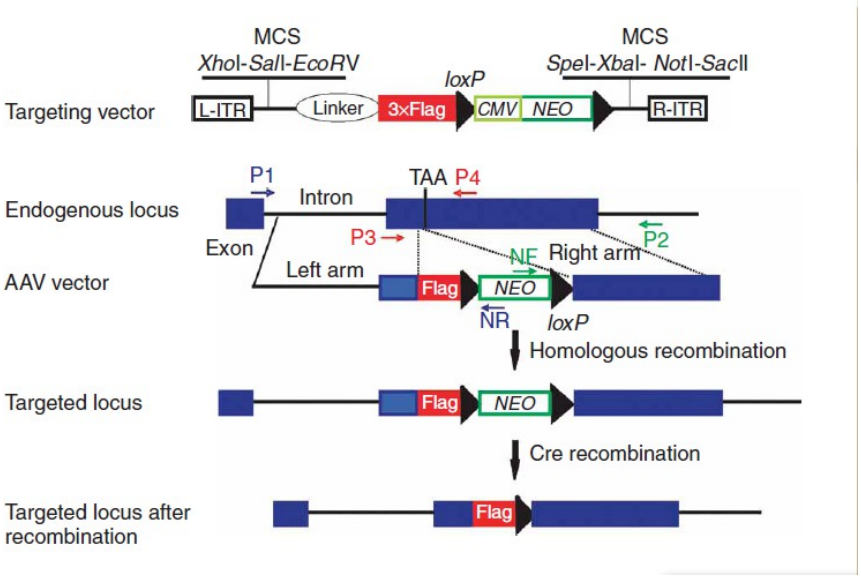
Supplementary Methods

Construction of knock-in targeting vectors

A ~1kb genomic fragment upstream of the stop colon was PCR amplified to be used as the left homologous arm and a ~ 1kb genomic fragment downstream of the stop colon was PCR amplified to be used as the right homologous arm. We first constructed a pAAV-lox P-Neo vector by ligating a fragment containing a neomycin resistance gene cassette flanked by two Lox P sites and left and right multiple cloning sites with the AAV vector consist of L and R-ITRs, bacterial replication origin and an ampicillin resistance gene. We then PCR amplified the 3xFlag tag with linker sequences from the pMZ3F plasmid using primers 5'-ggaattcgaaaagagaagatggaaa -3' and 5'-ggaattctcactacttgtcatcgtc-3'. The PCR products were digested with Eco RI and inserted in the pAAV-Lox P-Neo vector to construct a plasmid called pAAV-LoxP-Neo-3xFlag. For each target gene, a PCR fragment (~1-kb) extending from intron to the last exon before the stop codon was amplified from genomic DNA and cloned in-frame with the 3xFlag tag sequence as the left homologous arm. A PCR fragment from the sequence after the TGA stop codon extending to the 3' end of the targeted genes was also amplified from genomic DNA to be used as the right arm. Primers used to amplify homologous arms for each gene loci are available upon request.



Schematic of targeting vector and strategy (NEO-loxP-3Flag) vector.(Reproduced from Zhang et al, Nature Methods, Vol 5, 2. 2008).

Packaging of rAAV targeting constructs

The targeting AAV viruses were packaged in 293T cells (a T75 flask at 70% confluence) by transfecting equal amounts of the targeting vector, pHelper and pRC plasmids (3 µg each).

The targeting construct made above (2.5 µg) was mixed with pAAV-RC and pHelper plasmids (2.5 µg of each) from the AAV Helper-Free System (Stratagene) and transfected into HEK 293T cells (ATCC) using Lipofectamine (Invitrogen). The DNA was dissolved in Opti-MEM reduced-serum media (Invitrogen) to a total volume of 750 µl (i.e. if volume of DNA was 50 µl, volume of Opti-MEM was 700 µl). Similarly, 54 µl of Lipofectamine was dissolved in Opti-MEM to a total volume of 750 µl. The two tubes were combined and the DNA–Lipofectamine mix was incubated at room temperature for 15 min. HEK 293T cells at 70–80% confluence in a 75 cm2 flask were washed with Hank's Balanced Salt Solution (HBSS, HyClone) and then 7.5 ml Opti-MEM was added. To this, the 1.5 ml DNA–Lipofectamine mixture was added dropwise, and the cells were incubated at 37°C for 3–4 h. The Opti-MEM was replaced with 293 growth medium and the cells were allowed to grow for 72 h prior to harvesting virus. Virus was harvested according to the AAV Helper-Free System instructions with minor modifications. Briefly, the media was aspirated from the flask and the 293 cells were scraped into 1 ml of phosphate-buffered saline (Invitrogen), transferred to a 2 ml microfuge tube, and subjected to three cycles of freeze–thaw. Each cycle consisted of 10 min freeze in a dry ice–ethanol bath, and 10 min thaw in a 37°C water bath, vortexing after each thaw. The lysate was then clarified by centrifugation at 12 000 r.p.m. in a microfuge to remove cell debris and the supernatant containing rAAV was divided into three aliquots of 330 µl each and frozen at –80°C. The rAAV preparation generally contained ~3 x 108 genome particles/ml.

AAV titration assay.

The rAAV was titrated by real-time PCR. Briefly, 10 µl of rAAV stock was mixed with 10 µl of

salmon sperm DNA (1 mg/ml) and 20 µl of 2 M NaOH. The mixture was then incubated at 56oC for 30 min and then neutralized by adding 19 µl of 2 M HCl. The rAAV lysates was diluted 10 folds and 1µl of dilutant was mixed with 2 µl of 5 µM forward primer (5'-TGAATGAACTGCAGGACGAG-3'), 2 µl of 5 µM reverse primer (5'-CAATAGCAGCCAGTCCCTTC-3') and 12.5 µl of SYBR green PCR mix in a total volume of 25 µl. To calculate the copy number, the rAAV-NEO targeting vector was serially diluted in the range of 103 to 106 copies per µl as the real-time PCR standards. Gene targeting and isolation of recombinant cell lines. Cells (HCT116 and RKO) were grown in 25cm2 flasks and infected with rAAV when 75% confluent (~3x106). At the time of infection, medium was aspirated and 4 ml of medium containing 50–250 µl of rAAV lysate (0.2-1 x 108 viral particles) was added to each flask. Cells were washed with PBS buffer and detached with trypsin (Invitrogen) 24 h after infection. Cells were replated in eight 96-well plates in medium containing geneticin (Invitrogen) at a final concentration of 1mg/ml. Drug resistant colonies were grown for 10-14 day (~3, 000 G418 resistance clones/T25 flask). At the end of the selection period, genomic DNA was extracted from single clones growing in 96-well plates using the Lyse-N-Go reagent (Pierce). Locus-specific integration was assessed by PCR using a primer that annealed outside the homology region and another that annealed within neo. Positive clones were confirmed by PCR across both homology arms. Primers used for screening of individual targeted loci are available upon request.

Cre-mediated excision of the drug resistance marker in targeted cells.

To remove the drug resistance marker from correctly targeted clones, cells were infected with an adenovirus that expresses the Cre recombinase, as described2. Cells were plated at limiting dilution in nonselective medium, 24 h after infection. After 2 weeks, single cell clones were plated in duplicate and 0.4 mg/ml geneticin was added to one set of wells. After 1 week

of growth, clones that were geneticin-sensitive were expanded for further analysis.