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

Short title: 25-Kilobase Pair Traditional and CRISPR/Cas9-Stimulated Knock-Ins

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ABSTRACT

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

AUTHOR SUMMARY

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

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.

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

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

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

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

Despite the associated uncertainties, as described in the report that follows, we sought to expand the limits of CRISPR knock-in technology. Specifically, we attempted to increase the physical extent to which mouse genomic DNA could be replaced with donor (in this case, human) DNA at an orthologous locus. Driving our efforts was the desire to create a whole animal model that would replace 17 kbp of the mouse *Bcl2l1* gene with the corresponding segment of human *BCL2L1*, including a conditionally removable segment (2.9-kbp) of intron 2, a cryptic human exon immediately 3' of this, and a native human exon some 20 kbp downstream (28). Using two approaches, we first carried out the replacement by employing a combination of bacterial artificial chromosome (BAC) recombineering, classic ES cell targeting, dual selection, and recombinase-driven cassette removal (hereafter referred to as our traditional approach) (29). In the second approach, we used the same vector (devoid of its selectable marker cassettes), microinjecting it along with CRISPR RNA guides and Cas9 into mouse zygotes (hereafter referred to as our CRISPR approach). In both instances we were able to achieve humanization of *Bcl2l1* to the extent designed, remove all selection cassettes, and demonstrate the functionality of the conditionally removable, *loxP*-flanked, 2.9-kbp intronic segment.

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

METHODS

Husbandry

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

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

Preparation of the Targeting Vectors/Donor Molecules

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

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

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

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

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' -dCGCATAAGCTTTTGTGTTCCAGATTCC-3'	HinDIII
C	oTLD29new	+	Mouse	mm10	Chr 2 : 128129327 128129348	246	22	35	5' -dCGCATGCGGCCGCATAGTTAATAACCACCAAGGCA-3'	NotI
D	oTLD30	-	Mouse	mm10	Chr 2 : 128129528 128129548		21	32	5' -dCGCATAAGCTTAAGTACTGCTAGCCCAAGAA-3'	HinDIII
E	oTLD27	+	Human	hg38	Chr 2 : 111124898 111124917	742	20	31	5' -dCGCATAAGCTTTATGCTCAGAGGGTTTGG-3'	HinDIII
F	oTLD28	-	Human	hg38	Chr 2 : 111125598 111125617		20	31	5' -dCGCATGGATCTGATTACCTCACTGAAGCC-3'	BamHI
G	oTLD20	+	Human	hg38	Chr 2 : 111125618 111125641	765	24	35	5' -dCGCATGAATTCGGCAGGCTTTGCCATGTTATAG-3'	EcoRI
H	oTLD21new	-	Human	hg38	Chr 2 : 111126335 111126358		24	37	5' -dCGCATGGCGGCCCTACTTTACTTCACAGGTATAACC-3'	AscI
I	oTLD31	+	Mouse	mm10	Chr 2 : 128129549 128129573		25	38	5' -dCGCATGGCGGCCGTAGAAATTTCTAAAACTATATTC-3'	AscI
J	oTLD32	-	Mouse	mm10	Chr 2 : 128130340 128130367	843	28	39	5' -dCGCATGTGACGTATTAAAGACTTAATAGTCTCCAGAGG-3'	Sall
K	oTLD9B	+	Human	hg38	Chr 2 : 111127109 111127130		22	35	5' -dCGCATGGCGGCCCTCTCTTACACTCTGGGAGGAT-3'	NotI
L	oTLD10	-	Human	hg38	Chr 2 : 111128507 111128525	1441	19	30	5' -dCGCATGGATCCCAACAGCATGATGTTCCCC-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' -dCGCATGTGCTGACTGAGTGGGAAGATCAAGCC-3'	Sall
O	oTLD13	+	Mouse	mm10	Chr 2 : 128147195 128147214	478	20	33	5' -dCGCATGCGGCCGCGTAAAGACCTCTCCCATCC-3'	NotI
P	oTLD14	-	Mouse	mm10	Chr 2 : 128147618 128147646		20	33	5' -dCGCATGGCGGCCCAACAGGACAGCCAGCTAC-3'	AscI
Q	oTLD15	+	Human	hg38	Chr 2 : 111151266 111151285	842	20	33	5' -dCGCATGGCGGCCGTGACTGCTTCGCTAAAGG-3'	AscI
R	oTLD16	-	Human	hg38	Chr 2 : 111152064 111152083		20	31	5' -dCGCATGAATTCCTCCCACTTTGATCTCTGAA-3'	EcoRI
S	oTLD17	+	Mouse	mm10	Chr 2 : 128147689 128147710	510	22	33	5' -dCGCATGGATCCGCATCTTCAGAAAGCAGTGTGT-3'	BamHI
T	oTLD18	-	Mouse	mm10	Chr 2 : 128148155 128148176		22	33	5' -dCGCATGTGCTGCTCCTCAGTCCATTCATCAACAG-3'	Sall
Y	oTLD40	+	Mouse	mm10	Chr 2 : 128173955 128173974		20	31	5' -dCGCATAAGCTTATCAGGCCAGGGTTCTAGT-3'	HinDIII
Z	oTLD41	-	Mouse	mm10	Chr 2 : 128174299 128174318	388	20	33	5' -dCGCATGGCGGCCCATAGTGTCCCTGTCCCAAGG-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.

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

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

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

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

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

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

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

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

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

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

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

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

Electroporation

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

CRISPR sgRNA Design and Production

For our CRISPR approach, all single-guide RNAs (sgRNAs) were designed using an algorithm available at <http://crispr.mit.edu> (39). These sgRNAs, shown in Table 2, were designed along two concepts. In the first, the two highest scoring sgRNAs (one in each direction) within a 250-bp region were selected from both the 5' and 3' ends of the 17-kbp segment of the mouse *Bcl2l1* segment being replaced. In the second, two internal sgRNAs (one in each direction) closest to each end of the replaced segment were selected regardless of their overall score. Guides were produced according to the method of Briner, *et al.* (40). Cas9 mRNA (CRISPR associated protein 9 mRNA, 5-methylcytidine, pseudouridine) was purchased from TriLink Biotechnologies (San Diego, CA).

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	ACAACCTTTCCAGATCAGT	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	AGGGTAGCTGGCTGCTCTGT	TGG	20	CLOSEST TO END	DOWNSTREAM	Chr 2:	128147624 - 128147643	plus

Four guides were designed at each end of the mouse *Bcl2l1* 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.

Microinjection

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

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

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

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).

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' -dGAGTCAAAGCCTACATCCCCAA-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.

Sanger Sequencing

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

Genetic mapping

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

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

These backcross schemes can be annotated as follows:

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

FVB/NJ X FVBB6NF1/J-*BCL2L11*

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

RESULTS

Traditional Approach

Following electroporation of the pTLD39 vector into the JM8-A3 line of ES cells and selection on G418, we assayed 89 surviving clones for the presence of the puromycin resistance cassette by PCR. Of these, twenty-seven contained the puromycin cassette and were subjected to puromycin selection. Of these, four clones survived and were assessed for homology arm, insert, and neomycin resistance cassette count by quantitative PCR. One clone passed all of these tests for proper targeting of the central human *BCL2L11* segment to the endogenous mouse *Bcl2l11* gene. ES cells from this clone were microinjected into blastocysts resulting in nine high-quality chimeras. The four highest quality male chimeras were mated to C57BL/6NJ females resulting in two independent instances of germline transmission of the humanized allele. Although presumably identical, 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 *BCL2l11*.**

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
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								
		6	85.71%	No deletion of 17-kbp mouse segment; No mouse human junctions	N/A										
	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.

To further explore the inheritance of these genetic changes, we mated the human insertion/deletion-positive P_0 s from Experiments 2, 6, and 7 to C57BL/6J mice and genotyped their progeny. The results of these analyses are shown in Table 5. As shown, the human insertion-positive P_0 mouse (male) from Experiment 2 (guides closest to ends, 1 ng/ μ L donor DNA) failed to transmit the humanized allele to any of 29 of its N_1 progeny suggesting that the P_0 mouse is mosaic with a germline consisting primarily of unmodified wildtype cells.

In contrast, the human insertion- and deletion-positive P_0 mouse (male) from Experiment 7 (highest scoring guides, 10 ng/ μ L donor DNA) transmitted its deletion-bearing allele to four of its 21 N_1 progeny. This P_0 mouse, however, did not transmit the human insertion-bearing allele to any of these 21 mice again suggesting that the P_0 mouse is mosaic with a germline consisting of relatively few human insertion-bearing cells.

Interestingly, the human insertion- and deletion-positive P_0 mouse (female) from Experiment 6 (guides closest to ends, 5 ng/ μ L donor DNA) transmitted either a human insertion-bearing allele or a deletion-bearing allele to all of its 13 N_1 progeny, but never both, implying that this animal is breeding as a true heterozygote with a genotype of both human insertion- and deletion-bearing alleles at the *Bcl2l1* locus. Subsequent breeding of three select N_1 mice (two bearing the human insertion and one bearing the deletion) gave results consistent with Mendelian expectations. Mating males with B6N.Cg-Tg(*Sox2-Cre*)1Amc/J female mice resulted in progeny in which the *loxP*-flanked 2.9-kbp human intronic segment was deleted, as designed.

Genetic mapping

We used an outcross-backcross genetic mapping strategy as a means of localizing the insertion site of BAC-derived human *BCL2L1* sequences. Twenty-two N_2 progeny were analyzed from the C57BL/6NJ X (FVB/NJ X C57BL/6NJ) backcross and twenty-eight progeny from the FVB/NJ X (FVB/NJ X C57BL/6NJ) backcross. Analysis of the data demonstrates strong linkage between the human *BCL2L1* segment and several genetic markers on mouse Chromosome 2 (See Fig 3). In the backcross to C57BL/6NJ, the marker with strongest linkage, marker rs13476756, had a log-odds ratio (LOD) of 6.58 ($p < 0.004$). In the backcross to FVB/NJ, marker rs13476756 had a LOD score of 7.64 ($p < 0.0004$).

Analysis of individual haplotypes (specifically, points of recombination in samples 261, 263, 266, 303, and 319) further narrows the insertion-critical region to a 45.2-Mbp region from marker rs4223406 (nucleotide 113,827,352) to marker rs3689600 (nucleotide 159,014,253) on Mouse Chromosome 2 (GRC38/mm10), which is consistent with integration into the 36,510-bp mouse *Bcl2l1* gene that spans from nucleotide 128,126,038 to nucleotide 128,162,547. Put another way, this analysis shows that both the mouse *Bcl2l1* gene and the engineered human sequences must be colocalized within a region comprising less than 2% of the mouse genome. We conclude that integration of the human sequence has not occurred randomly, but has indeed occurred by homologous recombination as designed.

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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FIGURE CAPTIONS

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

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

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

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

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

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

Figure 1

[Click here to download Figure Fig1.tif](#)

		Special BPs No. 1-10		No. 11-20		No. 21-30		No. 31-40		No. 41-50		No. 51-60		No. 61-70		No. 71-80		No. 81-90		No. 91-100		No. 101-110		No. 111-120		No. 121-130		No. 131-140		No. 141-150		No. 151-160		No. 161-170		No. 171-180		No. 181-190		No. 191-200		No. 201-210		No. 211-220		No. 221-230		No. 231-240		No. 241-250		No. 251-260		No. 261-270		No. 271-280		No. 281-290		No. 291-300																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
2019-2020	2020-2021	2021-2022	2022-2023	2023-2024	2024-2025	2025-2026	2026-2027	2027-2028	2028-2029	2029-2030	2030-2031	2031-2032	2032-2033	2033-2034	2034-2035	2035-2036	2036-2037	2037-2038	2038-2039	2039-2040	2040-2041	2041-2042	2042-2043	2043-2044	2044-2045	2045-2046	2046-2047	2047-2048	2048-2049	2049-2050	2050-2051	2051-2052	2052-2053	2053-2054	2054-2055	2055-2056	2056-2057	2057-2058	2058-2059	2059-2060	2060-2061	2061-2062	2062-2063	2063-2064	2064-2065	2065-2066	2066-2067	2067-2068	2068-2069	2069-2070	2070-2071	2071-2072	2072-2073	2073-2074	2074-2075	2075-2076	2076-2077	2077-2078	2078-2079	2079-2080	2080-2081	2081-2082	2082-2083	2083-2084	2084-2085	2085-2086	2086-2087	2087-2088	2088-2089	2089-2090	2090-2091	2091-2092	2092-2093	2093-2094	2094-2095	2095-2096	2096-2097	2097-2098	2098-2099	2099-2100	2100-2101	2101-2102	2102-2103	2103-2104	2104-2105	2105-2106	2106-2107	2107-2108	2108-2109	2109-2110	2110-2111	2111-2112	2112-2113	2113-2114	2114-2115	2115-2116	2116-2117	2117-2118	2118-2119	2119-2120	2120-2121	2121-2122	2122-2123	2123-2124	2124-2125	2125-2126	2126-2127	2127-2128	2128-2129	2129-2130	2130-2131	2131-2132	2132-2133	2133-2134	2134-2135	2135-2136	2136-2137	2137-2138	2138-2139	2139-2140	2140-2141	2141-2142	2142-2143	2143-2144	2144-2145	2145-2146	2146-2147	2147-2148	2148-2149	2149-2150	2150-2151	2151-2152	2152-2153	2153-2154	2154-2155	2155-2156	2156-2157	2157-2158	2158-2159	2159-2160	2160-2161	2161-2162	2162-2163	2163-2164	2164-2165	2165-2166	2166-2167	2167-2168	2168-2169	2169-2170	2170-2171	2171-2172	2172-2173	2173-2174	2174-2175	2175-2176	2176-2177	2177-2178	2178-2179	2179-2180	2180-2181	2181-2182	2182-2183	2183-2184	2184-2185	2185-2186	2186-2187	2187-2188	2188-2189	2189-2190	2190-2191	2191-2192	2192-2193	2193-2194	2194-2195	2195-2196	2196-2197	2197-2198	2198-2199	2199-2200	2200-2201	2201-2202	2202-2203	2203-2204	2204-2205	2205-2206	2206-2207	2207-2208	2208-2209	2209-2210	2210-2211	2211-2212	2212-2213	2213-2214	2214-2215	2215-2216	2216-2217	2217-2218	2218-2219	2219-2220	2220-2221	2221-2222	2222-2223	2223-2224	2224-2225	2225-2226	2226-2227	2227-2228	2228-2229	2229-2230	2230-2231	2231-2232	2232-2233	2233-2234	2234-2235	2235-2236	2236-2237	2237-2238	2238-2239	2239-2240	2240-2241	2241-2242	2242-2243	2243-2244	2244-2245	2245-2246	2246-2247	2247-2248	2248-2249	2249-2250	2250-2251	2251-2252	2252-2253	2253-2254	2254-2255	2255-2256	2256-2257	2257-2258	2258-2259	2259-2260	2260-2261	2261-2262	2262-2263	2263-2264	2264-2265	2265-2266	2266-2267	2267-2268	2268-2269	2269-2270	2270-2271	2271-2272	2272-2273	2273-2274	2274-2275	2275-2276	2276-2277	2277-2278	2278-2279	2279-2280	2280-2281	2281-2282	2282-2283	2283-2284	2284-2285	2285-2286	2286-2287	2287-2288	2288-2289	2289-2290	2290-2291	2291-2292	2292-2293	2293-2294	2294-2295	2295-2296	2296-2297	2297-2298	2298-2299	2299-2300	2300-2301	2301-2302	2302-2303	2303-2304	2304-2305	2305-2306	2306-2307	2307-2308	2308-2309	2309-2310	2310-2311	2311-2312	2312-2313	2313-2314	2314-2315	2315-2316	2316-2317	2317-2318	2318-2319	2319-2320	2320-2321	2321-2322	2322-2323	2323-2324	2324-2325	2325-2326	2326-2327	2327-2328	2328-2329	2329-2330	2330-2331	2331-2332	2332-2333	2333-2334	2334-2335	2335-2336	2336-2337	2337-2338	2338-2339	2339-2340	2340-2341	2341-2342	2342-2343	2343-2344	2344-2345	2345-2346	2346-2347	2347-2348	2348-2349	2349-2350	2350-2351	2351-2352	2352-2353	2353-2354	2354-2355	2355-2356	2356-2357	2357-2358	2358-2359	2359-2360	2360-2361	2361-2362	2362-2363	2363-2364	2364-2365	2365-2366	2366-2367	2367-2368	2368-2369	2369-2370	2370-2371	2371-2372	2372-2373	2373-2374	2374-2375	2375-2376	2376-2377	2377-2378	2378-2379	2379-2380	2380-2381	2381-2382	2382-2383	2383-2384	2384-2385	2385-2386	2386-2387	2387-2388	2388-2389	2389-2390	2390-2391	2391-2392	2392-2393	2393-2394	2394-2395	2395-2396	2396-2397	2397-2398	2398-2399	2399-2400	2400-2401	2401-2402	2402-2403	2403-2404	2404-2405	2405-2406	2406-2407	2407-2408	2408-2409	2409-2410	2410-2411	2411-2412	2412-2413	2413-2414	2414-2415	2415-2416	2416-2417	2417-2418	2418-2419	2419-2420	2420-2421	2421-2422	2422-2423	2423-2424	2424-2425	2425-2426	2426-2427	2427-2428	2428-2429	2429-2430	2430-2431	2431-2432	2432-2433	2433-2434	2434-2435	2435-2436	2436-2437	2437-2438	2438-2439	2439-2440	2440-2441	2441-2442	2442-2443	2443-2444	2444-2445	2445-2446	2446-2447	2447-2448	2448-2449	2449-2450	2450-2451	2451-2452	2452-2453	2453-2454	2454-2455	2455-2456	2456-2457	2457-2458	2458-2459	2459-2460	2460-2461	2461-2462	2462-2463	2463-2464	2464-2465	2465-2466	2466-2467	2467-2468	2468-2469	2469-2470	2470-2471	2471-2472	2472-2473	2473-2474	2474-2475	2475-2476	2476-2477	2477-2478	2478-2479	2479-2480	2480-2481	2481-2482	2482-2483	2483-2484	2484-2485	2485-2486	2486-2487	2487-2488	2488-2489	2489-2490	2490-2491	2491-2492	2492-2493	2493-2494	2494-2495	2495-2496	2496-2497	2497-2498	2498-2499	2499-2500	2500-2501	2501-2502	2502-2503	2503-2504	2504-2505	2505-2506	2506-2507	2507-2508	2508-2509	2509-2510	2510-2511	2511-2512	2512-2513	2513-2514	2514-2515	2515-2516	2516-2517	2517-2518	2518-2519	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