All cloning was performed using standard methods. Phusion DNA polymerase was used in all PCRs. DH5α was used as the *E. coli* host in most cloning steps, but when insulator containing vectors were involved, the Stbl2 cells (Invitrogen 10268019) were used. All restriction enzymes, CIP, Klenow fragment, T4 DNA polymerase and T4 DNA ligase were purchased from the New England Biolabs. All primer sequences are listed in Supplementary File S2. All constructs were verified by restriction digestion and/or sequencing. For integrated fragments, both the cloned fragments and the original PCR products from genomic DNA were fully sequenced to ensure all polymorphisms were naturally occurring, and not introduced by PCR.

**1. Cloning of the targeting vectors**

1a. cloning of original version of the targeting vectors

The oligos MCS-L-5' and MCS-L-3' were annealed and ligated into HindIII and XhoI digested pBluescript II KS(+) to introduce the left half of the multiple cloning site (MCS), resulting in PBS-MCS-L. The HindIII site was eliminated after ligation. The oligos I-CreI-5' and I-CreI-3' were annealed and ligated into XbaI and BamHI digested PBS-MCS-L to introduce the first I-CreI site, resulting in PBS-I-CreI-MCS-L. The BamHI site was eliminated after ligation. The p[sChFP] construct (Abreu-Blanco *et al.* 2012), which was derived from pCaSpeR-4, was digested with EcoO109I, blunt ended with Klenow and then digested with EcoRI to release the 4.25 kb *mini-white* fragment, which was ligated into EcoRI and SmaI digested PBS-I-CreI-MCS-L to generate PBS-I-CreI-mini-W-MCS-L.

The FRT(+) fragment was amplified from the genomic DNA of Bloomington stock number 19139 using primers FRT(200)+5' and FRT(200)3', and the attB(+) fragment was amplified from pUASTattB (Bischof *et al.* 2007) using primers attB5' and attB+3'. 3 fragment ligation was performed to join NotI and ClaI digested FRT(+), ClaI and SpeI digested attB(+) and NotI and SpeI digested pBluescript II KS(+) to generate PBS-FRT(+)-attB(+). A unique NheI site was also introduced between the NotI site and the FRT(+) fragment.

The FRT(-) fragment was amplified from the genomic DNA of Bloomington stock number 19139 using primers FRT(200)-5' and FRT(200)3', and the attB(-) fragment was amplified from pUASTattB (Bischof *et al.* 2007) using primers attB5' and attB-3'. 3 fragment ligation was performed to join SpeI and ClaI digested FRT(-), ClaI and NotI digested attB(-) and NotI and SpeI digested pBluescript II KS(+) to generate PBS-attB(-)-FRT(-). A unique NheI site was also introduced between the NotI site and the attB(-) fragment.

The FRT(+)-attB(+) fragment and the attB(-)-FRT(-) fragment were released from PBS-FRT(+)-attB(+) and PBS-attB(-)-FRT(-) by digestion with NotI and SpeI, and then ligated into NotI and XbaI digested PBS-I-CreI-mini-W-MCS-L to generate PBS-(+)-I-CreI-W-MCS-L and PBS-(-)-I-CreI-W-MCS-L, respectively.

The oligos MCS-R-5' and MCS-R-3' were annealed and ligated into ApaI and KpnI digested pBluescript II KS(+) to introduce the right half of the MCS, resulting in PBS-MCS-R. The KpnI site was eliminated after ligation. The oligos I-SceI-5' and I-SceI-3' were annealed and ligated into SacI and SacII digested PBS-MCS-R to introduce the first I-SceI site, resulting in PBS-I-SceI-MCS-R. Unique Acc65I and AatII sites were introduced between the SacI site and the I-SceI site.

The 3xP3-RFP marker (containing the promoter, the gene ORF and the polyA signal) (Bischof *et al.* 2007) was amplified from the genomic DNA of Bloomington stock number 36313 using primers 3xP3-RFP-5' and 3xP3-RFP-3', digested with SacII and XbaI, and ligated into SacII and XbaI digested pBluescript II KS(+) to generate PBS-3xP3-RFP. The construct pUChsneo-Act (Thummel *et al.* 1988) was digested with NdeI, blunt ended with Klenow, and digested with HindIII to release the hs-neo marker, which was ligated into ClaI digested, Klenow blunt ended and then HindIII digested pBluescript II KS(+), resulting in PBS-hs-neo.

The 3xP3-RFP marker was released from PBS-3xP3-RFP by digestion with SacII and XbaI, and ligated into SacII and XbaI digested PBS-I-SceI-MCS-R to generate PBS-I-SceI-3xP3-RFP-MCS-R. The hs-neo marker was released from PBS-hs-neo by digestion with XbaI and SalI, and ligated into XbaI and SalI digested PBS-I-SceI-3xP3-RFP-MCS-R to generate PBS-I-SceI-3xP3-RFP-hs-neo-MCS-R. The I-SceI-3xP3-RFP-hs-neo-MCS-R cassette was released from PBS-I-SceI-3xP3-RFP-hs-neo-MCS-R by digestion with Acc65I and AscI, and ligated into Acc65I and AscI digested PBS-(+)-I-CreI-W-MCS-L and PBS-(+)-I-CreI-W-MCS-L to generate PBS-Targeting(+) and PBS-Targeting(-), respectively.

The oligos 2nd-I-CreI-5' and 2nd-I-CreI-3' were annealed and ligated into PacI digested PBS-Targeting(+) and PBS-Targeting(-) to introduce the second I-CreI site, generating PBS-Targeting(+)-2nd-I-CreI and PBS-Targeting(-)-2nd-I-CreI, respectively. After ligation, there was only a single PacI site at the end of the 2nd I-CreI that is proximal to the MCS, and the other PacI site was eliminated. The oligos 2nd-I-SceI-5' and 2nd-I-SceI-3' were annealed and ligated into PspXI digested PBS-Targeting(+)-2nd-I-CreI and PBS-Targeting(-)-2nd-I-CreI to introduce a second I-SceI site, generating PBS-Targeting(+)-II and PBS-Targeting(-)-II, respectively. After ligation, there was only a single PspXI site at the end of the 2nd I-SceI that is proximal to the MCS, and the other PspXI site was eliminated.

A second attB fragment was amplified from pUASTattB (Bischof *et al.* 2007) using primers 2nd-attB-5' and 2nd-attB-3', digested with Acc65I and AatII, and ligated into Acc65I and AatII digested PBS-Targeting(+) and PBS-Targeting(+)-II to generate PBS-Targeting-RMCE and PBS-Targeting-RMCE-II respectively.

1b. Cloning during marker optimization

In order to insert an insulator fragment between the FRT site and the *mini-white* marker, a 750 bp NheI-NsiI fragment covering part of the FRT sequence and part of the *mini-white* marker was first cloned into the pBluescript II KS(+) vector, and a gypsy insulator was inserted between FRT and *mini-white* using overlapping extension PCR. The resulting 1.2kb NheI-NsiI fragment was then inserted between the NheI and NsiI sites of the original vectors to replace the uninsulated NheI-NsiI fragment. NotI and PstI sites were added to the 5’ end of primers to facilitate cloning.

PBS-Targeting-RMCE was used as template, and FRT(+)-W-5’ and FRT-W-3’ were used as primers to amplify the 750bp FRT(+)-W fragment, which was then digested with NotI + PstI, and cloned into NotI + PstI digested pBluescript II KS(+), generating the PBS-FRT(+)-W construct.

PBS-Targeting-(-) was used as template, and FRT(-)-W-5’ and FRT-W-3’ were used as primers to amplify the 750bp FRT(-)-W fragment, which was then digested with NotI + PstI, and cloned into NotI + PstI digested pBluescript II KS(+), generating the PBS-FRT(-)-W construct.

pH-Stinger (Barolo *et al.* 2000) was used as template, and insulator-5’ and insulator-3’ were used as primers to amplify the 400bp gypsy insulator fragment, which was then digested with HindIII + EcoO109I, and cloned into HindIII + EcoO109I digested pBluescript II KS(+), generating the PBS-insulator construct.

PBS-FRT(+)-W was used as template, and FRT(+)-W-5’ and FRT-gypsy-3’ were used as primers to amplify the 530 bp FRT(+)-gypsy-5’ fragment. PBS-FRT(-)-W was used as template, and FRT(-)-W-5’ and FRT-gypsy-3’ were used as primers to amplify the 530 bp FRT(-)-gypsy-5’ fragment. PBS-insulator was used as template, and FRT-gypsy-W-5’ and FRT-gypsy-W-3’ were used as primers to amplify the 400bp FRT-gypsy-W fragment. PBS-FRT(+)-W was used as template, and gypsy-W-5’ and FRT-W-3’ were used as primers to amplify the 230 bp gypsy-W-3’ fragment.

Next, FRT(+)-gypsy-5’, FRT-gypsy-W and gypsy-W-3’ fragments were used as templates, and FRT(+)-W-5’ and FRT-W-3’ were used as primer to perform overlapping extension PCR to generate the 1.2kb FRT(+)-gypsy-W fragment. Similarly, FRT(-)-gypsy-5’, FRT-gypsy-W and gypsy-W-3’ fragments were used as templates, and FRT(-)-W-5’ and FRT-W-3’ were used as primer to perform overlapping extension PCR to generate the 1.2kb FRT(-)-gypsy-W fragment. The FRT(+)-gypsy-W and FRT(-)-gypsy-W fragments were digested with NotI + PstI, and cloned into NotI + PstI digested pBluescript II KS(+) vector, generating PBS-FRT(+)-gypsy-W and PBS-FRT(-)-gypsy-W constructs.

PBS-insulator was used as template to generate the following 400bp fragments using 3 different primer pairs: 1) AatII-ins with AatII-ins-5’ and AatII-ins-3’, 2) PacI-CreI-ins with PacI-CreI-ins-5’ and PacI-ins-3’, and 3) PspXI-SceI-ins with PspXI-SceI-ins-5’ and PspXI-ins-3’.

The AatII-ins fragment was digested with BstXI + AatII, and inserted into AatII digested and CIP treated PBS-Targeting-RMCE-II vector, generating PBS-Targeting-RMCE-II-ins vector. PCR was used to select the orientation of the insulator insertions such that a valid AatII site was present between the insulator and the attB site, and the AatII site between 3xP3-RFP and insulator was eliminated after ligation.

The 1.2kb NheI + NsiI fragment was released from PBS-FRT(+)-gypsy-W, and was ligated to NheI + NsiI digested PBS-Targeting-RMCE-II-ins, generating PBS-Targeting-RMCE-II-2xins constructs.

The MiMIC vector pMiLR-attP1-2-yellow-SA-EGFP (DGRC 1321) was used as template, and y-spacer-5’ and y-spacer-3’ were used as primers to amplify the 2 kb spacer, which was then digested with Acc65I + SalI, and was cloned into Acc65I + SalI digested pBluescript II KS(+), generating the PBS-y-spacer construct.

The PacI-CreI-ins fragment (see above) was digested with PvuI + PacI, and cloned into PacI digested PBS-y-spacer, generating PBS-ins-CreI-y. PCR was used to select the insertion orientation such that the PacI site between the yellow spacer and the insulator is reconstructed, and the PacI site on the other side of the insulator is eliminated after ligation.

PspXI + SalI digested PspXI-SceI-ins fragment was ligated into PspXI digested PBS-ins-CreI-y, generating PBS-ins-CreI-y-SceI-ins. Again, PCR was used to select the insertion orientation such that the PspXI site between the yellow spacer and the insulator is reconstructed, and the PspXI site on the other side of the insulator is eliminated after ligation.

SalI + DrdI were used to digest the PBS-ins-CreI-y-SceI-ins construct to release the 2.8kb spacer fragment, which was inserted into PacI + PspXI digested PBS-Targeting-RMCE-2xins, generating PBS-Targeting-RMCE-II-4xins.

pUAST (Brand and Perrimon 1993) was used as the template, and SV40pA-5’ and SV40pA-3’ were used as primers to amplify the 750 bp SV40pA fragment, which was digested with EcoRIHF + SpeI, and cloned into EcoRIHF + SpeI digested pBluescript II KS(+), generating PBS-SV40pA construct.

Finally, the SV40pA fragment was released from PBS-SV40pA by SpeI + NheI digestion, and was inserted into SpeI digested and CIP treated PBS-Targeting-RMCE-II, and PBS-Targeting-RMCE-II-4xins vectors, generating constructs pTargeting-RMCE and pTargeting-RMCE-insulated. PCR was used to ensure the correct orientation of the SV40pA insert.

pTargeting-RMCE was digested with KpnIHF + AatI, and blunt ended with Klenow fragment. This fragment was then self-ligated to generate pTargeting-attB(+). pTargeting-RMCE-insulated was generated similarly by digestion with AatII + Acc65I, followed by blunt ending with T4 DNA polymerase. This fragment was then self-ligated to generate pTargeting-attB(+)-insulated.

The SpeI + NheI SV40pA fragment from PBS-SV40pA was ligated into SpeI digested and CIP treated PBS-Targeting(-)-II, generating vector pTargeting-attB(-). PCR was used to ensure the correct orientation of the SV40pA insert.

The pTargeting-attB(-)-insulated was generated in a number of steps. The SpeI + NheI SV40pA fragment from PBS-SV40pA was ligated into SpeI digested and CIP treated PBS-Targeting(-), generating vector PBS-Targeting(-)-B. PCR was used to ensure the correct orientation of the SV40pA insert.

The AatII-ins fragment was digested with BstXI + AatII, and inserted into AatII digested and CIP treated PBS-Targeting(-)-B vector, generating PBS-Targeting(-)-B-ins vector. PCR was used to select the orientation of the insulator insertions such that a valid AatII site is present between the insulator and the attB site, and the AatII site between 3xP3-RFP and insulator is eliminated after ligation.

The 1.2kb NheI + NsiI fragment was released from PBS-FRT(-)-gypsy-W, and was ligated to NheI + NsiI digested PBS-Targeting(-)-B-ins, generating PBS-Targeting(-)-B-2xins constructs.

SalI + DrdI were used to digest the PBS-ins-CreI-y-SceI-ins construct to release the 2.8kb spacer fragment, which was inserted into PacI + PspXI digested PBS-Targeting(-)-B-2xins, generating pTargeting-attB(-)-insulated.

**2. Cloning of *Ubx* landing site donor plasmid**

A pCassette-ubiDsRed vector was first generated. The attP-MCS1 fragment was PCR amplified from the genomic DNA of the attP51C fly line (Bloomington #24482) using primers attP-MCS1-5’ and attP-MCS1-3’, and digested with SacI + SacII. This digested fragment was ligated into SacI + SacII digested pBluescript II KS(+), generating PBS-MCS1-attP.

The Act-mCD8-GFP fragment was released from the construct pUAST-Act-mCD8-GFP (unpublished construct) by PstI + StuI digestion, and was ligated into PstI + HincII digested PBS-MCS1-attP, generating PBS-MCS1-attP-Act-mCD8-GFP.

The attP-MCS2 fragment was PCR amplified the genomic DNA of the attP51C fly line (Bloomington #24482) using primers attP-MCS2-5’ and attP-MCS2-3’, and digested with ApaI + Acc65I. The digested fragment was ligated into ApaI + Acc65I digested PBS-MCS1-attP-Act-mCD8-GFP, which generated pCassette-Act-mCD8-GFP.

The UbiDsRed fragment was released from the construct pXLBacII-pUbDsRed-T3 (a gift from Al Handler) by XmaI + ClaI digestion, which was then ligated into XmaI + ClaI digested pBluescript II KS(+), generating PBS-UbiDsRed.

The UbiDsRed fragment was released from PBS-UbiDsRed by digestion with SacII + ApaI, which was ligated into pCassette backbone, generated by SacII + ApaI digestion of pCassette-Act-mCD8-GFP. This ligation generated the construct pCassette-UbiDsRed.

A 3.2 kb Ubx-N-L fragment was amplified from the genomic DNA of Bloomington line #28877 using primers Ubx-N-L-5’ and Ubx-N-L-3’. A 3.3kb Ubx-N-R fragment was amplified from the genomic DNA of Bloomington line #28877 using primers Ubx-N-R-5’ and Ubx-N-R-3’. Fragments Ubx-N-L and Ubx-N-R were digested with SalI + Acc65I and ligated into SalI + Acc65I digested pBluescript II KS(+), generating constructs PBS-Ubx-N-L and PBS-Ubx-N-R. The Ubx-N-L homologous arm was released from PBS-Ubx-N-L by AvrII + AscI digestion, and was ligated into AvrII + AscI digested pCassette-UbiDsRed, generating pCassette-UbiDsRed-Ubx-N-L. The Ubx-N-R homologous arm was released from PBS-Ubx-N-R by digestion with NheI + PacI, and was ligated into NheI + PacI digested pCassette-UbiDsRed-Ubx-N-L, generating the final donor plasmid pCassette-UbiDsRed-Ubx-N. This donor plasmid was used to make the injection mixture with the two TALEN mRNAs.

**3. Cloning of the *Antp* targeting plasmids**

The Antp1 fragment was amplified by PCR from the genomic DNA of Bloomington stock number 19139 using primers Antp1 5' and Antp1 3', digested with ApaI and EcoRI, and ligated into ApaI and EcoRI digested pBluescript II KS(+) to generate PBS-Antp1. A unique AscI site was introduced between the ApaI site and the 5' end of Antp1 fragment.

The Antp2 fragment was amplified by PCR from the genomic DNA of Bloomington stock number 19139 using primers Antp2 5' and Antp2 3', digested with XbaI and EcoRIHF, and ligated into XbaI and EcoRIHF digested pBluescript II KS(+) to generate PBS-Antp2.

The Antp3 fragment was amplified by PCR from the genomic DNA of Bloomington stock number 19139 using primers Antp3 5' and Antp3 3', digested with XbaI and NotIHF, and ligated into XbaI and NotIHF digested pBluescript II KS(+) to generate PBS-Antp3. A unique SbfI site was introduced between the 3' end of Antp3 fragment and the NotI site.

Construct PBS-Antp2 YPWM-->AAAA was generated by introducing the YPWM-->AAAA mutation into PBS-Antp2 by DpnI mediated mutagenesis: PBS-Antp2 was used as template to perform mutagenetic PCR using primers Antp YPWM-AAAA 5' and Antp YPWM-AAAA 3'. The PCR product was digested with DpnI overnight before being used to transform *E. coli* to obtain the mutant clones.

Fragment DmGFP was amplified from Addgene plasmid No. 26224 using primers DmGFP 5' and DmGFP 3'. Fragment Antp4-DmGFP was amplified from PBS-Antp2 YPWM-->AAAA using primers M13R and Antp4 3', and fragment DmGFP-Antp5 was amplified from PBS-Antp2 YPWM-->AAAA using primers Antp5 5' and M13.

Next, overlapping PCR was performed to generate fragment Antp2-DmGFP YPWM-->AAAA: Fragments DmGFP, Antp4-DmGFP, DmGFP-Antp5 were mixed to serve as the template, which was amplified by primers M13 and M13R. The resulting fragment, Antp2-DmGFP YPWM-->AAAA, was digested with EcoRI and XbaI, and ligated into EcoRI and XbaI digested pBluescript II KS(+) to generate PBS-Antp2-DmGFP YPWM-->AAAA. In this construct, DmGFP is fused in frame to the N terminus of the Antp coding region, and the fusion protein has a 15 amino acid linker (SerGlyGlyGlyGly SerGlyGlyGlyGly SerGlyGlyGlyGly) between GFP and Antp.

Fragment Antp4-3xFLAG was amplified from PBS-Antp2 YPWM-->AAAA using primers M13R and Antp4 3xFLAG 3', and fragment 3xFLAG-Antp5 was amplified from PBS-Antp2 YPWM-->AAAA using primers Antp5 3xFLAG 5' and M13. Overlapping PCR was then performed to generate fragment Antp2-3xFLAG YPWM-->AAAA: Fragments Antp4-3xFLAG and 3xFLAG-Antp5 were mixed to serve as the template, which was amplified by primers M13 and M13R. The resulting fragment, Antp2-3xFLAG YPWM-->AAAA, was digested with EcoRI and XbaI, and ligated into EcoRI and XbaI digested pBluescript II KS(+) to generate PBS-Antp2-3xFLAG YPWM-->AAAA.

Next, PBS-Antp1 was digested with ApaI and EcoRIHF to release Antp1 fragment, and PBS-Antp3 was digested with ApaI and XbaI to generate the Antp3-PBS fragment. PBS-Antp2-DmGFP YPWM-->AAAA was digested with XbaI, EcoRIHF and ScaI to release fragment Antp2-DmGFP YPWM-->AAAA. ScaI was used to cut the plasmid backbone so that the desired fragment (the insert) could be separated from the backbone on an agarose gel. 3 fragment ligation was then performed to join ApaI and EcoRIHF released Antp1 fragment, ApaI and XbaI generated Antp3-PBS fragment, and XbaI and EcoRIHF released fragment Antp2-DmGFP YPWM-->AAAA to generate PBS-Antp-DmGFP YPWM-->AAAA.

PBS-Antp2-3xFLAG YPWM-->AAAA were digested with XbaI and EcoRIHF to release fragment Antp2-3xFLAG YPWM-->AAAA. 3 fragment ligation was then performed to join ApaI and EcoRIHF released Antp1 fragment, ApaI and XbaI generated Antp3-PBS fragment, and XbaI and EcoRIHF released fragment Antp2-3xFLAG YPWM-->AAAA to generate PBS-Antp-3xFLAG YPWM-->AAAA.

Antp-DmGFP YPWM-->AAAA and Antp-3xFLAG YPWM-->AAAA were released from PBS-Antp-DmGFP YPWM-->AAAA and PBS-Antp-3xFLAG YPWM-->AAAA respectively by digestion with AscI and SbfIHF, and were ligated into AscI and SbfIHF digested pTargeting-RMCE-insulated to generate the final *3xFLAG-Antp* and *GFP-Antp* targeting plasmids.

**4. Cloning of the *Ubx* targeting plasmids**

The 3.3 kb Ubx-N1 fragment, the 1.8kb Ubx-N2 fragment and the 3.1kb Ubx-N3 fragment were amplified from the genomic DNA of Bloomington stock number #28877 using primers Ubx-N1-5’ + Ubx-N1-3’, Ubx-N2-5’ + Ubx-N2-3’, and Ubx-N3-5’ + Ubx-N3-3’. These 3 fragments were digested with XbaI + KpnI, and ligated into XbaI + KpnI digested pBluescript II KS(+), generating PBS-Ubx-N1, PBS-Ubx-N2, and PBS-Ubx-N3 constructs.

Fragment Ubx-N4-3xFLAG was amplified using M13 + Ubx-N4-3xFLAG-3’ as primers from PBS-Ubx-N2, and fragment 3xFLAG-Ubx-N5 was amplified from PBS-Ubx-N2 using primers 3xFLAG-Ubx-N5-5’ + M13R. Next, fragments Ubx-N4-3xFLAG and 3xFLAG-Ubx-N5 were used as templates to perform overlapping PCR using primers M13 + M13R to generate the Ubx-N2-3xFLAG fragment. Fragment Ubx-N2-3xFLAG was digested with XbaI + KpnI, and ligated into XbaI + KpnI digested pBluescript II KS(+) to generate construct PBS-Ubx-N2-3xFLAG.

The YPWM-AAAA mutation was introduced into PBS-Ubx-N2-3xFLAG using DpnI mediated mutagenesis, as described above, using primers Ubx-YPWM-AAAA-5’ + Ubx-YPWM-AAAA-3’, and the resulting construct was PBS-Ubx-3xFLAG-N2-m.

The Ubx-N1 fragment was released from PBS-Ubx-N1 by XbaI + EcoRI digestion, and the Ubx-3xFLAG-N2-m fragment was released from PBS-Ubx-3xFLAG-N2-m by digestion with EcoRI + HindIII. These two fragments were ligated into XbaI + HindIII digested PBS-Ubx-N3 in a 3-fragment ligation reaction, generating construct PBS-3xFLAG-Ubx-N-m.

The entire integrated fragment, 3xFLAG-Ubx-N-m, was released from PBS-3xFLAG-ubx-N-m by PacI + AgeI digestion, and was ligated into PacI + AgeI digested pTargeting-RMCE-insulated, generating the final *3xFLAG-Ubx* targeting plasmid.

**5. Cloning of the *Gr28b* targeting plasmids**

The *Gr28b* left arm was amplified from genomic DNA of line *MI11240* using primers Gr28b-LA-5' + Gr28b-LA-3' and digested with NotI + BamHI. The *Gr28b* right arm was amplified from genomic DNA of isogenic *w1118* line (Bloomington #5905) using primers Gr28b-RA-5' + Gr28b-RA-3', and digested with BamHI + SalI. The two digested fragment were ligated into NotI + SalI digested pBluescript II KS(+) in a 3-fragment ligation reaction, generating construct PBS-GR28b-del. The Gr28b-del fragment was released from PBS-Gr28b-del by AscI + AvrII digestion, and ligated into AscI + AvrII digested pTargeting-RMCE-insulated, generating the final *Gr28b* targeting plasmid.

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