SUPPLEMENTARY DATA FOR:

High-efficiency genome editing via 2A-coupled co-expression of fluorescent proteins and zinc finger nucleases or CRISPR/Cas9 nickase pairs

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Supplementary Table S1. DNA sequence of the zinc finger moieties of the *RSK2*, *RSK4* and *PRMT1* ZFNs.

RSK2 ZFNL
5'-GGTACCCGCCGCTATGGCTGAGAGGCCCTTCCAGTGTCGAATCTGCATGCGTAAGTT
TGCCGACCGCTCCAACCGCATCAAGCATACCAAGATACACACGGGCGAGAAGCCCTTC
CAGTGTCGAATCTGCATGCGTAACTTCAGTCAGTCCGGCGCCCTGGCCCGCCACATCCG
CACCCACACCGGCGAGAAGCCTTTTGCCTGTGACATTTGTGGGAGGAAATTTGCCACCT
CCTCCAACCGCAAGACCCATACCAAGATACACACGGGCGGAGGCGGATCTCAGAAGC
CCTTCCAGTGTCGAATCTGCATGCGTAACTTCAGTCAGTCCTCCGACCTGTCCCGCCAC
ATCCGCACCCACACCGGCGAGAAGCCTTTTGCCTGTGACATTTGTGGGAGGAAATTTG
CCCAGTCCGGCTCCCTGACCCGCCATACCAAGATACACCTGCGGGGATCC-3'

RSK2 ZFNR
5'-GGTACCCGCCGCTATGGCTGAGAGGCCCTTCCAGTGTCGAATCTGCATGCGTAACTT
CAGTCAGTCCGGCGCCCTGGCCCGCCACATCCGCACCCACACCGGCGAGAAGCCTTTT
GCCTGTGACATTTGTGGGAGGAAATTTGCCCGCCTGGACAACCGCACCGCCCATACCA
AGATACACACGGGCGGACAACGGCCCTTCCAGTGTCGAATCTGCATGCGTAACTTCAG
TCGCTCCGCCGCCCTGTCCCGCCACATCCGCACCCACACCGGCGAGAAGCCTTTTGCCT
GTGACATTTGTGGGAGGAAATTTGCCCAGTCCGGCGACCTGACCCGCCATACCAAGAT
ACACACGGGCGGAGGCGGATCTCAGAAGCCCTTCCAGTGTCGAATCTGCATGCGTAAC
TTCAGTACCTCCGGCTCCCTGTCCCGCCACATCCGCACCCACACCGGCGAGAAGCCTTT
TGCCTGTGACATTTGTGGGAGGAAATTTGCCCAGTCCGGCCACCTGTCCCGCCATACCA
AGATACACCTGCGGGGATCC-3'

RSK4 ZFNL
5'-GGTACCCGCCGCTATGGCTGAGAGGCCCTTCCAGTGTCGAATCTGCATGCGTAACTT
CAGTGAGCGCGGCACCCTGGCCCGCCACATCCGCACCCACACCGGCGAGAAGCCTTTT
GCCTGTGACATTTGTGGGAGGAAATTTGCCCAGTCCGGCCACCTGTCCCGCCATACCAA
GATACACACGGGCGAGAAGCCCTTCCAGTGTCGAATCTGCATGCGTAAGTTTGCCCAG
TCCGGCGCCCTGGCCCGCCATACCAAGATACACACGCACCCGCGCGCCCCGATCCCGA
AGCCCTTCCAGTGTCGAATCTGCATGCGTAACTTCAGTCGCTCCGACAACCTGTCCGAG
CACATCCGCACCCACACCGGCGAGAAGCCTTTTGCCTGTGACATTTGTGGGAGGAAAT
TTGCCCAGCGCTCCAACCTGAAGGTGCATACCAAGATACACCTGCGGGGATCC-3'

RSK4 ZFNR
5'-GGTACCCGCCGCTATGGCTGAGAGGCCCTTCCAGTGTCGAATCTGCATGCGTAAGTT
TGCCCAGCGCTCCAACCTGGACTCCCATACCAAGATACACACGGGCGAGAAGCCCTTC
CAGTGTCGAATCTGCATGCGTAACTTCAGTCGTAGTGACGTCCTGAGCGCACACATCCG
CACCCACACAGGCGAGAAGCCTTTTGCCTGTGACATTTGTGGGAAGAAATTTGCCGAC
AGGAGCAACCGCATAAAGCATACCAAGATACACACGGGATCTCAGAAGCCCTTCCAGT
GTCGAATCTGCATGCGTAACTTCAGTCAGTCCGGCGACCTGACCCGCCACATCCGCACC
CACACCGGCGAGAAGCCTTTTGCCTGTGACATTTGTGGGAGGAAATTTGCCACCTCCG
GCAACCTGACCCGCCATACCAAGATACACCTGCGGGGATCC-3'

PRMT1 ZFNL
5'-GGTACCCGCCGCTATGGCTGAGAGGCCCTTCCAGTGTCGAATCTGCATGCGTAACTT
CAGTGACCGCTCCAACCTGTCCCGCCACATCCGCACCCACACCGGCGAGAAGCCTTTT

GCCTGTGACATTTGTGGGAGGAAATTTGCCCGCTCCGACGCCCTGACCCAGCATACCA
AGATACACACGGGCGGAGGCGGAAGCCAACGGCCCTTCCAGTGTCGAATCTGCATGCG
TAACTTCAGTACCTCCGGCAACCTGACCCGCCACATCCGCACCCACACCGGCGAGAAG
CCTTTTGCCTGTGACATTTGTGGGAGGAAATTTGCCACCTCCGGCTCCCTGACCCGCCA
TACCAAGATACACACGGGCGAGAAGCCCTTCCAGTGTCGAATCTGCATGCGTAAGTTT
GCCACCTCCGGCCACCTGTCCCGCCATACCAAGATACACCTGCGGGGATCC-3’

PRMT1 ZFNR
5’-GGTACCCGCCGCTATGGCTGAGAGGCCCTTCCAGTGTCGAATCTGCATGCGTAACTT
CAGTCGCTCCGCCAACCTGTCCGTGCACATCCGCACCCACACaGGCGAGAAGCCTTTTG
CCTGTGACATTTGTGGGAGGAAATTTGCCGACCGCGCCAACCTGTCCCGCCATACCAA
GATACACACGGGCGGACAACGGCCCTTCCAGTGTCGAATCTGCATGCGTAAGTTTGCC
CGCTCCGACAACCTGCGCGAGCATACCAAGATACACACGGGCGAGAAGCCCTTCCAGT
GTCGAATCTGCATGCGTAACTTCAGTGAGCGCGGCACCCTGGCCCGCCACATCCGCAC
CCACACCGGCGAGAAGCCTTTTGCCTGTGACATTTGTGGGAGGAAATTTGCCACCTCCT
CCAACCGCAAGACCCATACCAAGATACACCTGCGGGGATCC-3’

Supplementary Table S2. Sequence of plasmid and ssODN donors. ZFN and gRNA target sites are underlined. Protospacer-adjacent motifs (PAMs) are indicated in red. Mutations to be introduced into the genomic loci are in bold. Of these, codon conversion mutations are lower case bold. Diagnostic restriction endonuclease sites resulting from the mutagenesis are indicated in italics. Other mutations introduced in ZFN and gRNA target sites aim to prevent re-targeting of the locus once the donor has been knocked in. The size of the donors is given as numbers of nucleotides in parentheses after the name of the donor, and '**S**' or '**AS**' stands for ssODN used as a sense or an antisense donor.

ssODN donors:

RSK4 Cys443Val/BamHI (114, **S**)
5'-TTGGTGAAGTATATGAATTGAAGGAGGATATTGGTGTT*GGATCC*TACTCTGTT**gtt**AA
GCGATGCATACATGC**T**ACTACCAACATGGAATTTGCAGTGAAGGTATTGTCTCTGA-3'

RSK2 Cys436Val/SfcI (122, **S**)
5'-GTAAAAGAAGATATTGGAGTTGGCTCCTACT*CTGTA**g**tt*AAGAGATGTATTCATAAAG
CCACAAACATGGAGTTTGCTGTGAAGGTCAATTTTTTTTATTTAAAATGCAATTCATAC
AGTTC-3'

PRMT1 ScaI (75, **S**)
5'-GGCTACTGCCTCTTCTACGAGTCCATGCTCAACAC*AGTACT*CTATGCCCGGGACAAG
TGGCTGGTGAGGCCCCAG-3'

EMX1 gRNA 1/2 EcoRV (119, **AS**)
5'-CCTGGCCAGCAGCAAGCAGCACTCTGCCCTCGTGGGTTTCTGGATCCCCACCCTAGT
CATTGGAGGTGACGTC*GATATC*TTCCCCATTGGCCTGCTTCGTGGCAATGCGCCACCGG
TTG-3'

EMX1 gRNA 1/3 EcoRV (104, **AS**)
5'-GCAGCACTCTGCCCTCGTGGGTTTGTGGTTGCCCACCCTAGTCATTAGATGTCACGT
C*GATATC*TTCCCCATTGGCCTGCTTCGTGGCAATGCGCCACCGGTTG-3'

Plasmid donor:

RSK2 Cys436Val/BamHI (1680)
5'-CATGGATGTCTCATATCAGTATAGCCAAGGGCAGCACAACTTTCCTCAGCCTGTTTA
TGGAATCAGTAAGTTGCTGTTCATCATCTACTGTGTGTTATTAGTCCCTAACCCATGAC
GAGCTTCTTTAGCATAAGTTGTGTAAGGCTATGACTTCAGGGTGAAGAAAGGAGGGAG
TGGCTACTGAAAGTCCAAAATGGCGATAACAGGTATTGCTTGCAAATACATTATTTTTA
TTTAATCTTCACCATTACCCTCTGAGAAAATACAGGCTCAAAGTTACATAGCCTGTAAG

TGGTGTACCTGAGACAAGAATTCAGATATTCTAATTCTGAATCCTCCTAAATCCTTTCC
TTCTATACCATAGGTTTCAAACTTCTCATTTGCAAAGGTACTTGAAAACCTATAGCCGA
CTGGGGACTGGAATAAGGCTGAATCACAGACAAAGATGTAGGCCCCATATCAAGTAGC
TCTGCTTTTTCTGTTGTGCAAATTCAATTTTTGTTTGTAACATAAGCTTTTTTAAGTCAT
ATTTCTTGCTGTACACTTCATTGTAAACACTTTCAAAATCACTTGTTTGTAGAATAATAA
GTACTATAAAGATGTATTTTTATTCTTATTCATTTATATGTAGGGCATCAACAATGCAT
GCTGAGTAACATACTTCCCTATTATAAAATGCACTAAAGGCATTATGGGACTCTTCACC
AATTTATAATTTTTTGTAGCAGTTACACAGGAACAGTATTCAGTTTACTGATGGATATG
AAGTAAAAGAAGATATTGGAGTT*GGATCC*TACTCTGTT**gtt**AAGAGATGTATACATAAA
GC**A**ACAAACATGGA**A**TTTGC**T**GTGAAGGTAAATTTTTTTTATTTAAAATGCAATTCATA
CAGTTCTTGTTCATGCATGTCAGTACCAGTTAAAAATTACACTCCCCTTGTTGTTAAAA
GTGCCTTTTGTTATAAAAAAGTTAAATATCTGGCTAGTGATCTTCAGAGATCTTAATCT
AGAACCCTGTGAGCTAAAGGTAAGGTGGTTATATATCTAGTTTTCCCAGAGCAGTAGC
AGTTTACACCTCAAGTGATTTTTTTTCTTTTTTTACCTCAAGTGATTTTTAAAGTATCTTT
TTACTCTGAGAAGTCCCCATTTTATGCTCAGGGTGTCAGCAAATTCCTCAAAATTGTGT
GCAAAATTTTGTATGTTTACATCTTCTCTGGCAAAAAGTTCATACCTTTCTTTGCATCTA
AAAGGATCTGGGAACTAATGAATAAAAACCACTATCATAAAATTCGTTATCATTGGCT
GTTTTAATTTCATCAAGTATCCTAAGACAGAATTTTCTAAGCCAAAAACCAAGGAACCT
AGCTCTACGATAAAGTTTGAATTTCAAACCTTTAGTCGTAAGTGAACAAGCACTATGTT
CAAGTCTCTTTTCACTTCTTGGAATAGTAACCTCTGCTCTCCAATCTCAAGCAATTCAA
ACAATGTCTAAAGCTTTCAACTTGTTTGAACCTTAGGACTGGTTTATAAATATACATAG
CTCATGTTTAGAGAGATAGTGACATTAGTTGTTATCACTCTTCTAAACTTAGATGTAAC
TGCTTTAAAGTCGTCAGTGTCTATAGGGCTTTCTGTCAGATAGAAGGATGAACCATAAA
AATACATTTAATTTGCTTCATTGGAAGGA-3’

Supplementary Table S3. gRNA target site sequences.

Target site	Gene	Target sequence 5' to 3'	PAM 5' to 3'	Target strand
1*	*EMX1*	GACATCGATGTCCTCCCCAT	TGG	-
2†	*EMX1*	GGGCAACCACAAACCCACGA	GGG	+
3‡	*EMX1*	GTCACCTCCAATGACTAGGG	TGG	+
	KRAS	TAGTTGGAGCTGGTGGCGT	AGG	+
§	*VEGFA*	GACCCCCTCCTCCCCGCCTC	CGG	-

**EMX1* target site 20 in reference (12).
†*EMX1* target site 22 in reference (12).
‡*EMX1* target site 21 in reference (12).
§*VEGFA* target site 2 in reference (36).

Supplementary Table S4. Sequence of QuickChange primers used to generate Cas9-D10A nickase mutant and to swap *EMX1* gRNA target sequences into pCMV-Cas9-GFP.

Gene	Primer sequence (the sense sequence is shown). For the gRNA swapping primers this means that the U6 promoter is located upstream of the gRNA, given in red. The sequences that aneal to pCMV-Cas9-GFP are underlined. Diagnostic restriction enzyme sites are in italics	Introduced mutation/gRNA	Diagnostic QuickChange restriction enzyme site
Cas9	GACAAGAAGTACAGCATCGGCC*TGGCCA*TCGGCACCAACTCTGTGGGCTG	D10A	MscI
EMX1	CTTGTGGAAAGGACGAAACACCGGAC*ATCGAT*GTCCTCCCCATGTTTTAGAGCTAGAAATAGCAAGTTAA	gRNA 1*	ClaI
EMX1	CTTGTGGAAAGGACGAAACACCGGGGCAACCACAAACC*CACGAG*TTTTAGAGCTAGAAATAGCAAGTTAA	gRNA 2†	BssSI
EMX1	CTTGTGGAAAGGACGAAAC*ACCG**GT*CACCTCCAATGACTAGGGGTTTTAGAGCTAGAAATAGCAAGTTAA	gRNA 3‡	AgeI

**EMX1* target site 20 in reference (12).
†*EMX1* target site 22 in reference (12).
‡*EMX1* target site 21 in reference (12).

Supplementary Table S5. Primers, PCR conditions, diagnostic RFLP sites and diagnostic fragment sizes for RFLP and CEL-I assays. Primers used for sequencing of PCR products are in bold.

Target	Forward primer	Reverse primer	Annealing temp (°C)	PCR additives	PCR product size (bp)	Diagnostic RFLP site	Diagnostic fragment sizes (bp)*
ZFN on-target analysis							
PRMT1	TAGAAGGGTTCATGGCTTCTGC	GCTACGGATTTCCTGTTCCTACG	62	1.6 mM $MgCl_2$	483	ScaI	251+232
RSK2 **(with ssODN donor)**	CCTTCTATACCATAGGTTTCAAACTTC	ATGGAGGCAAGACACATCCT	61	1.6 mM $MgCl_2$	1510	SfcI	(55+244)† +398+813
RSK2 **(with plasmid donor)**	CCTTCTATACCATAGGTTTCAAACTTC	ATGGAGGCAAGACACATCCT	61	1.6 mM $MgCl_2$	1510	BamHI	1068 + 442
RSK4	ATGTACTAAGCAATGAGGATGCAG	AACATTATATCCTATTTCACTGAAATTGTAC	60	1.6 mM $MgCl_2$	1207	BamHI	840 + 367
ZFN off-target analysis							
RSK2	AATTTGCTGACACCCTGAGC	CCAAAATGGCGATAACAGGT	63	1.6 mM $MgCl_2$	977	-	333 + 644
RSK2 **off-target 1**	**TCTTCTGAAGGGTCTCGCTTAC**	TGCTTTATTATGTTGCCTGTGG	63	1.6 mM $MgCl_2$	833	-	292 + 541
RSK2 **off-target 2**	TTCCTTTCCTTGCCAAATTCTA	**TCTTCTTCTCTTGGTCCCTCTG**	62	1 M betaine, 1.6 mM $MgCl_2$	620	-	134 + 486
RSK2 **off-target 3**	**CGTAACCGCAGTCGTATCCA**	TGACGTTGTCATGCACCAGA	62	0.8 mM $MgCl_2$	976	-	199 + 777
RSK2 **off-target 4**	**TCAGTGTGGGAAGGACTTGC**	GAGAACCAGAGGAGGCAGTG	62	1 M betaine, 1.6 mM $MgCl_2$	621	-	289 + 332
RSK2 **off-target 5**	ATGTTGGCAGAAGGGATTTCTA	AATTAGCAAAAGCATCACAGCA	62	1.6 mM $MgCl_2$	1163	-	474 + 689
RSK2 **off-target 6**	TGCAACCATACAGAGCTCCT	TGTTGTCCCATGAAAGCAGC	63	1.6 mM $MgCl_2$	692	-	204 + 488
RSK2 **off-target 7**	TGATGAGCGAGACCTGTCTAAA	CTAAGCTTGTTTTCCTGCGACT	62	1.6 mM $MgCl_2$	745	-	124 + 621
RSK2 **off-target 8**	GTGGTGGGTGAAAGATTAGCTC	TTTCTCAGTTTGAGTGGGGAAT	62	1.6 mM $MgCl_2$	827	-	212 + 615
RSK2 **off-target 9**	ACAGTTCACATGCTTTGCTCAT	CTAGACCTCTCTGGGCCTGTAA	62	1.6 mM $MgCl_2$	641	-	130 + 511
RSK2 **off-target 10**	AGGAAGCAAGTTGTCAGGAGAG	CTGAGGATGTACGAGCACAGAC	62	1M betaine, 1.6 mM $MgCl_2$	1077	-	327 + 750
RSK4	TCTTCTGAAGGGTCTCGCTTAC	TGCTTTATTATGTTGCCTGTGG	63	1.6 mM $MgCl_2$	833	-	265 + 568
RSK4 **off-target 1**	**AATTTGCTGACACCCTGAGC**	CCAAAATGGCGATAACAGGT	63	1.6 mM $MgCl_2$	977	-	358 + 619
RSK4 **off-target 2**	**CCAGTTGCCGTCCATTAGGT**	CAATGTTGATGCCGGTGGTG	62	1M betaine, 1.6 mM $MgCl_2$	831	-	283 + 548
RSK4 **off-target 3**	TGAATCACCAACAAGCCAGTG	**CGTTCAAATGGCACTCCACC**	63	1.6 mM $MgCl_2$	928	-	317 + 611
RSK4	**CAGCAAATTGCCAAGCAGTA**	TTCTGAAAGGGTGGGATCTG	62	1.6 mM	822	-	327 + 485

off-target 4				$MgCl_2$			
***RSK4* off-target 5**	ACAAGGTCCCTGGCATAGTG	ATTGGGGCCCATTCTAATTC	62	1 M betaine, 1.6 mM $MgCl_2$	1132	-	293 + 839
***RSK4* off-target 6**	CTGCACATCCAACTGCTATCG	CTCTGCTTCCCCTTTGGTTGA	62	1.6 mM $MgCl_2$	631	-	286 + 345
***RSK4* off-target 7**	AGCTCTGGCTCTAGCAAACG	GTGCTCTTGGGAAAAGCAAG	62	1M betaine, 1.6 mM $MgCl_2$	1146	-	445 + 701
***RSK4* off-target 8**	CAATGGTGACCCGAAGTTCT	ATGCCTGCATGGGGTTAATA	62	1.6 mM $MgCl_2$	1151	-	558 + 593
***RSK4* off-target 9**	GCTTATCGACACCAGCCAGT	AGGTCTTATGGCCCACAACG	63	1.6 mM $MgCl_2$	1225	-	598 + 627
***RSK4* off-target 10**	AGCCCATCTTTTCTCACACAGT	CTCTGCTCAGGTGTTTTGTGAC	62	1M betaine, 1.6 mM $MgCl_2$	1103	-	350 + 753
CRISPR/Cas9 on-target analysis							
EMX1	CCATCCCCTTCTGTGAATGT	GGAGATTGGAGACACGGAGA	62	1M betaine, 1.6mM $MgCl_2$	639	EcoRV	302 + 337
***EMX1* (for gRNA targets 1 and 2)**	CCATCCCCTTCTGTGAATGT	GGAGATTGGAGACACGGAGA	62	1M betaine, 1.6mM $MgCl_2$	639	-	265 + 374
***EMX1* (for gRNA targets 1 and 3)**	CCATCCCCTTCTGTGAATGT	GGAGATTGGAGACACGGAGA	62	1M betaine, 1.6 mM $MgCl_2$	639	-	286 + 353
***VEGFA*‡**	CAAAGTGAGTGACCTGCTTTTG	CAGCAGAAAGTTCATGGTTTCG	65	1M betaine, 1.6 mM $MgCl_2$	640	-	203 + 437
CRISPR/Cas9 off-target analysis							
***VEGFA* off-target 1§**	AAGGGGCTGCTGGGTAGGAC	CGTGATTCGAGTTCCTGGCA	64	1M betaine, 1.6 mM $MgCl_2$	197	-	78 + 119
***VEGFA* off-target 2§§**	CCCATGAGGGGTTTGAGTGC	TGAAGATGGGCAGTTTGGGG	62	1M betaine, 1.6 mM $MgCl_2$	285	-	74 + 211

*For CEL-I analyses, the diagnostic fragment sizes may vary slightly depending on the mutagenesis outcome.

†Within the parentheses are shown non-diagnostic RFLP fragments common to both wild-type and mutant PCR products.

‡ *VEGFA* gRNA target site 2 in reference (36).

§ *VEGFA* off-target 6 for gRNA target site 2 in reference (36).

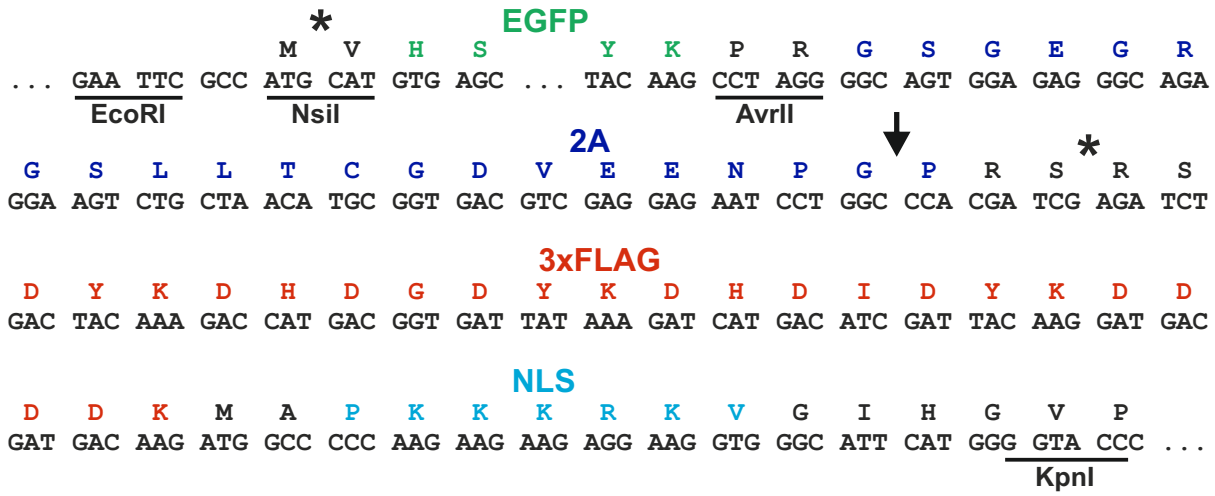
§§*VEGFA* off-target 17 for gRNA target site 2 in reference (36).

Supplementary Table S6. Primary chromosomal location and gene copy number of the major genes targeted in this study.

Gene	Chromosomal location	Copy number
K562		
RSK2	ChrX: 20,168,029-20,285,523	2*†
RSK4	ChrX: 83,318,984-83,442,933	2*†
VEGFA	Chr6: 43,737,921-43,754,224	4*†
EMX1	Chr2: 73,143,389-73,162,020	3*†
PRMT1	Chr19: 50,179,043-50,192,286	3*†
Jurkat		
RSK2	ChrX: 20,168,029-20,285,523	3*
RSK4	ChrX: 83,318,984-83,442,933	3*
MCF-10A		
RSK2	ChrX: 20,168,029-20,285,523	2‡

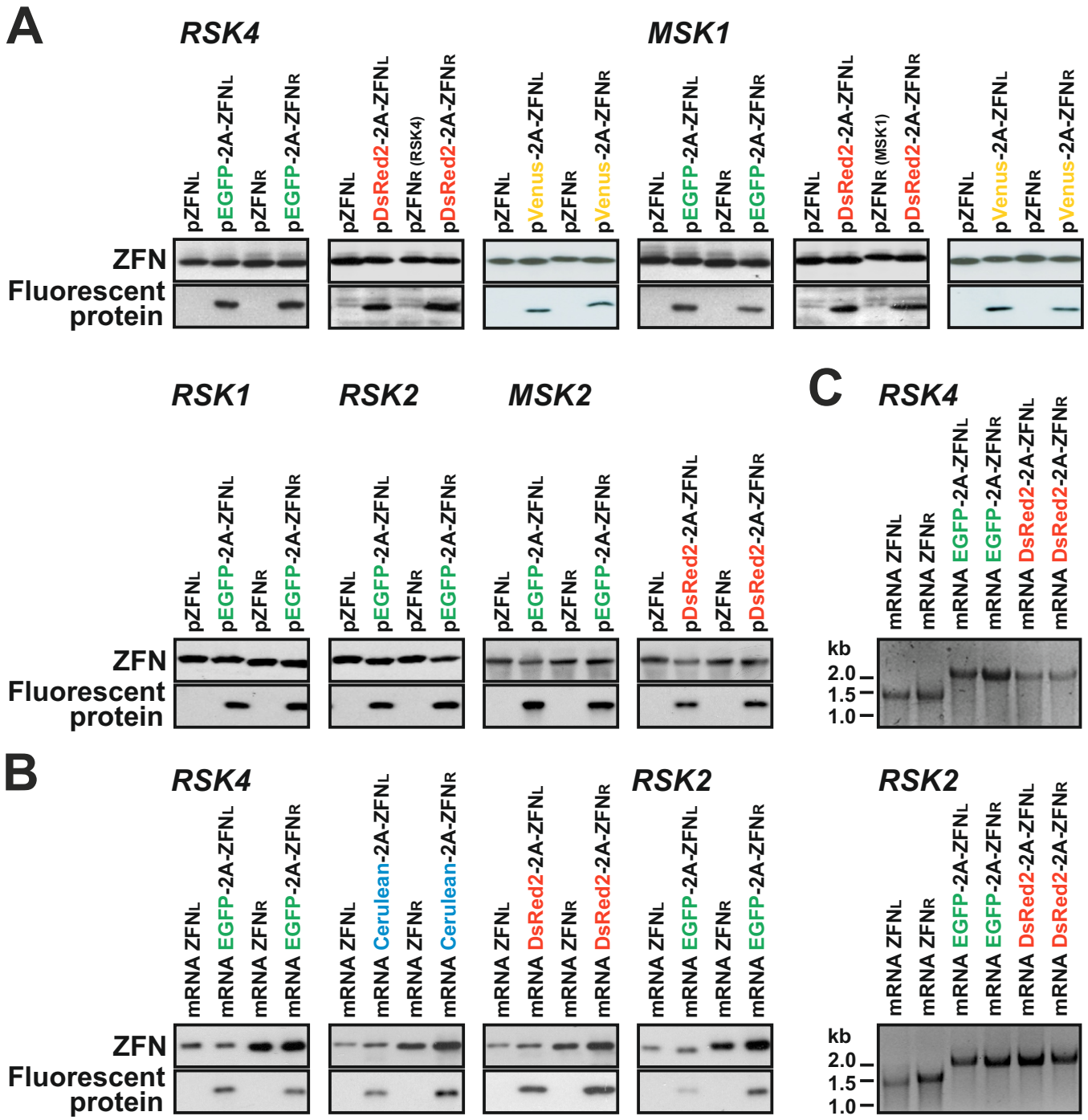
* The data was obtained experimentally by analysis of genomic DNA from the indicated cell lines using a CytoScan HD array from Affymetrix, as described in Materials and Methods.
†The data was independently obtained by the Wellcome Trust Sanger Institute Cancer Genome Project (http://www.sanger.ac.uk/genetics/CGP).
‡The data was obtained from reference (34).

Supplementary Figure S1



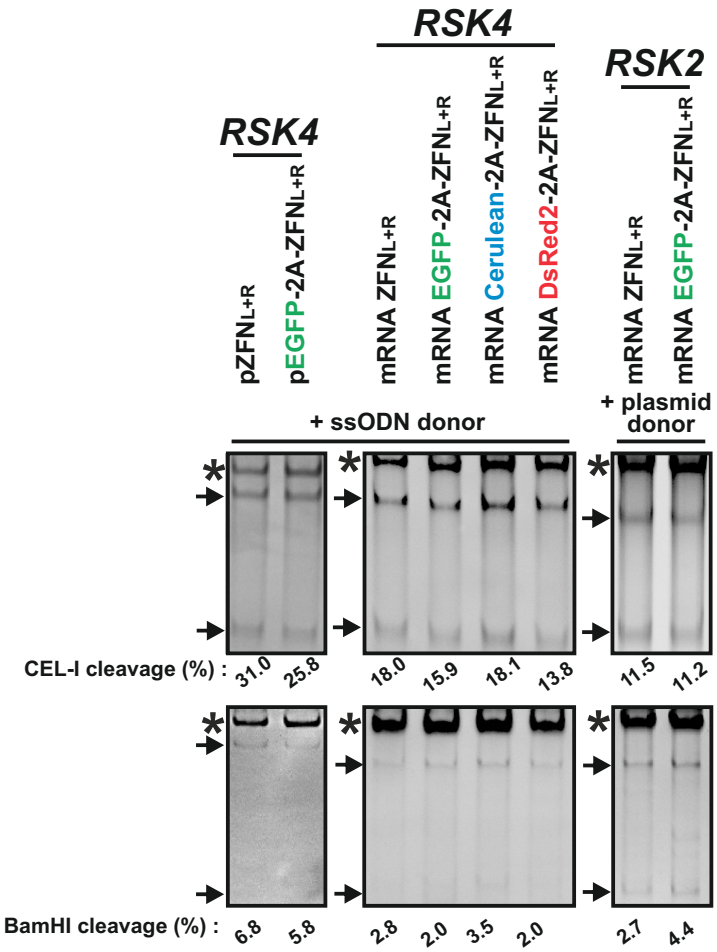
Supplementary Figure S1. Sequence introduced into pZFN to generate pFluo-2A-ZFN. Nucleotide and amino acid sequence of pFluo-2A-ZFN between the EcoRI and KpnI sites indicated in the schematic of pFluo-2A-ZFN given in Figure 1A. The sequence introduced in pZFN, the standard CompoZr ZFN expression construct, in order to generate pFluo-2A-ZFN is located between the two asterisks. The arrow in the 2A sequence indicates the position of translational skipping, which leaves the ZFN modified only by a Pro residue from the 2A peptide.

Supplementary Figure S2



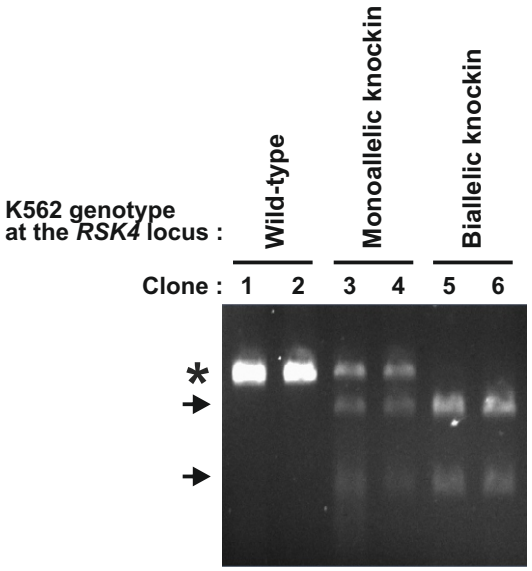
Supplementary Figure S2. Fluo-2A-ZFN constructs express fluorescent proteins and ZFNs as separate entities and at normal levels. (**A**) HCT116 cells were transfected with pFluo-2A-ZFN encoding various fluorescent proteins and ZFNs for various genes or transfected with pZFN for the same genes. (**B**) HCT116 cells were nucleofected with mRNA transcribed *in vitro* from pFluo-2A-ZFN encoding various fluorescent proteins and ZFNs for various genes or from pZFN for the same genes. For (A,B), the cells were lysed two d post-transfection and equal aliquots of the cell lysates were subjected to immunoblotting with antibodies against the FLAG epitope tag of the ZFNs or against the relevant fluorescent protein. (**C**) Equal aliquots of various *in vitro* mRNA transcription reactions using pFluo-2A-ZFN or pZFN as template were analysed for production yields by gel electrophoresis. In all of the above panels ZFNL and ZFNR denote left and right ZFN, respectively, of the ZFN pair. The experiments were repeated 3 times with similar results.

Supplementary Figure S3



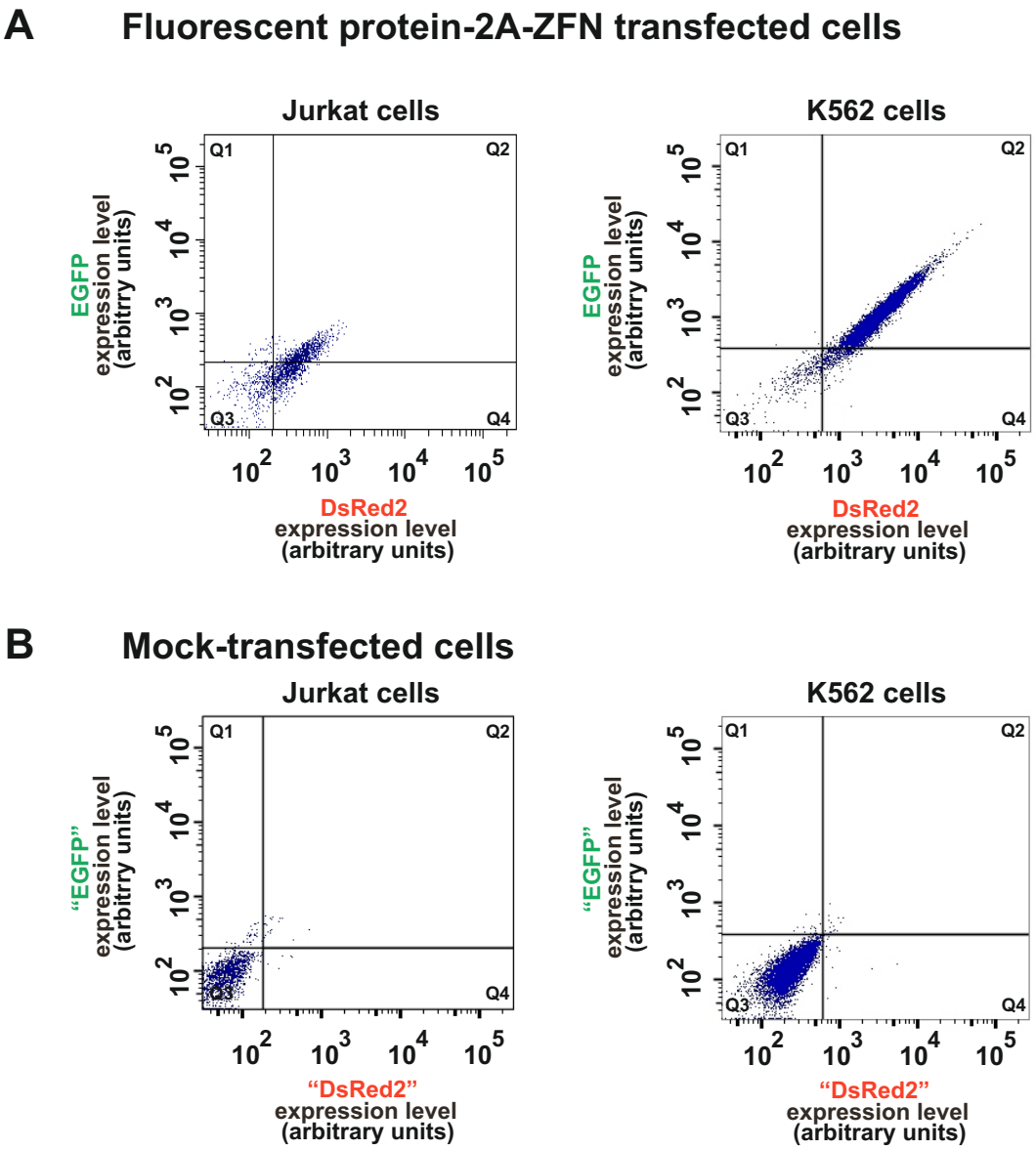
Supplementary Figure S3. Fluo-2A-ZFN constructs delivered as plasmid or mRNA express ZFNs that function effectively. K562 cells were transfected with pEGFP-2A-ZFN or pZFN for *RSK4* or with mRNA transcribed *in vitro* from pFluo-2A-ZFN encoding various fluorescent proteins or pZFN for *RSK4* or *RSK2*. An ssODN (*RSK4* Cys443Val/BamHI) or a plasmid (*RSK2* Cys436Val/BamHI) donor was co-transfected, as indicated, designed to introduce a codon-swap mutation and a diagnostic restriction endonuclease site in *RSK4* or *RSK2*. Three d post-transfection, the cells were lysed and genomic DNA was subjected to CEL-I or RFLP assay. PCR products containing amplicons derived from mutant alleles or from wild-type alleles only, are indicated by arrows and asterisk, respectively. The experiments were repeated several times with similar results. N.D., none detected.

Supplementary Figure S4



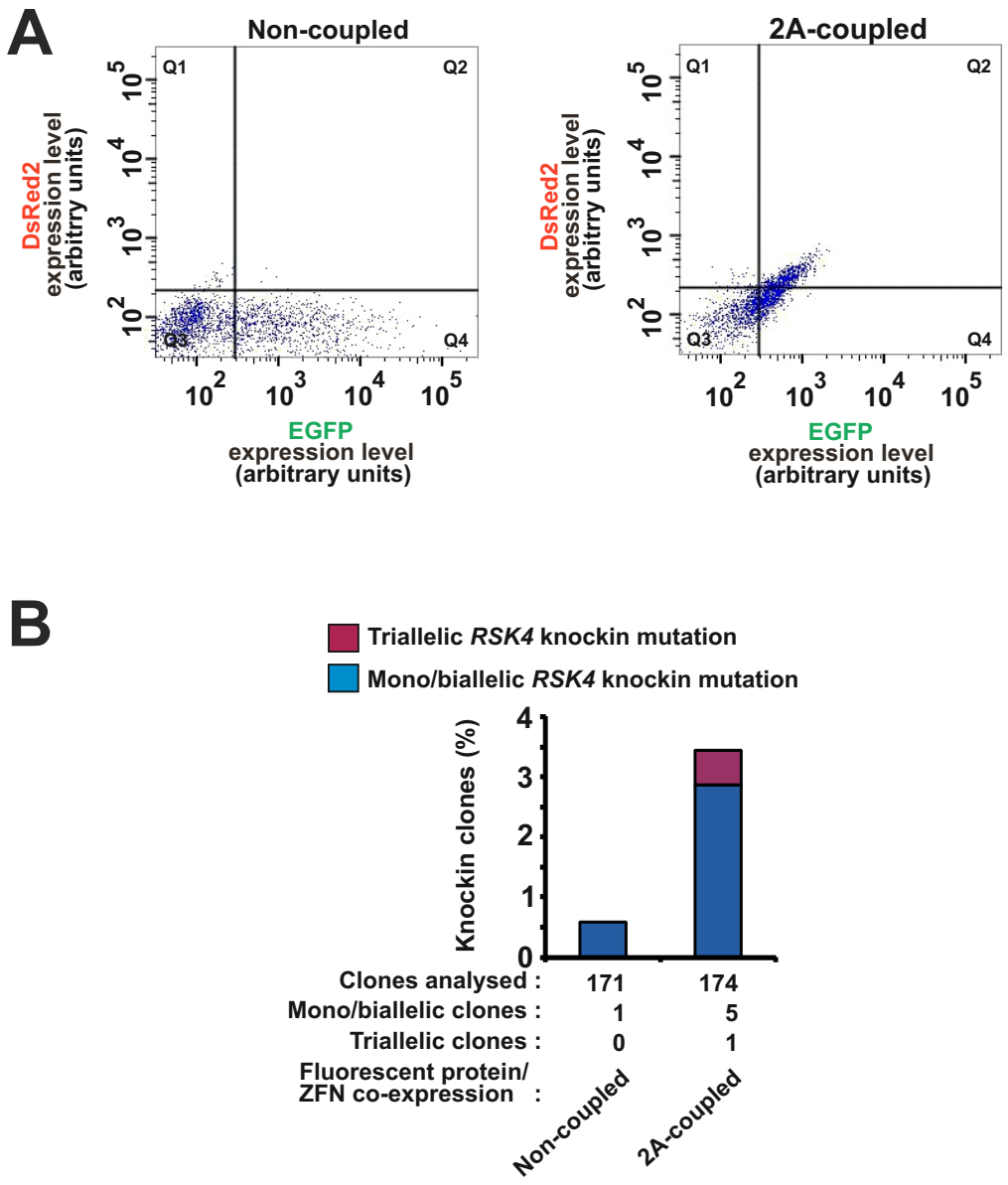
Supplementary Figure S4. Example of RFLP identification of monoallelic and biallelic knockin modification of the *RSK4* locus. Six clonal K562 cell lines derived from the experiment depicted in Figure 1E showing wild-type, monoallelic or biallelic knockin modification of the *RSK4* locus as revealed by RFLP analysis. Unmodified clones show a non-digested 1207 bp PCR product amplified from the wild-type alleles (indicated by an asterisk). Clones with monoallelic modification show a non-digested 1207 bp PCR product derived from the wild-type allele as well as 840 bp and 367 bp diagnostic fragments of PCR products amplified from the modified allele (indicated by arrows). Clones with biallelic modification only show the 840 bp and 367 bp diagnostic fragments of PCR products amplified from the modified alleles.

Supplementary Figure S5



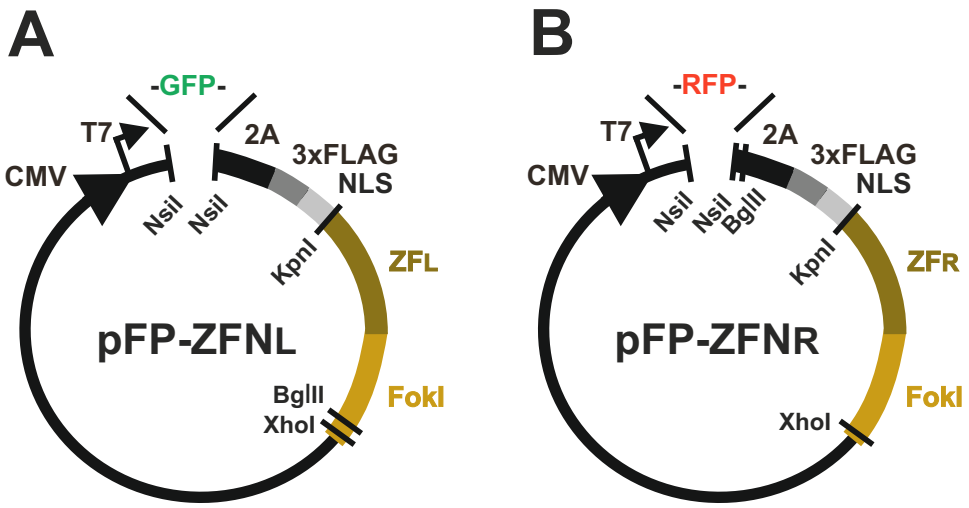
Supplementary Figure S5. FACS profiles of Jurkat and K562 cells. FACS profiles of Jurkat and K562 cells 3 d after transfection with mRNA for EGFP-2A-ZFNL and DsRed2-2A-ZFNR along with an ssODN knockin donor (*RSK4* Cys443Val/BamHI) (**A**) or 3 d after transfection with irrelevant mRNA not encoding fluorescent protein (**B**). The profiling in (B) helped define the fraction of cells (Q3) devoid of detectable fluorescence signal derived from the transfected constructs encoding EGFP or DsRed2. Based on this data, cell populations with specific EGFP signal (Q1), specific DsRed2 signal (Q4) or specific signals for both EGFP and DsRed2 (Q2) were assigned in the FACS profiles in (A).

Supplementary Figure S6



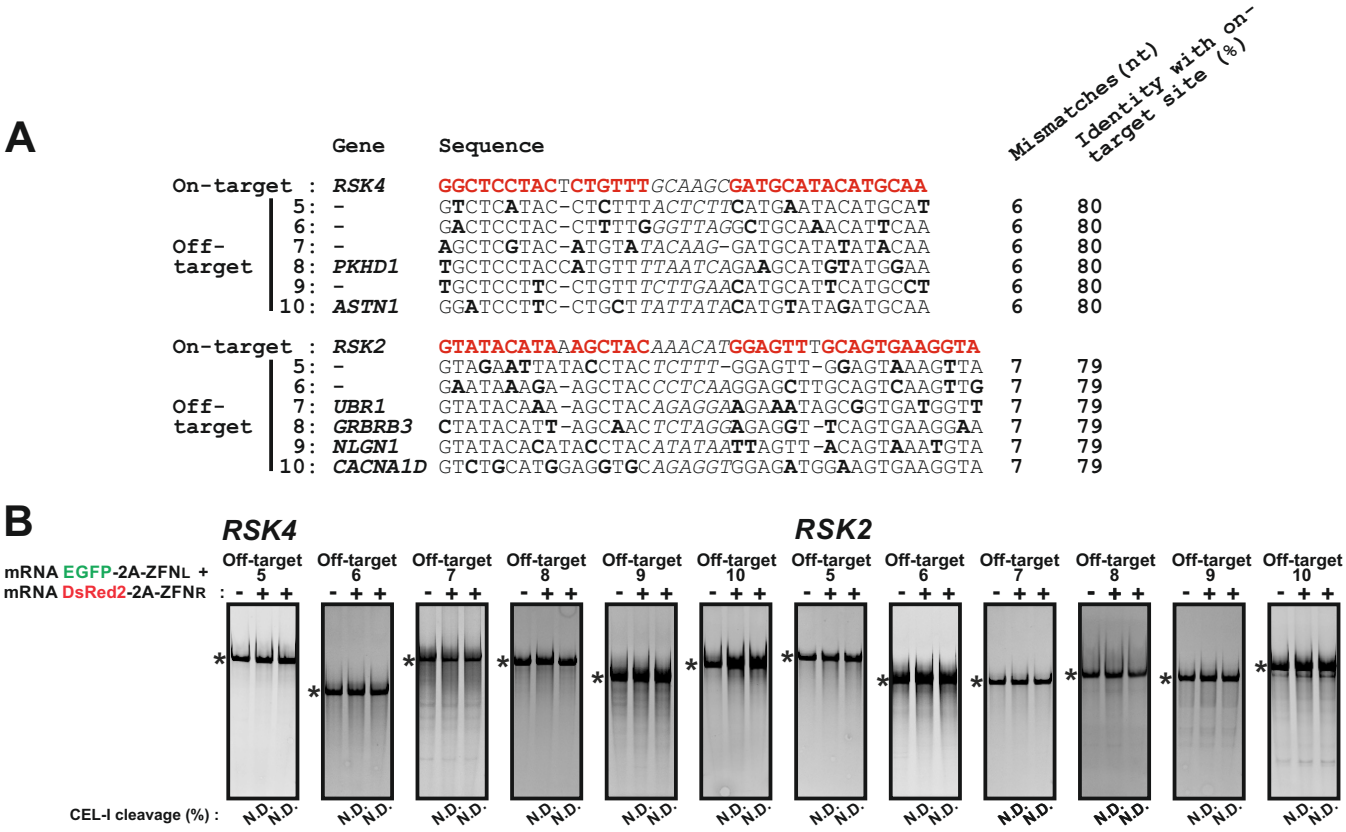
Supplementary Figure S6. Non-coupled versus 2A-coupled co-expression of fluorescent proteins and ZFNs. Jurkat cells were either transfected with mRNA for ZFNL and ZFNR along with a plasmid expressing EGFP (co-expression: non-coupled) or with mRNA for EGFP-2A-ZFNL and DsRed2-2A-ZFNR (co-expression: 2A-coupled). In both cases, the ZFNs targeted the *RSK4* locus and the ssODN donor *RSK4* Cys443Val/BamHI was co-transfected along with the ZFNs. (**A**) FACS profiles of the two cell populations 3 d after transfection. (**B**) Three d post-transfection, the two cell populations were sorted for EGFP and EGFP/DsRed2 fluorescence, respectively, cells were plated out singly, expanded to clones and analysed for mono/bi- or triallelic knockin at the *RSK4* locus. The data are summed from 3 independent experiments.

Supplementary Figure S7



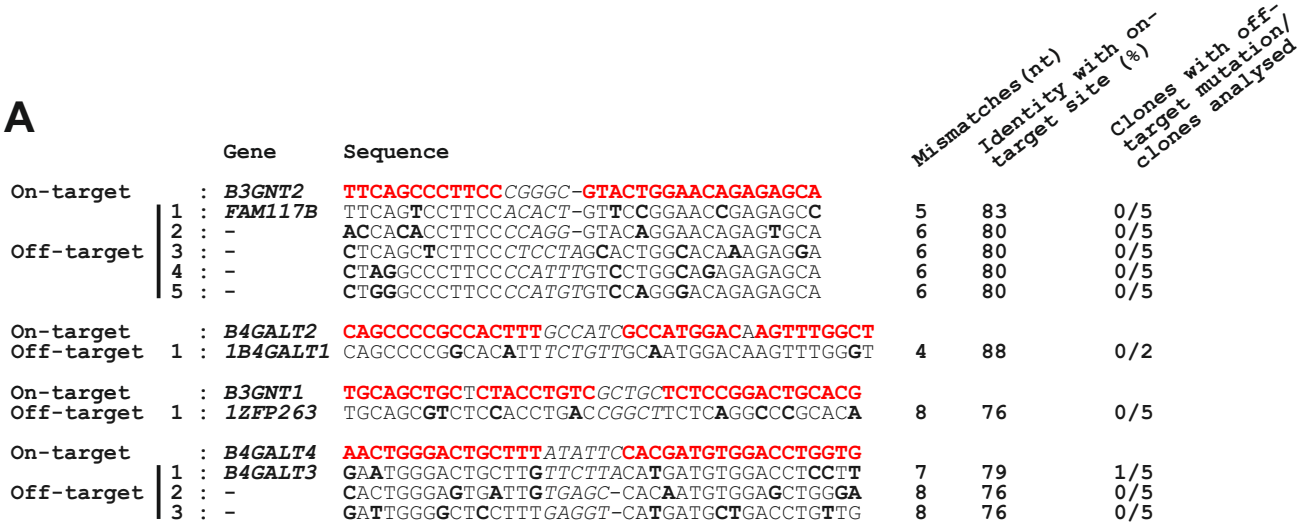
Supplementary Figure S7. pFP-2A-ZFN constructs that can be converted into FP-ZFNL-ZFNR single vector constructs. Schematic of pFP-ZFN constructs that couple ZFNL and ZFNR monomers of a pair to GFP and RFP, respectively. Fluorescent protein and ZFN can be exchanged by using the NsiI and KpnI/XhoI restriction sites, respectively. Furthermore, the ZFNL and ZFNR of the pair can be combined into a pFP-ZFNL-ZFNR single vector construct (Figure 3A) by using the BglII and XhoI sites present in both of the individual pFP-ZFN plasmids.

Supplementary Figure S8



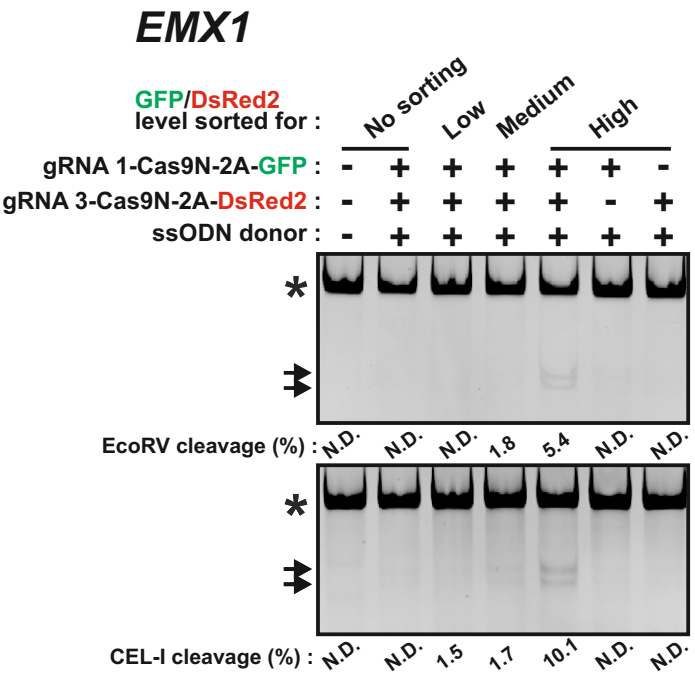
Supplementary Figure S8. Off-target cutting associated with FACS for high ZFN levels in K562 cells. (**A**) Sequence of the top-ranking candidate off-target sites 5-10 for *RSK4* and *RSK2* ZFNs. The on-target site is indicated in red and mismatches in off-target sites relative to the on-target site are boldface. The spacer sequence between the individual ZFN monomers is in italics. (**B**) K562 cells were transfected with EGFP-2A-ZFNL and DsRed2-2A-ZFNR for *RSK4* or *RSK2* along with ssODN donors for either gene. Three d later, genomic DNA was isolated from the 20% most highly fluorescent cell population and analysed by CEL-I assay for modification at the 6 off-target sites shown in (A). PCR products containing amplicons derived from mutant alleles and PCR products derived from wild-type alleles only, are indicated by arrows and asterisk, respectively. The experiment was repeated 3 times with similar results. N.D., none detected.

Supplementary Figure S9



Supplementary Figure S9. Off-target cutting in CHO clones established using FACS isolation of cells with high ZFN levels. (**A**) CHO clones with biallelic, NHEJ-derived knockout of various genes were established by transfection with the appropriate EGFP-2A-ZFNL and DsRed2-2A-ZFNR constructs followed by FACS isolation for the top-2 to -18% most highly double-fluorescent cells and single-cell plating for clonal expansion. Derived clones were analysed for mutations in top-ranking, candidate off-target sites by Sanger sequencing. Only one monoallelic mutation was identified: a 4bp insertion (shown in C) in off-target 1 in a clone modified by *B4GALT4* ZFNs. The ZFN on-target site is indicated in red and mismatches in off-target sites relative to the target site are boldface. The spacer sequence between the individual ZFN monomers is in italics. (**B**) CHO cells were transfected with plasmid expressing EGFP-2A-ZFNL and DsRed2-2A-ZFNR for *B4GALT4*. Three d later, genomic DNA was isolated from non-sorted cells or from cells FACS isolated for medium or high fluorescence intensities and analysed by CEL-I assay for modification of off-target site 1 in *B4GALT3*. PCR products containing amplicons derived from mutant alleles and PCR products derived from wild-type alleles only, are indicated by arrows and asterisk, respectively. The experiment was repeated 3 times with similar results. N.D., none detected. (**C**) Genomic sequence of the monoallelic mutation in *B4GALT4* off-target 1 (isolated by TOPO cloning), indicating the 4 bp insertion (boxed) and the ZFN binding sites (black bars).

Supplementary Figure S10



Supplementary Figure S10. **Genome editing by CRISPR/Cas9 nickase pairs is enhanced by using the 2A-coupled fluorescent protein/FACS strategy.** K562 cells were transfected with Cas9N-FP constructs targeting the *EMX1* locus as a nickase pair or as individual nickases coupled to GFP or dsRed2, as indicated. An ssODN donor (*EMX1* gRNA 1/3 EcoRV) for knockin of an EcoRV restriction site in *EMX1* was co-transfected. Three d after transfection, genomic DNA was isolated from non-sorted cells or from cells FACS isolated for low, medium or high GFP/dsRed2 double-fluorescence intensities and analysed by RFLP or CEL-I assay, as indicated. In all experiments, repeated 3 times with similar results, PCR products containing amplicons derived from mutant alleles or from wild-type alleles only, are indicated by arrows and asterisk, respectively. N.D., none detected.