**Fig. 4 Targeting and conditional modification of the mir-106a~363 cluster**

a. The mir-106a~363 cluster was removed as a 932bp deletion using the Puro\_tk cassette. The predicted sizes of PCR products generated by PCR (internal to the targeting vector) across the mir-106a-363 cluster or modified alleles are indicated in brackets. An RMCE cassette containing a cloned genomic sequence for the wildtype mir-106a~363 cluster (pMA_F3RoxNeoRox_miR106a~363_LoxPFRT) was inserted into the targeted locus by co-transfected with PGK-FlipO followed by FIAU and G418 selection. The PGK-Neo<sup>R</sup> deletion was by Dre recombinase transfection. Cre deletion successfully deleted the conditional allele, although the mir-106a~363 clone analysed in this figure was derived by Cre deletion of the mir-106a~363<sup>Puro</sup>tk clone.  

b, Long-range PCR between primers specific for the selection cassette and the region external to the 5’ and 3’ homologous arms of the targeting vector.  
c, PCR between primers (internal to the targeting vector) specific for flanking sequences of mir-106a~363 locus and the allelic variants.  
d, Comparative quantitative PCR for miRNAs within the mir-106a~363 cluster and miR-294 as a control. The TaqMan MicroRNA assays used are color coded. n=3; error bars show SD.

**ONLINE METHODS**

**Vector design** The miRNA targets were selected using the miRBase registry (http://www.mirbase.org)<sup>5,34</sup>. Manually annotated files of predicted sequences for all of the targeting vectors and targeted alleles were generated using the Vector NTI software (Invitrogen). The 50bp homology arms for vector construction were selected using the WTSI high throughput gene targeting (htgt) software (http://www.sanger.ac.uk/htgt/welcome). Oligonucleotides homologous sequences with appended PCR primer sequences were ordered from Sigma-Genosys Ltd.
As standard the vectors were designed to have homology arms of 5.5kb (+/- 500bp) and 3.3kb (+/-300bp), and with deletions as small as possible surrounding the region corresponding to the stem-loop sequence of the pre-miRNA.

**Recombineering fragment preparation** The neomycin/kanamycin selection cassette pHpmK3 (GenBank accession JN195814) was derived from the plasmid pL452. The flanking recombinase recognition sites were modified to include an FRT and F3 inverted relative to each by cloning of annealed complementary oligonucleotides. PL611 was used as vector backbone for retrieval of the targeting vector from the BAC clone. The puromycin/thymidine kinase selection cassette pHpmF1 (GenBank accession JN195815) was made by cloning the bacterial EM7 promoter within a 465bp AgeI/NcoI fragment and subcloned at the same restriction sites 5’ to the puromycin resistance gene in YTC37.

In preparation for recombineering (i) pHP-mK3 was digested with NotI and Sall to release a FRT-loxP-PGK-EM7-Neo-bpA-F3-loxP fragment, (ii) pL611 was digested with BamHI and EcoRI and (iii) pHP-mF1 was digested with HindIII and XhoI to release a PGK-EM7-Puro-Dtk-bpA fragment. The digests were electrophoresed and the appropriate fragments were excised from the agarose gel and the digests were and electrophoreses were repeated. The final eluted products were test transformed into electrocompetent DH10B (Invitrogen), spread on appropriate selective agar plates, to check for plasmid contamination. The pHP-mK3 and pPL611 isolated fragments were used as templates for PCR of recombineering fragments using the same conditions as previously described except that the annealing temperature for PCR of PL611 was 46°C, 40 cycles were used and the PCR products were purified by passing the reaction through an ultrafiltration membrane (Qiagen).

**Recombineering reaction.** Bacterial artificial chromosome (BAC) clones from the indexed mouse C57BL/6J libraries RPCI23 or RPCI24 were selected using the htgt software. Four different BAC clones were selected for each targeting vector construction. High throughpout targeting vectors production was modified from the method described using the λ defective prophage containing plasmid pSim18. However, PCR fragments for recombineering were purified by using a 96 well ultrafiltration membrane (Qiagen) by washing three times with and then resuspending in HPLC grade water. The recombineering fragments were electroporated into bacteria in 96 well format using an ECM630 (BTX Harvard Apparatus).

**Kanamycin/neomycin selective targeting vectors (Neo-TV)** were created entirely in liquid phase. Initially the HpmK3 cassette was PCR amplified with 20bp or 21bp primers appended respectively to 50bp gene specific recombineering oligonucleotides (designated as U and D). The HpmK3 cassette appended PCR product was recombineered into the BAC clone thus deleting the miRNA target. The targeting vector insert was retrieved by recombineering into the PL611 vector backbone PCR amplified with 22bp and 20bp primers appended to 50bp gene specific recombineering oligonucleotides (designated as G5 and G3). Following miniprep and retransformation the Neo-TV were restriction
digested and sequenced and those that passed quality control were archived as frozen bacterial glycerol stocks.

**PuroΔtk selective targeting vectors (Puro-TV)** were created by a third round of recombineering in which the PGK-EM7-PuroΔtk-bpA fragment was inserted into the targeting vector in place of the kanamycin cassette using the promoter (PGK-EM7) and 3’UTR (bpA) sequences as homology arms. Following 2 days growth in puromycin-TB (Autogen Bioclear) and 50 μg/ml carbenocillin the bacteria were minipreped and plasmid retransformed. The bacteria were spread on puromycin-AGAR (Autogen Bioclear), 50μg/ml ampicillin selective plates and grown for 2 days at 37°C. Approximately 12 puromycin resistant *E.coli* colonies were picked and individually streaked on a 20μg/ml Kanamycin, 50μg/ml ampicillin Agar plate and a puromycin, 50μg/ml ampicillin plate. *E.coli*. Clones that grew on puromycin but failed to grow on kanamycin plates were considered as candidates for as puromycin selective targeting vectors. They were picked and arrayed in 96 deep well plates with 2xTY containing 50μg/ml ampicillin, and were inoculated into 4 replicates for overnight culture. The overnight cultures of puromycin resistant clones were pooled and the pelleted 4ml of bacterial growth were minipreped using 96 Well TurboPrep columns (Qiagen). The DNA preps were restriction digested and submitted for sequence verification as previously described. Individual targeting vector clones that had passed quality control were picked into 850ml of Puromycin TB with 50μg/ml carbenocillin to create a “Puromycin Master-Plate” from which a glycerol stock was made. The Puro-TV were maxipreped from 500ml of 2xTY with 50μg/ml Ampicillin using the Qiagen kit to provide a stock of DNA for ES cell targeting.

**ES cell culture** JM8.F6 and JM8.A3 cells are C57Black/6N derived feeder dependent ES cell clones that were grown on neomycin and puromycin resistant SNL7 fibroblasts in DMEM media containing 15% Fetal Calf serum as previously described. The Puro-TV were linearized by FseI or Ascl digest. Linearized DNA was ethanol precipitated, washed with 70% ethanol, air dried and 20μg was electroporated into 1x10^7 ES cells (Biorad) 230V, 500μF. Selection with Puromycin (3μg/ml) was begun after 24h and continued for a further 8 days with daily media changes. As a routine 32 colonies were picked for each transfection, although this was often increased to 48 or 96 colonies for where initial transfections yielded very few or no positive clones so that repeated transfection was required. The ES cell clones were expanded and frozen in 96 well formats in 10% DMSO and 50 % Fetal Calf Serum and genomic DNA was extracted from a replicate. Following primary screen PCR amplification positive clones were thawed into a single well of a 24 well plate and then expanded up to the equivalent of a single well of a 6 well plate. Finally each ES cell clone was frozen into Matrix vials (Thermo). In general up to six independent clones of each targeted miRNA gene were archived.

ES cells were differentiated using the embryoid body method as previously described. Approximately 600 ES cells were suspended in 20μl hanging drops in differentiation medium. After 2 days the aggregated cells were washed into bacterial dishes with differentiation medium and cultured for a further 4 days. The embryoid bodies were then seeded onto gelatinized tissue culture plates.
**PCR genotyping of ES cell clones** As a routine ES cell clones were genotyped by long range PCR. PCR primers were selected using the Primer 3 software (http://frodo.wi.mit.edu/primer3/). Standard primers were selected at the 5’ end (LR2: 5’-tctagagaattagacactctggtc-3’) and 3’ end (LR3: 5-attataagatatgatgtagttagaattggtc-3’) end of the selection cassette. Primers (LR1 and LR4) were chosen that lay external to the homology arms so that appearance of the expected length of PCR product was indicative of a correctly targeted ES cell clone. Either the Sequa Prep (Invitrogen) or Longamp (NEB) kits were used for the long range PCR. During the primary genotyping screen from 96 well plates only the product for the short arm was amplified. During the secondary screen following amplification of the ES cell clones the short arm PCR was repeated in order to confirm that the correct ES cell had been expanded, but in addition the clone was analysed for the correct long arm PCR product.

**Post targeting modification.** Puro/Δtk selection cassette removal was achieved in tissue culture by transfecting 1x10⁶ ES cells with 20μg of pCAG-Cre grown for 5 days, reseeded at 3x10⁵ per 10 cm diameter dish and selected with FIAU (200nM) for a further 10 days. Short range PCR protocol was developed to amplify across the miRNA deleted region both to verify removal of the selection cassette from the Cre treated ES cells and in order to genotyping of knockout mice. The short-range primers for short range PCR of the mir-210 deleted locus were 5'-AGGTGAAATAGAAGGGTTACAAGGTT-3' and 5'-AACCCTAATACACCTAAGAAGAGTTCC-3' yielding products of 527bp for the wildtype and 443 bp for the deleted mir-210 alleles.

The polylinker and recombinase sites within the plasmid pMA_F3RoxNeoRoxLoxPFRT (GenBank accession JN195816) were created by de novo synthesis by Geneart AG. The pTd-tomato fluorescent reporter cDNA was cloned as a BamHI/NotI fragment into the polylinker of pMA_F3RoxNeoRoxLoxPFRT at the BamHI and NotI restriction sites. For the creation of conditional constructs PCR products were amplified from appropriate BAC clones using Extensor Hi-Fidelity PCR Master Mix (Thermo Scientific) and primers with appended restriction enzyme sites. One copy of the mir-106a~363 cluster was amplified with the primers 5’-AAAAAAAAGGCGCGCCCATCCTATGAGATGCTTGCGCC-3’ and 5’- AAAAAAAAAAAGCTTGCACTTGAAGACGGCTTTCCAC-3’, digested with Ascl and HindIII and cloned into the Ascl/HindIII sites of pMA_F3RoxNeoRoxLoxPPolyFRT to generate pMA_F3RoxNeoRoxLoxP_mir-106a~363_FRT.

For RMCE 27, 40 1x10⁷ ES cells were co-transfected with 20μg pHpmK3 pMA_F3RoxNeoRoxLoxPFRT and 40μg of the pPGK-FLPo expression vector29, 41 (available from www.addgene.org) or pBluescript (pBS) (Agilent Technologies), as negative control, and seeded upon a 10cm diameter dish. Selection was for 4 days with G418 (200μg/ml) followed by alternative selections (i) continued G418 for a further 6 days, (ii) FIAU + G418 for a further 6 days, (iii) trypsinizing and re-seeding single cells suspension on fresh 10cm dish with G418 + FIAU selection for a further 10 days. To catalyze recombination of Rox sites pCAGGS-Dre (20μg) was transfected and cells
plated at low density. The status of the ES cells as to whether the Neomycin or Puromycin cassettes were present in the locus were determined by PCR either across the cassette insertion site, or using neomycin or puromycin cassette specific and locus specific primers to amplify to across the insertion junctions. The primer sequences for amplification across the mir-21 locus were 5’-TGTTCATTTTGGTTCTGTTAGG-3’ and 5’-CCATGATGCTGGATATGGTGGTTAAT-3’. The primer sequences for amplification across the mir-290~295 loci were 5’-CTTTGATTTCCAGGGTTTCTTCT-3’ and 5’-TCCCAAAAGGGTCCCTATTACTTGTTT-3’. The primer sequences for amplification across the mir-106a~363 loci were 5’-TTAGACGGAGGGAGGAGTCCAAAATC-3’ and 5’-CACTCCAAATGTGTCAAGCAATG-3’.

**Fluorescence imaging.** Images of fluorescent reporter expression were taken using a Leica inverted microscope equipped with a DFC420 camera. Images were processed using Adobe Photoshop CS2.

**Expression analysis.** The expression levels of the miRNAs within the mir-106a~363 locus were determined using the comparative C\textsubscript{T} method using the RNA from the parental JM8.A3 ES cell clone as calibrator. RNA was isolated using the miReasy kit and reverse transcription and quantitative PCR was performed using the TaqMan MicroRNA reverse transcription kit and MicroRNA assays (Applied Biosystems) for mmu-miR-106a, mmu-miR-18b, hsa-miR-20b, hsa-miR-19b, hsa-miR-92 has-miR-363 with snoRNA202 as an endogenous control, and mmu-miR-294 as a control non-targeted miRNA.

**Blastocyst micro-injection.** ES cell clones were micro injected as previously described into C57BL/6J-Tyr\textsuperscript{c2} blastocysts in the case of JM8.F6 and C57BL/6N blastocysts in the case of JM8.A3. Mouse production and husbandry was conducted in accordance with UK Home Office regulation.