTAGAAATTTCTAAAACTATATTC-3'	AscI
J	oTLD32	-	Mouse	mm10	Chr 2 : 128130340 128130367		28	39	5' -dCGCATGTCGACGTATTAAGACTCTAATAGTCTCCAGAGG-3'	SalI
K	oTLD9B	+	Human	hg38	Chr 2 : 111127109 111127130		22	35	5' -dCGCATGCGCGCGCTCTCTTACACTCTGGGAGGAT-3'	NotI
L	oTLD10	-	Human	hg38	Chr 2 : 111128507 111128525	1441	19	30	5' -dCGCATGGATCCAACAGCATGATGGTTCCCC-3'	BamHI
M	oTLD11	+	Human	hg38	Chr 2 : 111128526 111128545	501	20	31	5' -dCGCATGAATTCCTCCATAGAGGCTGTGCCAT-3'	EcoRI
N	oTLD12	-	Human	hg38	Chr 2 : 111128985 111129004		20	31	5' -dCGCATGTCGATGAGTGGGAGAGCAAGCC-3'	SalI
O	oTLD13	+	Mouse	mm10	Chr 2 : 128147195 128147214	478	20	33	5' -dCGCATGCGCGCGGTAAAGACTCTCCCCATCC-3'	NotI
P	oTLD114	-	Mouse	mm10	Chr 2 : 128147618 128147646		20	33	5' -dCGCATGGCGCGCCCAACAGGACAGCCAGCTAC-3'	AscI
Q	oTLD115	+	Human	hg38	Chr 2 : 111151266 111151285	842	20	33	5' -dCGCATGGCGCGCGGTACTGCTTCCGCTAAAGG-3'	AscI
R	oTLD116	-	Human	hg38	Chr 2 : 111152064 111152083		20	31	5' -dCGCATGAATTCCTCCCACTTTGATCTCTGAA-3'	EcoRI
S	oTLD117	+	Mouse	mm10	Chr 2 : 128147689 128147710	510	22	33	5' -dCGCATGGATCCGCATCTTCAGAAGCAGTGTGTT-3'	BamHI
T	oTLD118	-	Mouse	mm10	Chr 2 : 128148155 128148176		22	33	5' -dCGCATGTCGACTCTCAGTCCATTCATCAACAG-3'	SalI
Y	oTLD40	+	Mouse	mm10	Chr 2 : 128173955 128173974	388	20	31	5' -dCGCATAAGCTTATCAGGCCAGGGTTCTAGT-3'	HinDIII
Z	oTLD41	-	Mouse	mm10	Chr 2 : 128174299 128174318		20	33	5' -dCGCATGCGCGCGCATAGTGTCCCTGCCAAGG-3'	NotI

Oligonucleotides used in the construction of donor vectors. Restriction enzyme sites have been incorporated within 11- to 13-base segments of non-homology at the 5' end of each primer.

2
3
4 Segments KL and MN were cloned along with the neomycin resistance gene- (*Neo^R*-) containing
5 *EcoRI/BamHI* fragment of PL452, into a pBluescript II vector (Agilent Technologies, Santa Clara, CA USA)
6 modified to contain an R6K γ origin of replication (33, 34). This plasmid is named pTLD01.

7
8 Segments CD, EF, GH, and IJ were cloned along with the neomycin resistance gene- (*Neo^R*-) containing
9 *EcoRI/BamHI* fragment of PL451, into a pBluescript II (Agilent Technologies, Santa Clara, CA USA) vector
10 modified to contain an R6K γ origin of replication (33, 34). This plasmid is named pTLD02.

11
12 Segments OP, QR, and ST were cloned along with the blasticidin resistance gene- (*Bsd^R*-) containing
13 *EcoRI/BamHI* fragment of pTLD08 (a PL452 derivative carrying *attB*, *attP*, and *Bsd^R*), into a pBluescript II
14 vector (Agilent Technologies, Santa Clara, CA USA) modified to contain an R6K γ origin of replication (33, 34).
15 This plasmid is named pTLD03.

16
17 Segments AB and YZ were cloned into a pBR322-based vector along with the negatively selectable
18 thymidine kinase (*tk*) gene (35, 36). This plasmid is named pTLD11.
19

1 To begin the assembly of our humanized donor vector proper, pTLD01 was used with standard
2 recombineering approaches to place a *loxP*-flanked neomycin resistance cassette (*Neo^R*) just distal to the
3 2,903-bp deletion region in the human *BCL2L11*-containing BAC (29). After transferring the modified BAC to
4 the *Cre*-expressing *E. coli* strain, SW106, the *Neo* cassette was removed by exposing cells to arabinose,
5 leaving a single *loxP* site remaining (32).

6
7 Next, plasmid pTLD02 was used with standard recombineering techniques to place the EF segment of
8 human DNA, a *loxP* site, an FRT-flanked *Neo* cassette, and the GH segment of human DNA just distal to
9 mouse Exon 2 in the mouse *Bcl2l11*-containing BAC.

10
11 Next, plasmid pTLD03 was used with standard recombineering techniques to place the QR segment of
12 human DNA, and an *attB/attP*-flanked blasticidin resistance (*Bsd^R*) cassette, slightly distal to mouse Exon 4 in
13 the pTLD02-modified, mouse *Bcl2l11*-containing BAC described above.

14
15 Next, plasmid pTLD11 was linearized with *HindIII* and used with standard recombineering procedures to
16 retrieve the AB to YZ segment of the mouse *Bcl2l11* gene from the pTLD02/pTLD03-modified BAC, becoming
17 pTLD14.

18
19 At this point, plasmid pTLD14 was purified, digested with *AsclI*, and its two major fragments resolved by
20 agarose gel electrophoresis. The larger of the two linear fragments was gel-purified and electroporated into
21 recombinogenic *E. coli* cells containing the *loxP*-modified human BAC clone described above, thus capturing
22 the 27,282-bp human segment between flanking mouse homology arms, becoming plasmid pTLD15.

23
24 After experiencing some difficulty with blasticidin-based embryonic stem (ES) cell selection, we replaced
25 the open reading frame (ORF) of *Bsd^R* with that of *Puro^R* through a negatively selectable *rpsL* intermediate (37).

26
27 This completed vector, pTLD39, performed well in embryonic stem cells subjected to sequential
28 neomycin/puromycin selection.

1 For the purpose of CRISPR/Cas9-based zygotic microinjection, the final *Neo^R/Bsd^R*-containing vector
2 (plasmid pTLD15) was electroporated; first, into the FLP-expressing *E. coli* strain SW105 to remove *Neo^R*
3 (making plasmid pTLD66), and next, into a ϕ C31 recombinase-expressing *E. coli* strain (an SW105 derivative)
4 to remove *Bsd^R* (32). The final vector was named pTLD67.

5 6 Electroporation

7 For our traditional approach, we electroporated 25 μ g of linear pTLD39 DNA into 1.5×10^7 cells of the JM8-
8 A3 (Strain: C57BL/6N) line of mouse embryonic stems cells. ES cells were then plated in ES+2i medium with
9 sequential gentamycin (G418, 200 μ g/ml, Gibco, Fisher Thermo Scientific, Waltham, MA, USA) and puromycin
10 (0.75 μ g/ml, Sigma-Aldrich, St. Louis, MO, USA) selection (38). Surviving ES cell clones were propagated on
11 ES+2i medium, karyotyped, further tested for the presence of the puromycin resistance cassette by PCR, and
12 assessed for homology arm, insert, and neomycin resistance cassette count by quantitative PCR. Properly
13 targeted clones were microinjected into 3.5-days *post coitum* (dpc) blastocysts (see below).

14 15 CRISPR sgRNA Design and Production

16 For our CRISPR approach, all single-guide RNAs (sgRNAs) were designed using an algorithm available at
17 <http://crispr.mit.edu> (39). These sgRNAs, shown in Table 2, were designed along two concepts. In the first, the
18 two highest scoring sgRNAs (one in each direction) within a 250-bp region were selected from both the 5' and
19 3' ends of the 17-kbp segment of the mouse *Bcl2l11* segment being replaced. In the second, two internal
20 sgRNAs (one in each direction) closest to each end of the replaced segment were selected regardless of their
21 overall score. Guides were produced according to the method of Briner, *et al.* (40). Cas9 mRNA (CRISPR
22 associated protein 9 mRNA, 5-methylcytidine, pseudouridine) was purchased from TriLink Biotechnologies
23 (San Diego, CA).

1 **Table 2. Single Guide RNAs (sgRNAs).**

End	Guide name	Guide Rank	Guide sequence	PAM Sequence	Guide length	Design parameter	PAM location	Chr	mm 10 coordinates	Strand
5'	BC1L1	Guide #1	AGTTGTACCAGGCATCACCG	TGG	20	TOP SCORE	UPSTREAM	Chr 2:	128129677 - 128129696	minus
5'	BC1R1	Guide #5	AAAATATCCACGGTGATGCC	TGG	20	TOP SCORE	DOWNSTREAM	Chr 2:	128129667 - 128129686	plus
5'	BC1L3	Guide #6	TGTGGAAGTGGACGAGTTTG	AGG	20	CLOSEST TO END	UPSTREAM	Chr 2:	128129606 - 128129625	minus
5'	BC1R3	Guide #10	ACAACITTTCCAGATCAGT	TGG	20	CLOSEST TO END	DOWNSTREAM	Chr 2:	128129623 - 128129642	plus
3'	BC2L1	Guide #1	TACGTGGAGAAGCACCTTAC	AGG	20	TOP SCORE	UPSTREAM	Chr 2:	128147456 - 128147475	minus
3'	BC2R1	Guide #4	TGTAAGGTGCTTCTCCACGT	AGG	20	TOP SCORE	DOWNSTREAM	Chr 2:	128147455 - 128147474	plus
3'	BC2L3	Guide #20	TTATTTAAATAAATACCAAC	AGG	20	CLOSEST TO END	UPSTREAM	Chr 2:	128147642 - 128147661	minus
3'	BC2R3	Guide #14	AGGGTAGCTGGCTGTCTGT	TGG	20	CLOSEST TO END	DOWNSTREAM	Chr 2:	128147624 - 128147643	plus

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Four guides were designed at each end of the mouse *Bcl2l11* gene segment to be replaced including two (one in each orientation) with the top design score, and two (one in each orientation) located closest to the outermost ends.

4 **Microinjection**

5 For our traditional approach, properly targeted ES clones were microinjected into 3.5-dpc blastocysts, and
6 the blastocysts transferred to pseudopregnant host dams, by standard techniques (41). The resulting embryos
7 were allowed to go to term; the pups were delivered naturally and reared by the dams until weaning at four
8 weeks of age.

9
10 For our CRISPR approach, microinjection mixes were prepared as shown in Table 3. Approximately 80
11 C57BL/6NJ zygotes were microinjected (in one to two technical replicates with each microinjection mix
12 described above), transferred to pseudopregnant females by standard techniques, and allowed to go to term
13 where they were reared by the dams until weaning at four weeks of age.

1 **Table 3. Microinjection Mixes.**

Experiment	Number	1	2	3	4	5	6	7	8
Guides	BC1L1			50 ng/μL	50 ng/μL			50 ng/μL	50 ng/μL
	BC1R1			50 ng/μL	50 ng/μL			50 ng/μL	50 ng/μL
	BC1L3	50 ng/μL	50 ng/μL			50 ng/μL	50 ng/μL		
	BC1R3	50 ng/μL	50 ng/μL			50 ng/μL	50 ng/μL		
	BC2L1			50 ng/μL	50 ng/μL			50 ng/μL	50 ng/μL
	BC2R1			50 ng/μL	50 ng/μL			50 ng/μL	50 ng/μL
	BC2L3	50 ng/μL	50 ng/μL			50 ng/μL	50 ng/μL		
	BC2R3	50 ng/μL	50 ng/μL			50 ng/μL	50 ng/μL		
Other	Cas9 mRNA	100 ng/μL	100 ng/μL	100 ng/μL	100 ng/μL	100 ng/μL	100 ng/μL	100 ng/μL	100 ng/μL
Reagents	Donor vector	5 ng/μL	1 ng/μL	5 ng/μL	1 ng/μL	10 ng/μL	5 ng/μL	10 ng/μL	5 ng/μL
Embryos	Rep 1	~80	~80	~80	~80	~80		~80	
Injected	Rep 2						~80		~80

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Microinjection mixes contained four guides (either those with the highest scores or those with the most terminal positions within the mouse Bcl2l11 segment to be replaced) and varying concentrations of donor DNA (1, 5, or 10 ng/μL).

4 Genotyping

Potentially chimeric mice, arising from the microinjection of 3.5-dpc blastocysts (traditional approach) or 1-celled zygotes (CRISPR approach), and their progeny were genotyped at designed Cas9 binding sites using the oligonucleotide primers described in Table 4. As shown (See Fig 2), these primers were used in pairs, in separate PCR reactions designed to amplify DNA across: 1) the Cas9 binding sites of intact (or small INDEL-containing) mouse alleles, 2) the mouse/human junctions of humanized alleles (or randomly integrating transgenes), and 3) the breakpoints of any deletion-bearing alleles.

Table 4. Genotyping oligonucleotides.

Primer Name	Sequence	Length (nt)	Forward/Reverse	Chromosome	Coordinates (mm10/hg38)
oTLD56	5' -dATCTGTGGCCTTCTAGCCAA-3'	20	Forward	Mouse Chr 2	128129240- 128129259
oTLD57	5' -dAGAATGCCCTAACTCAGCCA-3'	20	Reverse	Mouse Chr 2	128130445- 128130464

oTLD239	5' -dTGCATCTAAGGGTTTGGCTT-3'	20	Forward	Mouse Chr 2	128147294- 128147313
oTLD338	5' -dGAGTCAAAGCCTACATCCCAA-3'	22	Reverse	Mouse Chr 2	128147780- 128147801
oTLD335	5' -dGGAACAGCAAGTCGATCAACAC-3'	22	Reverse	Human Chr 2	111125334- 111125355
oTLD337	5' -dGGTGTTTGAGGAGAGTGCTGTA-3'	22	Forward	Human Chr 2	111151728- 111151749

Standard PCR primers were designed to amplify the junctions flanking the original mouse *Bcl2l11* allele, the humanized *BCL2L11* allele, and the deletion-bearing allele.

1

2 Sanger Sequencing

3 For more detailed analysis of specific alleles, PCR products from genotyping reactions were purified and
4 sequenced by JAX Scientific Services according to the method developed by Sanger (42). PCR products were
5 purified using HighPrep PCR magnetic beads (MagBio Genomics, Gaithersburg, MD USA). Cycle sequencing
6 was performed using a BigDye Terminator Cycle Sequencing Kit, version 3.1 (Applied Biosystems, Foster City,
7 CA USA). Sequencing reactions contained 5µl of purified PCR product (3-20 ng) and 1µl of primer at a
8 concentration of 5 pmol/µl. Sequencing reaction products were purified using HighPrep DTR (MagBio
9 Genomics, Gaithersburg, MD USA). Purified reactions were run on an Applied Biosystems 3730xl DNA
10 Analyzer (Applied Biosystems, Foster City, CA USA). Sequence data were analyzed using Sequencing
11 Analysis Software, version 5.2 (Applied Biosystems, Foster City, CA USA). Resulting sequence (.abi) files
12 were imported into Sequencher, version 5.0.1 (Gene Codes Corporation, Ann Arbor, MI USA), for further
13 analysis.

14

15 Genetic mapping

16 To show that the human segment of *BCL2L11* had replaced its mouse counterpart in the orthologous
17 *Bcl2l11* locus, we used genetic mapping to localize the humanized segment of the *BCL2L11/Bcl2l11* gene

1 (See Fig 3). Two backcrosses were established using the following approach. First, FVB/NJ females were
2 crossed to C57BL/6NJ males carrying the humanized segment to obtain F₁ hybrid (FVBB6NF1/J) progeny.
3 These progeny were then genotyped for the presence of the humanized segment. Males carrying the human
4 sequence (FVBB6NF1/J-*BCL2L11*) were backcrossed to either FVB/NJ females or C57BL/6NJ females to
5 generate N₂ progeny.

6
7 These backcross schemes can be annotated as follows:

8
9 C57BL/6NJ X FVBB6NF1/J-*BCL2L11*

10
11 FVB/NJ X FVBB6NF1/J-*BCL2L11*

12
13 N₂ progeny from each backcross (along with appropriate controls) were genotyped using KASP-chemistry
14 (LGC Limited, Teddington, UK) across a set of approximately 150 single-nucleotide polymorphism (SNP)
15 markers distributed roughly equally across the mouse genome. Concordance between each marker in the set
16 and the humanized segment was calculated by chi-square (χ^2) analysis.

17 18 RESULTS

19 Traditional Approach

20 Following electroporation of the pTLD39 vector into the JM8-A3 line of ES cells and selection on G418, we
21 assayed 89 surviving clones for the presence of the puromycin resistance cassette by PCR. Of these, twenty-
22 seven contained the puromycin cassette and were subjected to puromycin selection. Of these, four clones
23 survived and were assessed for homology arm, insert, and neomycin resistance cassette count by quantitative
24 PCR. One clone passed all of these tests for proper targeting of the central human *BCL2L11* segment to the
25 endogenous mouse *Bcl2l11* gene. ES cells from this clone were microinjected into blastocysts resulting in nine
26 high-quality chimeras. The four highest quality male chimeras were mated to C57BL/6NJ females resulting in
27 two independent instances of germline transmission of the humanized allele. Although presumably identical,
28 independent lines (genetic background: C57BL/6JN) were developed from each instance. Mating males with

1 B6N.Cg-Tg(*Sox2-Cre*)1Amc/J female mice resulted in progeny in which the *loxP*-flanked 2.9-kbp human
2 intronic segment was deleted, as designed.

3 4 CRISPR Approach

5 At term, a total of 94 pups were born; six were stillborn and six did not survive to four weeks of age. Eighty-
6 two mice were weaned and distributed among experiments as shown in Table 4.

7
8 Both Experiment 3 (highest scoring guides, 5 ng/μL donor DNA) and Experiment 5 (guides closest to ends,
9 10 ng/μL donor DNA) resulted in no viable pups remaining at wean-age. Despite these results Experiment 7
10 (conducted with a donor DNA concentration equal to that of Experiment 5, *i.e.*, 10 ng/μL) and Experiment 8 (a
11 replicate of Experiment 3) resulted in seven and 21 pups, respectively, suggesting that the lack of pups in
12 Experiments 3 and 5 was due to technical failure rather than anything systematically wrong with the
13 experimental design.

14
15 To genotype these 82 progeny PCR assays were designed to span each of the proximal and distal
16 mouse/human junctions and to span the 17-kbp mouse region to be replaced. The results of these experiments
17 are shown in Table 5. As noted, PCR assays designed to span each of the proximal and distal mouse/human
18 junctions identified three founders that were positive for both (Experiment 2, guides closest to ends, 1 ng/μL
19 donor DNA; Experiment 6, guides closest to ends, 5 ng/μL donor DNA; and Experiment 7, highest scoring
20 guides, 10 ng/μL donor DNA). PCR assays designed to span the 17-kbp mouse region to be replaced
21 identified two of the three founders described above (Experiment 6, guides closest to ends, 5 ng/μL donor
22 DNA; and Experiment 7, highest scoring guides, 10 ng/μL donor DNA).

1

Table 5. Summary of the CRISPR-stimulated replacement of 17-kilobase pairs of mouse *Bcl2l11* with 25-kilobase pairs of human *BCL2111*.

Guide Sets	[Donor]	P _{is} ¹	Percentage	PCR Results	N _s	Percentage	PCR Results	N _s crossed to	N _s	Percentage	PCR Results	F _{is} crossed to	N _s	Percentage	PCR Results
CLOSEST TO ENDS	5 ng/μL	12	100.00%	No deletion of 17-kbp mouse segment; No mouse human junctions	N/A										
	1 ng/μL	1	12.50%	Positive for 5' and 3' mouse/human junctions	50	100.0%	No deletion of 17-kbp mouse segment; No mouse human junctions								
		7	87.50%	No deletion of 17-kbp mouse segment; No mouse human junctions	N/A										
HIGHEST SCORES	5 ng/μL	0	N/A												
	1 ng/μL	16	100.00%	No deletion of 17-kbp mouse segment; No mouse human junctions	N/A										
CLOSEST TO ENDS	10 ng/μL	0	N/A												
	5 ng/μL	1	5.56%	Deletion of 17-kbp mouse segment; Positive for 5' and 3' mouse/human junctions	14	45.2%	Positive for 5' and 3' mouse/human junctions	X FVB/NU (to generate B6FVBf1s)	23	35.4%	Positive for 5' and 3' mouse/human junctions	X C57BL/6j	3	33.3%	Positive for 5' and 3' mouse/human junctions
													6	66.7%	Wildtype
												X C57BL/6j	9	56.3%	Positive for 5' and 3' mouse/human junctions
													7	43.8%	Wildtype
												X FVB/NU	7	TBD	Not yet genotyped
												X FVB/NU	19	TBD	Not yet genotyped
													42	64.6%	Wildtype
												X C57BL/6j	10	43.5%	Positive for 5' and 3' mouse/human junctions
													13	56.5%	Wildtype
												X C57BL/6j	9	36.0%	Positive for 5' and 3' mouse/human junctions
													16	64.0%	Wildtype
												X C57BL/6j	10	55.6%	Deletion of 17-kbp mouse segment; No mouse human junctions
													8	44.4%	Wildtype
												X C57BL/6j	6	37.5%	Deletion of 17-kbp mouse segment; No mouse human junctions
													10	62.5%	Wildtype
												X C57BL/6j	7	38.9%	Deletion of 17-kbp mouse segment; No mouse human junctions
	11	61.1%	Wildtype												
X C57BL/6j	9	56.3%	Deletion of 17-kbp mouse segment; No mouse human junctions												
	7	43.8%	Wildtype												
	17	94.44%	No deletion of 17-kbp mouse segment; No mouse human junctions	N/A											
HIGHEST SCORES	10 ng/μL	1	14.29%	Deletion of 17-kbp mouse segment; Positive for 5' and 3' mouse/human junctions	14	25.0%	Deletion of 17-kbp mouse segment								
		42	75.0%	No deletion of 17-kbp mouse segment; No mouse human junctions											
	5 ng/μL	21	100.00%	No deletion of 17-kbp mouse segment; No mouse human junctions	N/A										
TOTALS		82	3.66%	3 of 82 showed evidence of humanization; 2 of 82 showed evidence of 17-kbp deletion; around 30% showed evidence of small INDELS											

¹Possible mosaicism

2

Germline transmission from each of the founders and subsequent Mendelian inheritance of the humanized and deletion-bearing allele are shown. See text for more detail.

3

1 To further explore the inheritance of these genetic changes, we mated the human insertion/deletion-
2 positive P₀s from Experiments 2, 6, and 7 to C57BL/6J mice and genotyped their progeny. The results of these
3 analyses are shown in Table 5. As shown, the human insertion-positive P₀ mouse (male) from Experiment 2
4 (guides closest to ends, 1 ng/μL donor DNA) failed to transmit the humanized allele to any of 29 of its N₁
5 progeny suggesting that the P₀ mouse is mosaic with a germline consisting primarily of unmodified wildtype
6 cells.

7
8 In contrast, the human insertion- and deletion-positive P₀ mouse (male) from Experiment 7 (highest scoring
9 guides, 10 ng/μL donor DNA) transmitted its deletion-bearing allele to four of its 21 N₁ progeny. This P₀ mouse,
10 however, did not transmit the human insertion-bearing allele to any of these 21 mice again suggesting that the
11 P₀ mouse is mosaic with a germline consisting of relatively few human insertion-bearing cells.

12
13 Interestingly, the human insertion- and deletion-positive P₀ mouse (female) from Experiment 6 (guides
14 closest to ends, 5 ng/μL donor DNA) transmitted either a human insertion-bearing allele or a deletion-bearing
15 allele to all of its 13 N₁ progeny, but never both, implying that this animal is breeding as a true heterozygote
16 with a genotype of both human insertion- and deletion-bearing alleles at the *Bcl2l1* locus. Subsequent
17 breeding of three select N₁ mice (two bearing the human insertion and one bearing the deletion) gave results
18 consistent with Mendelian expectations. Mating males with B6N.Cg-Tg(*Sox2-Cre*)1Amc/J female mice resulted
19 in progeny in which the *loxP*-flanked 2.9-kbp human intronic segment was deleted, as designed.

20 21 Genetic mapping

22 We used an outcross-backcross genetic mapping strategy as a means of localizing the insertion site of
23 BAC-derived human *BCL2L1* sequences. Twenty-two N₂ progeny were analyzed from the C57BL/6NJ X
24 (FVB/NJ X C57BL/6NJ) backcross and twenty-eight progeny from the FVB/NJ X (FVB/NJ X C57BL/6NJ)
25 backcross. Analysis of the data demonstrates strong linkage between the human *BCL2L1* segment and
26 several genetic markers on mouse Chromosome 2 (See Fig 3). In the backcross to C57BL/6NJ, the marker
27 with strongest linkage, marker rs13476756, had a log-odds ratio (LOD) of 6.58 (p<0.004). In the backcross to
28 FVB/NJ, marker rs13476756 had a LOD score of 7.64 (p<0.0004).

1 Analysis of individual haplotypes (specifically, points of recombination in samples 261, 263, 266, 303, and
2 319) further narrows the insertion-critical region to a 45.2-Mbp region from marker rs4223406 (nucleotide
3 113,827,352) to marker rs3689600 (nucleotide 159,014,253) on Mouse Chromosome 2 (GRC38/mm10), which
4 is consistent with integration into the 36,510-bp mouse *Bcl2l11* gene that spans from nucleotide 128,126,038
5 to nucleotide 128,162,547. Put another way, this analysis shows that both the mouse *Bcl2l11* gene and the
6 engineered human sequences must be colocalized within a region comprising less than 2% of the mouse
7 genome. We conclude that integration of the human sequence has not occurred randomly, but has indeed
8 occurred by homologous recombination as designed.

10 DISCUSSION

11 Contemporary CRISPR technology is revolutionizing genetic engineering and has contributed [along with
12 zinc-finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) technologies] to the
13 newly emergent field of gene editing (43-56). The greater CRISPR technique is in a period of rapid expansion,
14 its methodology now being applied across dozens of species in thousands of laboratories around the globe.
15 Moreover, the seminal core technology continues to diversify with additional enzymatic reagents, novel
16 applications, and technical improvements under robust investigation. This, in turn, has led to a rapid expansion
17 of CRISPR knowledge and the publication of CRISPR reports and reviews on a daily basis.

18
19 In the experiments reported here, we set out to explore the feasibility of using CRISPR technology to
20 replace large (10s of kbp) segments of the mouse genome with human DNA from orthologous loci. Current
21 CRISPR approaches aimed at knocking experimental DNAs into a locus of interest by homologous
22 recombination have generally involved relatively small genomic expanses from single nucleotides to a few
23 kilobase pairs. Moreover, these experiments routinely make use of long oligonucleotides, or targeting vectors
24 with sub-kilobase homology arms, as donor molecules. Only more rarely are targeting vectors used of the
25 sizes routinely employed in studies involving mouse ES cells.

26
27 In contrast to these common practices, we surmised that experimentally altered DNAs, of 10s to 100s of
28 kilobase pair lengths, might be directed into a locus of interest if the DNA were outfitted with homology arms

1 15-30 times longer than those in common use today. Accordingly, we used a traditional approach with bacterial
2 artificial chromosomes containing both human and mouse genomic DNA to prepare a donor molecule with 25-
3 kbp of human *BCL2L11* genomic sequence flanked by 15-kbp and 30-kbp mouse homology arms. We have
4 demonstrated by PCR, sequence, and linkage analysis that replacement of a minimum of 25-kbp of mouse
5 genomic DNA can be achieved using human DNA from the corresponding locus.

6
7 Given this proof-of-principle, future studies can now begin to explore questions of efficiency and
8 optimization. In our experiment we performed microinjection into mouse zygotes to see if mice could be
9 recovered, with any degree of humanization of the mouse *Bcl2l11* gene, and if these mice were capable of
10 transmitting the humanized allele through the germline to their offspring. These experiments have
11 demonstrated the feasibility of this CRISPR/BAC technology to introduce experimental DNA in a directed
12 fashion to the zygotic genome and the ability of the specifically targeted DNA to be transmitted through the
13 germline to progeny. However, due to the small number of data points in whole animal experiments, one can
14 only speculate on the impact of guide selection and donor DNA concentration variables on overall success
15 rates.

16
17 Among the experiments in which donor DNA was detected in P₀ mice (Experiments 2, 6, and 7), DNA
18 donor concentrations of 1, 5, and 10 ng/μL were represented but the resulting mice show varying degrees of
19 mosaicism. In Experiment 2, where donor DNA concentration was at its lowest (1 ng/μL), donor DNA was not
20 detected among N₁ progeny (0/50) suggesting that integration of the donor DNA occurred at multicellular stage
21 of embryonic development and that those cells that did acquire the donor DNA did not contribute to the
22 germline at an appreciable level.

23
24 In Experiment 6, where donor DNA concentration was at an intermediate level (5 ng/μL), donor DNA was
25 detected among nearly half of all N₁ progeny (14/31) suggesting that integration of the donor DNA occurred at
26 the one-cell (zygotic) stage of embryonic development, that that cell gave rise to all cells of the germline, and
27 that the donor DNA was passed, during meiosis, into half of the population of mature spermatozoa. This result
28 is consistent with our hypothesis that a deletion, of the 17-kbp mouse segment to be replaced, occurred at the

1 *Bcl2l11* locus in the homologous chromosome in the zygote, and was transmitted, in repulsion to the DNA
2 insertion, to all remaining progeny (17/17). This result is entirely congruent with the optimal desired outcome,
3 *i.e.*, where the P₀ zygote undergoes biallelic modification, develops into a mouse with no mosaicism, and
4 transmits one or the other variant alleles in equal numbers (50%:50%) to the population of mature
5 spermatozoa.

6
7 In Experiment 7, where donor DNA concentration was at the highest level tested (10 ng/μL), the 17-kbp
8 deletion was detected in only 25% of all N₁ progeny (14/56), and the donor DNA, present in the P₀ mouse, was
9 not transmitted to the N₁ generation at all (0/56). These results can be explained assuming a scenario
10 whereby a deletion occurred in one *Bcl2l11* allele, in a single blastomere, at or near the two-cell stage, and that
11 this deletion-bearing cell gave rise to roughly half of the developing premeiotic germline and a fourth of all
12 mature (postmeiotic) germcells. At some later point in blastogenesis, one can hypothesize that an insertion of
13 donor DNA occurred, but in so few cells as to not contribute to the germline in an appreciable way.

14
15 A number of aspects in Experiment 7 may have contributed to its less than optimal result. First, due to its
16 viscosity, a donor DNA preparation with a DNA concentration that is too high may not be efficiently delivered
17 through the microinjection needle to the zygote, or delivered in a form less conducive to promoting Cas9
18 activity and/or HDR. Moreover, the guides designed for this experiment, although designed to have an optimal
19 score, did not have what we surmised to be an optimal position, near the ends of the mouse DNA segment to
20 be replaced. It may be that, in experiments of this type, guide position represents a more significant design
21 parameter than guide score alone. It is interesting to note that, among all experiments using guides designed
22 for high score optimization, only in Experiment 7, where donor DNA concentration was at the highest level
23 tested (10 ng/μL), was any evidence of donor DNA incorporation seen, and even here it was at a level
24 apparently so low in the P₀ founder mouse as to not transmit the modified allele to N₁ mice. You may recall that,
25 in the previously mentioned Experiment 6, where an optimal result was achieved, donor DNA concentration
26 was only 5 ng/μL. It is entirely possible that the successful result seen in that instance was driven by superiorly
27 performing/positioned (nearest the end) guides even at what could prove to be a suboptimal donor DNA
28 concentration. Comparing Experiment 6 with Experiment 7, it is interesting to note that the experiment with the

1 higher donor DNA concentration (Experiment 7, 10 ng/ μ L) did achieve a higher rate of incorporation (as a
2 percentage of live born mice, 14.3% versus 5.6%) but a lower quality of allele modification in the single founder
3 recovered (mosaicism/transmission of only one modified allele at low frequency compared to
4 nonmosaicism/transmission of both modified alleles at maximum frequency). One may speculate that DNA
5 concentration may be the most important parameter related to the introduction of DNA into individual zygotes;
6 whereas, guide design may prove to be the most important factor for promoting more frequent deletion
7 formation and more efficient HDR once donor DNA has entered the cell. Further experimentation, performed in
8 large numbers of cells *in vitro*, is likely to be a productive avenue for optimization of this technique.

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- 20
21
22

1 **FIGURE CAPTIONS**

2 **Fig 1. Construction of the *Bcl2l11/BCL2L11* targeting vector/donor molecule.**

3 A gene-targeting vector/donor molecule was constructed placing a 25-kbp segment of the human *BCL2L11*
4 gene between mouse homology arms, placing removable selectable marker cassettes at each end of the
5 human segment, and placing *loxP* sites around a 2.9-kbp segment of human DNA deleted in 12% of the East
6 Asian population. See text for details.

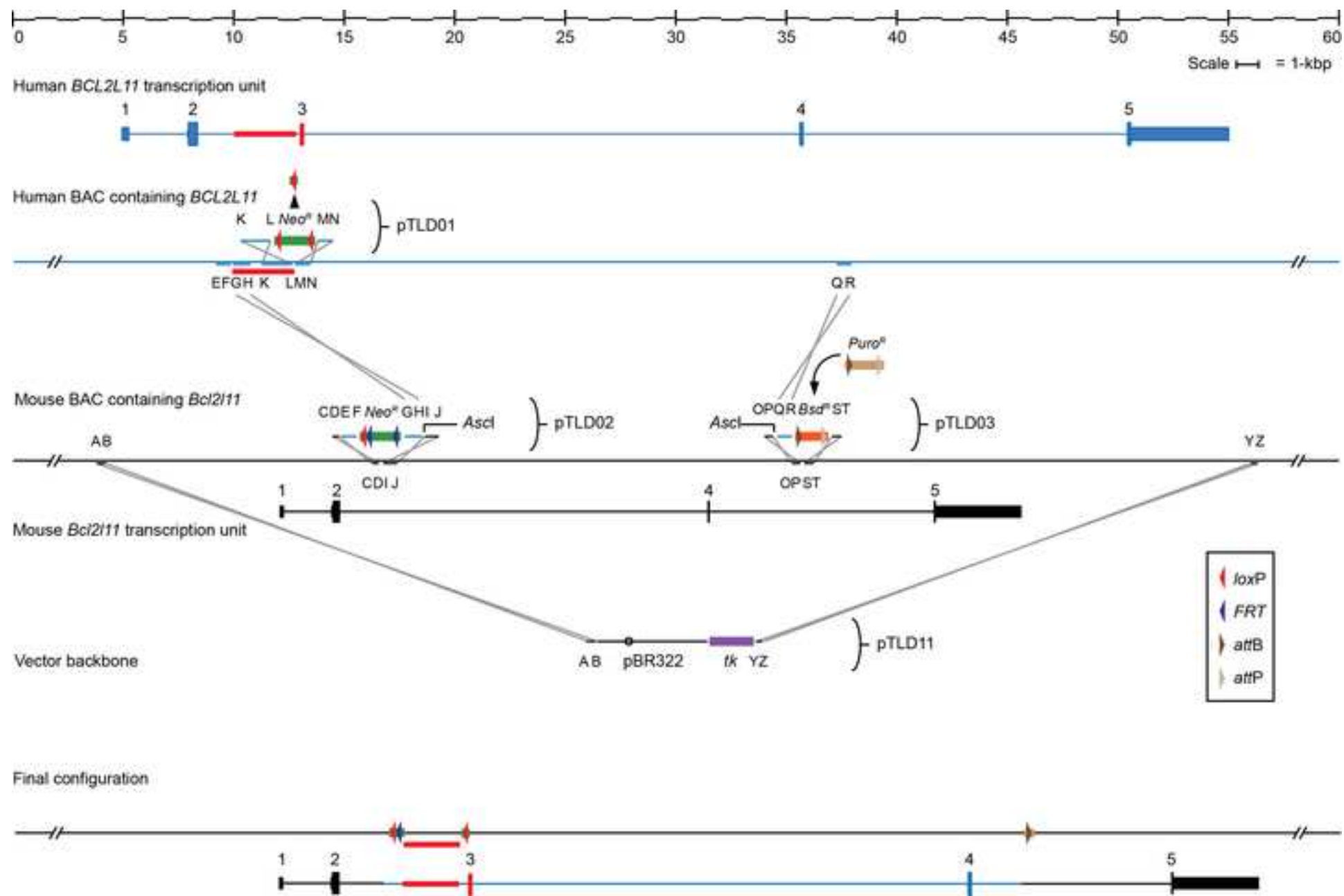
7
8 **Fig 2. Organization of genotyping primers for mouse (M), humanized (M/H), and deletion-bearing (Δ M)
9 alleles of *BCL2L11/Bcl2l11*.**

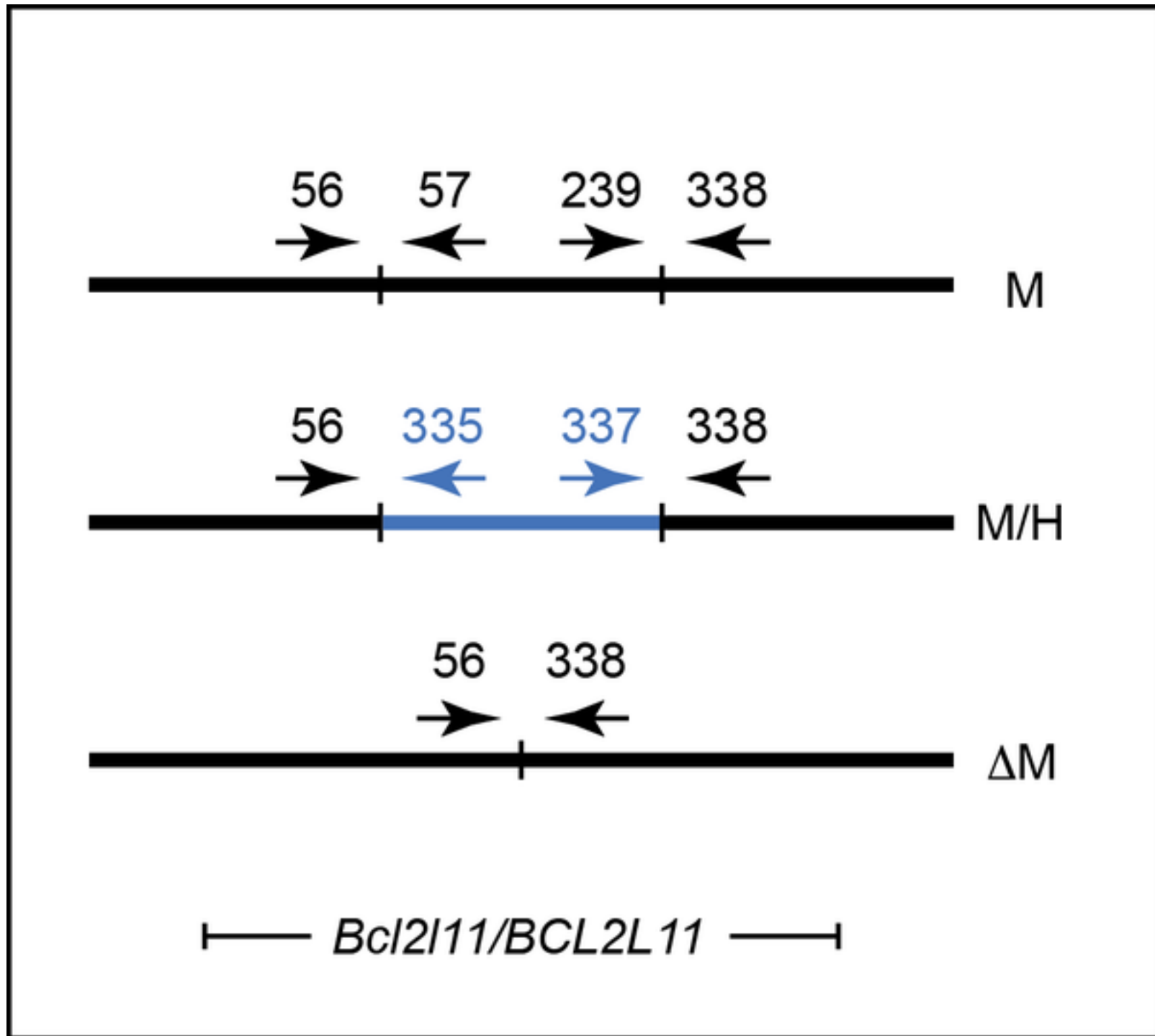
10 Schematic showing the organization of genotyping primers. Numbers, primer designation as in Table 4; left
11 and right segments of horizontal black lines, flanking regions of the mouse *Bcl2l11* region; central segment of
12 top horizontal black line, central (to be replaced) region of the mouse *Bcl2l11* gene; blue line, central segment
13 of human *BCL2L11* gene. See text for details.

14
15 **Fig 3. Linkage analysis of the *BCL2L11* integration site following CRISPR-stimulated homologous
16 recombination in mouse zygotes.**

17 Shown are the linkage analyses for 22 F2 progeny of a C57BL/6NJ X FVBB6NF1/J-*BCL2L11* backcross
18 (upper panel) and 28 F2 progeny of an FVB/NJ X FVBB6NF1/J-*BCL2L11* backcross (lower panel). Linkage
19 and haplotype analysis indicate that the *BCL2L11* vector's integration has occurred between markers
20 rs4223406 and rs3689600 and its segregation is fully concordant with markers rs13476756 and rs3662211.
21 This result is entirely consistent with integration of the human *BCL2L11* segment within the endogenous
22 mouse *Bcl2l11* gene as designed.

23





Chromosome	Ref. ID	Gene	Strand	Region 1																					
				100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	
chr1	101347511	A	T	A/A	A	A	A/A	A	A	A	A	A	A	A	A/A	A	A/A	A	A/A	A	A/A	A			
chr1	101347610	T	A	T/C	T	T	T/C	T	T	T	T	T	T	T	T/C	T	T	T	T	T	T	T			
chr1	101347688	L	A	A/G	A	A	A/A	A	A	A	A	A	A	A	A/A	A	A/A	A	A/A	A	A/A	A			
chr1	101347711	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T			
chr1	101347748	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T			
chr1	101347791	T	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T			
chr1	101347840	L	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C			
chr1	101347882	C	R	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C			
chr1	101347911	C	R	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C			
chr1	101347931	C	R	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C			
chr1	101347987	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T			

Chromosome	Ref. ID	Gene	Strand	Region 2																					
				100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	
chr1	101347911	A	C	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A			
chr1	101347925	T	C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C			
chr1	101347931	T	A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A			
chr1	101347988	C	T	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C			
chr1	101348011	C	T	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C			
chr1	101348040	T	C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C			
chr1	101348040	T	A	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C			
chr1	101348081	C	A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A			
chr1	101348091	C	A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A			
chr1	101348111	C	T	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C			