



Supplementary Materials

# Using a Dual CRISPR/Cas9 Approach to Gain Insight into the Role of LRP1B in Glioblastoma

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## Supplementary Tables

Table S1. sgRNA target sequences used and their respective *off-* and *on-target* scores

Target exon	Position within the gene (bp) <sup>a</sup>	Strand	sgRNA ID	sgRNA target sequence (5' → 3')	PAM sequence	Off-target score <sup>b</sup>	On-target score <sup>c</sup>
1	6720	Minus	sgRNA1	GACATTGTGGTCGCCCCGGTA	AGG	96.6	51.9
	6800	Plus	sgRNA2	CGTGGGAGCCGACCGAGGTA	AGG	90.2	50.3
85	1 862 943	Minus	sgRNA3	TTTGGTCCTTCATAGCGCGT	TGG	94.8	32.1
	1 862 993	Minus	sgRNA4	TTATAATGCAGTGCCCCCCA	TGG	80.5	44.2

<sup>a</sup> Human LRP1B RefSeqGene NG\_051023.1, NCBI Reference Sequence [32]. <sup>b</sup> Hsu et al. [37]. <sup>c</sup> Doench et al. [57]. Off- and on-target scores range from 0 to 100, with higher scores indicating lower off-target potential and higher predicted Cas9 activity at the target site, respectively.

Table S2. Potential off-target sequences within the human genome for each selected sgRNA

sgRNA Name	Sequence	PAM	Score	Gene	Locus
sgRNA 1	GACATTGTGGTCGCCCCGTA	AGG	100.0	LRP1B (ENSG00000168702)	Chr2:+142888311
	TAGAGTGTGGTCGCCCCGTA	CGG	0.7		Chr9:-98949462
	GAAATCGTGCTCGCCCCAGTA	GGG	0.4	METTL23 (ENSG00000181038)	Chr17:-74723151
	GACATTGTGGTACCAAGTA	AGG	0.4		Chr11:+121529897
	GACAAGGTGGCGCCCCGGT	CGG	0.3	UGCG (ENSG00000148154)	Chr9:-114659411
	GACATGGAAGTCTCCCGGTA	TAG	0.2		Chr2:-14296956
	GCCATTGTGGAAGCCCTGTA	AGG	0.2		Chr11:+121358605
	GACATTGTGATCGCCAGGAA	GGG	0.1		Chr5:-5572203
	GTCATTGTGTTAGCCTGGTA	AGG	0.1		Chr13:+44381193
	GACAATGAGGTGCGCCAGGCA	AGG	0.1		Chr22:+28198148
	GAGATGTGGCCCGCCTGGTA	GGG	0.1		Chr21:+46184543
sgRNA 2	CGTGGGAGCCGACCGAGGTA	AGG	100.0	LRP1B (ENSG00000168702)	Chr2:+142888217
	GAGGGGAGCGCACCGAGGTA	CAG	0.9		Chr16:+2683345
	GAGGGGAGCGCACCGAGGTA	CAG	0.9		Chr16:+2616850
	CGAAAGAGCCGCGGAGGTA	GAG	0.8		Chr15:+82042456
	CCTGGGAACAGACCGAGGGA	GAG	0.6		Chr19:+4454107
	CGAAGGGGCCGACCGAGGTC	AAG	0.6		Chr9:+34376639
	CTTGGCAGCTGCCCGAGGTA	GAG	0.5		Chr2:+67487160
	CGGAGGAGCCGCGGAGGTC	CAG	0.4		Chr2:+219922477
	CGTCCGAGCCGCGGAGGTC	GAG	0.4	FOXO4 (ENSG00000184481)	ChrX:+70316526
	CTGTGGGAGCTGCCCGAGGTT	GAG	0.4	GNG7 (ENSG00000176533)	Chr19:+2513099
	CGTGGGAGCCGACCGAGGTA	TAG	0.4		Chr7:+157252678
sgRNA 3	TTTGGTCCTTCATAGCGCGT	TGG	100.0	LRP1B (ENSG00000168702)	Chr2:+141032088
	GATGGTCTTTTATAGCGCGT	AAG	0.9		Chr15:+60777206
	GTTTGTCATTCATAGCCCGT	GAG	0.8		Chr10:+77379183
	TTGGGCTTTCATAGCACGT	AGG	0.4		Chr18:+47828723
	TTTGCTCTTCATAGCACCT	TAG	0.2		Chr1:-144870905
	TTTGTTCTTCATAGCACAT	TGG	0.2		Chr3:+29951857
	TCTGGTCCTCCATAGCTCTT	GGG	0.2		Chr8:+22995922
	TCTGGTCCTGCATAGCTCTT	GGG	0.2		Chr8:-22938350
	TGTGTTCTTCATAGCGGGA	TGG	0.2		Chr10:+32865797
	TTTGGTCAATCACAGCGCGC	CAG	0.2		Chr9:+33076306
	TTTGGCCATTCTTAGCGCCT	GAG	0.2		Chr14:-33848061
sgRNA 4	TTATAATGCAGTGCCCCCA	TGG	100.0	LRP1B (ENSG00000168702)	Chr2:+141032038
	TTCCCATCCAGTGCCCCCA	CAG	1.4		Chr20:+59999886
	TTGCCATGCTGTGCCCCCA	AAG	1.3		Chr10:-130874277
	CTATAATACAGTCCCCCA	AAG	1.3		Chr7:-13572699
	TTAGAATGCAGTGACCCCA	AGG	1.3		ChrY:-16857284
	TACAAAGCCGTGCCCCCA	GAG	1.0		Chr20:+29596954
	TTCTAATCCAGTGCTCCCA	AGG	0.9		Chr5:+107228315
	GGCTACTGCAGTGCCCCCA	AGG	0.8		Chr13:+113761336
	TTCTTCTCCAGTGCCCCCA	TGG	0.8	MICB (ENSG00000204516)	Chr6:+31474805
	TTCTTCTCCAGTGCCCCCA	TGG	0.8	PERB11.1 (ENSG00000204520)	Chr6:+31379730
	TGAGAAAGCAGTGCCCTCCA	AGG	0.5		Chr2:+109576512

Only the the first 10 off-target sequences for each sgRNA are presentes, ordered based on their Score values. In these sequences, mismatches are denoted by red letters.

**Table S3. Oligonucleotides used for sgRNA cloning**

Oligonucleotide duplex name	Oligonucleotide name	Sequence (5' → 3')
Duplex-1	1-sense	<b>CACCGACATTGTGGTCGCCCGGTA</b>
	1-antisense	<b>AAACTACCGGGCGACCACAATGTC</b>
Duplex-2	2-sense	<b>CACCGCGTGGGAGCCGACCGAGGTA</b>
	2-antisense	<b>AAACTACCTCGGTCGGCTCCACGC</b>
Duplex-3	3-sense	<b>CACCGTTGGTCCTTCATAGCGCGT</b>
	3-antisense	<b>AAACACGCGCTATGAAGGACCAAAC</b>
Duplex-4	4-sense	<b>CACCGTTATAATGCAGTGCCCCCA</b>
	4-antisense	<b>AAACTGGGGGGCACTGCATTATAAC</b>

**Table S4. Oligonucleotide primers used for genomic DNA analysis**

Oligonucleotide name	Position within the gene (bp) <sup>a</sup>	Strand	Sequence (5' → 3')
LRP1B-01-Fw	6574	Plus	GCTGACTGGCTGGACTCATT
LRP1B-01-Rv	6869	Minus	TTATCTGCAAGCATCGCCCA
LRP1B-01-In	6774	Minus	ACCCTGGCAATCGGCAATAA
LRP1B-85-Fw	1 862 483	Plus	AGGTGTGAAGGAGGCAACAA
LRP1B-85-Rv	1 862 145	Minus	GCAATGGGCACAATACGGAA
LRP1B-85-In	1 862 966	Minus	ACCTTACACACTTGTCAACCTCA

<sup>a</sup> Human *LRP1B* RefSeqGene NG\_051023.1, NCBI Reference Sequence [32].

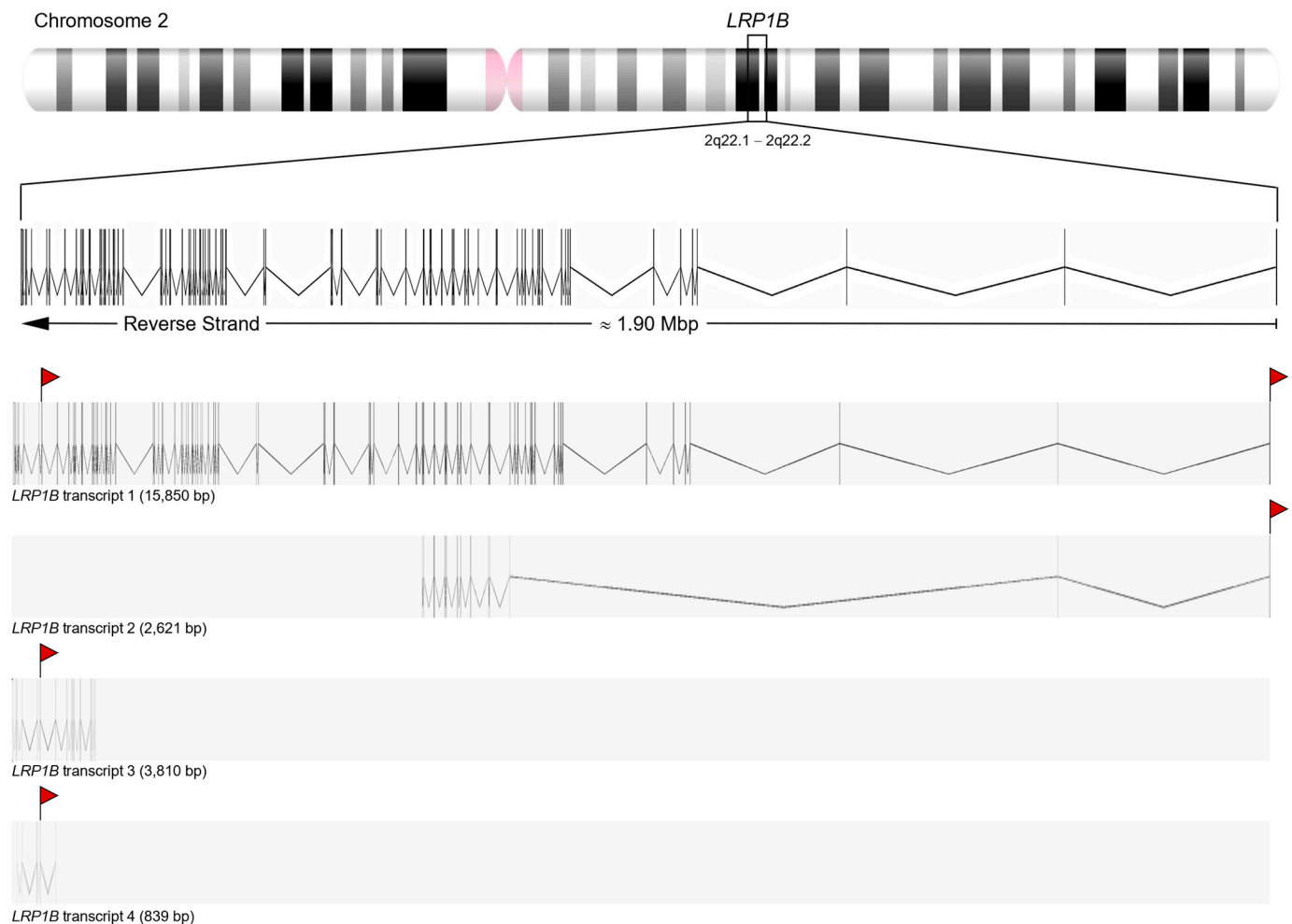
**Table S5.** List of differentially expressed proteins (DESPs) found in the secretomes of clones B9, E6 and H7, when compared to secretomes from mock cells.

				Abundance	Abundance	
				Ratio:	Ratio Adj. P-	
				(clone) /	Value: (clone) /	
				Mock	Mock	
Clone B9 vs Mock	Up-regulated	Q9UNZ2	NSFL1 cofactor p47	NSFL1C	100	1.7206E-16
		Q96KG7	Multiple epidermal growth factor-like domains protein 10	MEGF10	100	1.7206E-16
		P05231	Interleukin-6	IL6	100	1.7206E-16
		P08254	Stromelysin-1	MMP3	11.524	2.6517E-05
		P48307	Tissue factor pathway inhibitor 2	TFPI2	9.668	0.0002
		P03956	Interstitial collagenase	MMP1	9.409	0.0002
		Q7Z304	MAM domain-containing protein 2	MAMDC2	8.76	0.0004
		P21741	Midkine	MDK	7.771	0.0013
		P17301	Integrin alpha-2	ITGA2	6.729	0.0045
		P05120	Plasminogen activator inhibitor 2	SERPINB2	6.53	0.0057
		Q16363	Laminin subunit alpha-4	LAMA4	6.387	0.0068
	Down-regulated	P02452	Collagen alpha-1(I) chain	COL1A1	0.361	0.0379
		P58215	Lysyl oxidase homolog 3	LOXL3	0.359	0.0366
		P41222	Prostaglandin-H2 D-isomerase	PTGDS	0.317	0.0152
		Q13449	Limbic system-associated membrane protein	LSAMP	0.308	0.0120
		P21810	Biglycan	BGN	0.288	0.0075
		P08123	Collagen alpha-2(I) chain	COL1A2	0.286	0.0071
		P29279	CCN family member 2	CCN2	0.269	0.0044
		P05156	Complement factor I	CFI	0.258	0.0032
		Q9BRK3	Matrix remodeling-associated protein 8	MXRA8	0.254	0.0028
		P05109	Protein S100-A8	S100A8	0.228	0.0011
		Q9BQT9	Calsyntenin-3	CLSTN3	0.221	0.00081
		Q9UI42	Carboxypeptidase A4	CPA4	0.21	0.00051
		P06276	Cholinesterase	BCHE	0.185	0.0001
		Q9BXX0	EMILIN-2	EMILIN2	0.156	2.4687E-05
		P01009	Alpha-1-antitrypsin	SERPINA1	0.149	1.5161E-05
		Q14126	Desmoglein-2	DSG2	0.133	4.1205E-06
		Q8IX30	Signal peptide. CUB and EGF-like domain-containing protein 3	SCUBE3	0.01	1.7206E-16
Clone E6 vs Mock	Up-regulated	Q96KG7	Multiple epidermal growth factor-like domains protein 10	MEGF10	100	1.7171E-16
		P05231	Interleukin-6	IL6	100	1.7171E-16
		Q9C0C2	182 kDa tankyrase-1-binding protein	TNKS1BP1	100	1.71716E-16
		Q9UNZ2	NSFL1 cofactor p47	NSFL1C	100	1.71716E-16

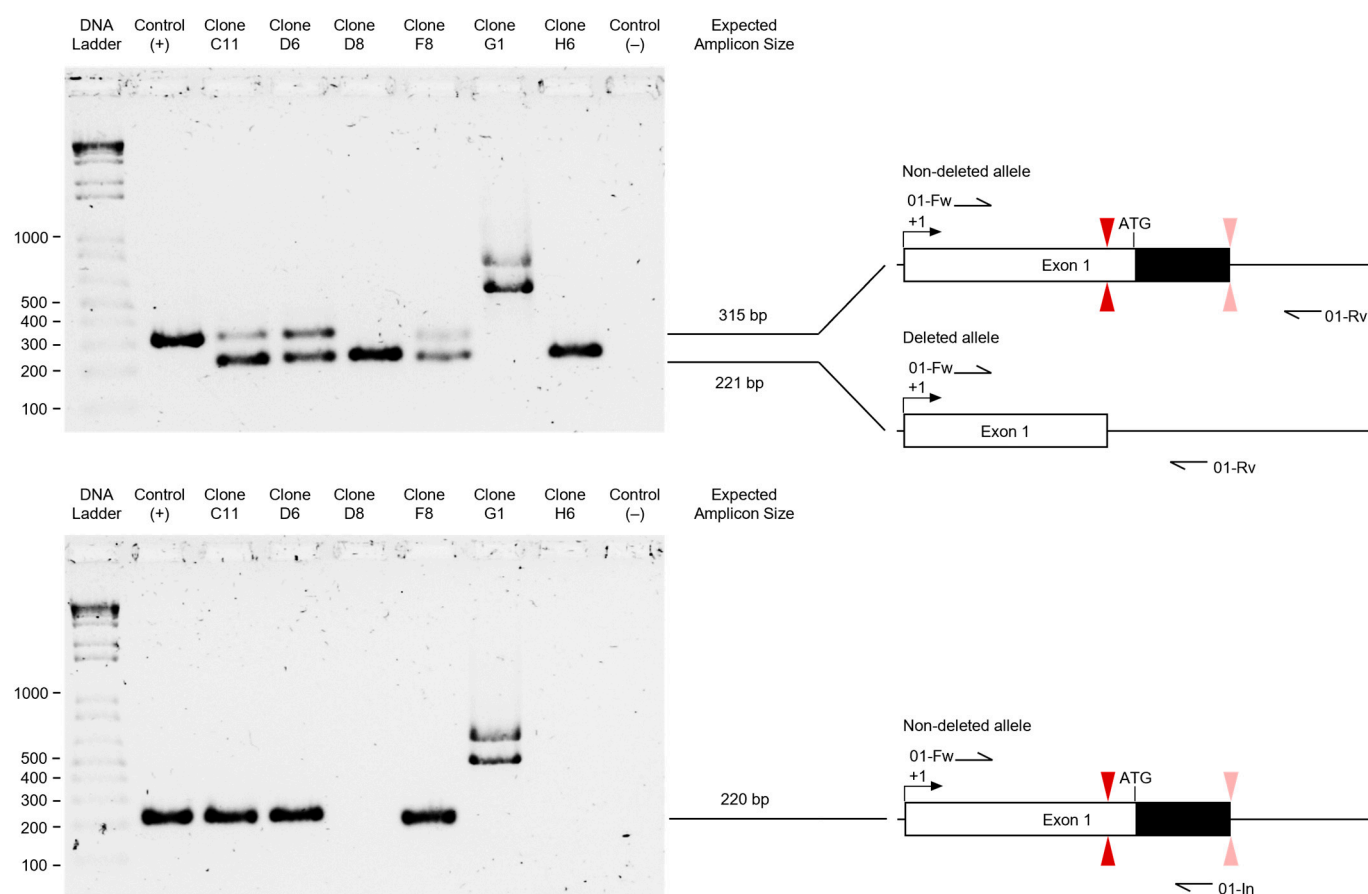
Table S5. (cont.)

					Abundance Ratio: (clone) / Mock	Abundance Ratio Adj. P- Value: (clone) / Mock
Accession	Description	Gene Symbol				
<b>Clone E6 vs Mock</b>	<b>Down - regu- lated</b>	P21589	5'-nucleotidase	NT5E	0.173	0.0400
		Q13449	Limbic system-associated membrane protein	LSAMP	0.139	0.013337294
		Q9BXX0	EMILIN-2	EMILIN2	0.1	0.002047735
<b>Clone H7 vs Mock</b>	<b>Up- regu- lated</b>	P05231	Interleukin-6	IL6	100	1.5956E-16
		Q9UNZ2	NSFL1 cofactor p47	NSFL1C	100	1.5956E-16
		Q96KG7	Multiple epidermal growth factor-like domains protein 10	MEGF10	100	1.5956E-16
		Q9C0C2	182 kDa tankyrase-1-binding protein	TNKS1BP1	100	1.5956E-16
		P36222	Chitinase-3-like protein 1	CHI3L1	17.201	1.25923E-07
		Q15782	Chitinase-3-like protein 2	CHI3L2	10.62	3.37175E-05
		Q7Z304	MAM domain-containing protein 2	MAMDC2	9.764	7.90558E-05
		P00751	Complement factor B	CFB	9.3	0.0001
		P43235	Cathepsin K	CTSK	7.39	0.0011
		P06702	Protein S100-A9	S100A9	7.346	0.0011
		P05156	Complement factor I	CFI	7.108	0.0015
		Q9BU40	Chordin-like protein 1	CHRD1	6.741	0.0024
		P49908	Selenoprotein P	SELENOP	6.686	0.0025
		P05109	Protein S100-A8	S100A8	6.415	0.0036
		Q9UKU9	Angiopoietin-related protein 2	ANGPTL2	5.138	0.0186
		Q15274	Nicotinate-nucleotide pyrophosphorylase [carboxylating]	QPRT	4.797	0.0297
		Q7Z7M9	Polypeptide N-acetylgalactosaminyltransferase 5	GALNT5	4.771	0.0307
		Q16363	Laminin subunit alpha-4	LAMA4	4.705	0.0338
	<b>Down- regu- lated</b>	P55290	Cadherin-13	CDH13	0.305	0.0365
		O95388	CCN family member 4	CCN4	0.294	0.0287
		P29279	CCN family member 2	CCN2	0.273	0.0173
		Q99715	Collagen alpha-1(XII) chain	COL12A1	0.253	0.0101
		O15240	Neurosecretory protein VGF	VEGF	0.194	0.0012
		Q96AM1	Mas-related G-protein coupled receptor member F	MRGPRF	0.19	0.0010
		P15692	Vascular endothelial growth factor A	VEGFA	0.167	0.0003
		Q13449	Limbic system-associated membrane protein	LSAMP	0.144	7.52018E-05
		Q9BXX0	EMILIN-2	EMILIN2	0.113	5.36226E-06
		P21810	Biglycan	BGN	0.1	1.29275E-06
		Q8N6T3	ADP-ribosylation factor GTPase-activating protein 1	ARFGAP1	0.01	1.5956E-16
		Q9NT68	Teneurin-2	TENM2	0.01	1.5956E-16
		O75635	Serpin B7	SERPINB7	0.01	1.5956E-16
		Q86UN3	Reticulon-4 receptor-like 2	RTN4RL2	0.01	1.5956E-16

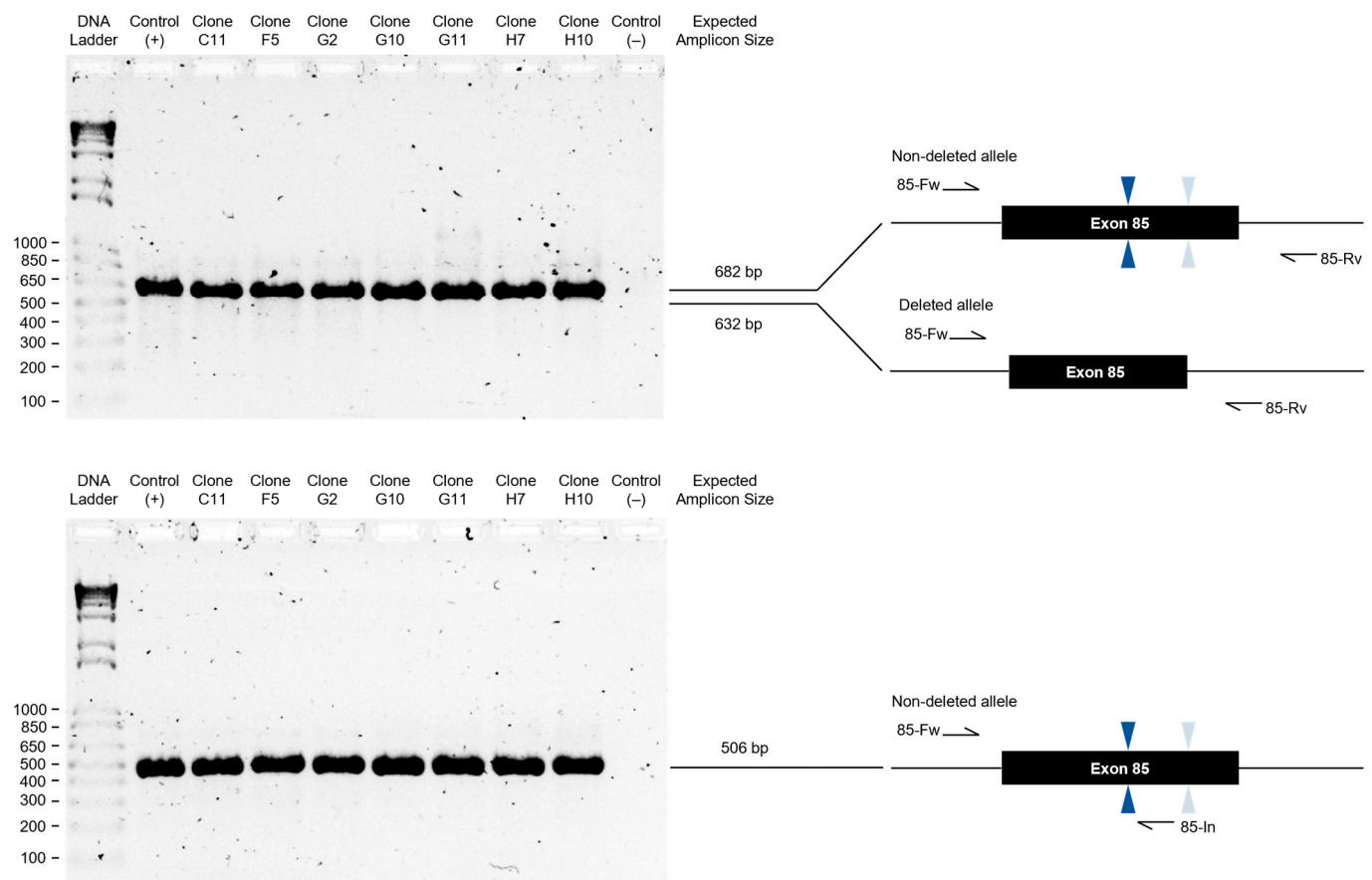
## Supplementary Figures



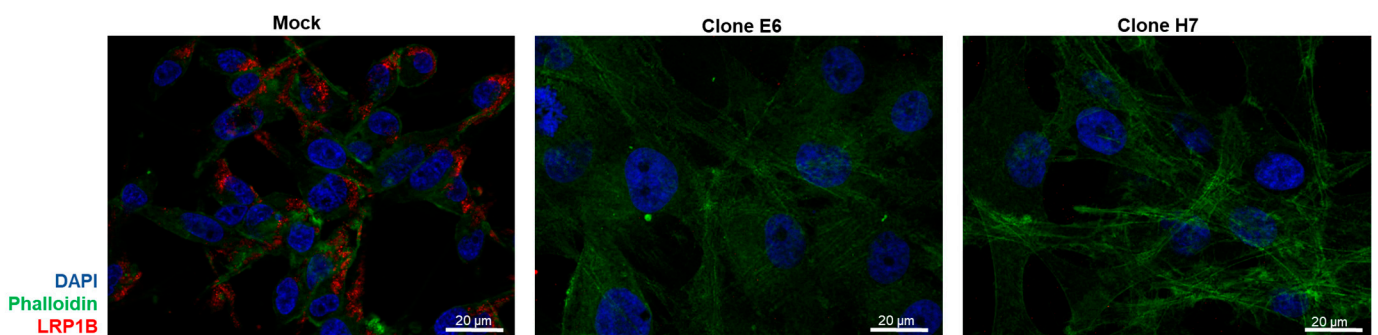
**Figure S1. Schematic representation of *LRP1B* gene and targeted regions.** Chromosomal location of the *LRP1B* locus. The chromosome ideogram was obtained from the NCBI online tool [31]. Human *LRP1B* is located on the long arm of chromosome 2 at position 2q22.1-2q22.2. It spans from 140.231.423 to 142.131.016 bp, according to the human genome assembly GRCh38.p13 from the Genome Reference Consortium [78] being approximately 1.90 megabase pairs (Mbp) long and composed of 91 exons that comprise 13.80 kilobase pairs (kbp) of coding sequence (CDS). Schematic representation of *LRP1B* gene structure depicting the exon-intron structure of *LRP1B*. The human *LRP1B* (ID: ENSG00000168702) has four putative protein-coding transcripts [33]: (i) full-length *LRP1B* transcript (***LRP1B* transcript 1**; ID: ENST00000389484.8), which includes all 91 annotated exons that together encode a 4599-amino acid membrane-bound protein (LRP1B) and (ii) three other predicted alternatively spliced *LRP1B* transcripts (***LRP1B* transcripts 2, 3 and 4**) that have their CDS incomplete on the 3' end, 5' end, or both ends. Specifically, *LRP1B* transcript 2 contains 14 exons (1,2,18-29; ID: ENST00000434794.1), which encode a putative 781-amino acid soluble LRP1B isoform. *LRP1B* transcripts 3 and 4 encode two putative membrane-bound LRP1B isoforms of 789 and 280 amino acids, respectively. *LRP1B* transcript 3 contains 17 exons (74-89, 91; ID: ENST0000043-7977.5), whereas *LRP1B* transcript 4 contains 7 exons (84-89, including an extra 114 bp exon between exons 86 and 87; ID: ENST00000442974.1). The gene and transcripts schemes were obtained from Ensembl release version 100 [33,35]. The most upstream exons shared by the majority of the *LRP1B* predicted transcripts are signaled with red flags.



**Figure S2. Representative images of PCR analysis of transfected selected clones.** Exon 1 analysis for some clones derived from U87 cells transfected with paired PX459-sgRNA1 and -2 vectors. *Upper panel.* the CRISPR/Cas9-targeted exon 1 was amplified from genomic DNA using the primers forward (01-Fw) and reverse (01-Rv). Expected sizes of the PCR amplicons for *LRP1B* non-deleted and deleted alleles are 315 bp and 221 bp, respectively. *Lower panel.* the CRISPR/Cas9-targeted exon 1 was amplified from genomic DNA using the primers forward (01-Fw) and reverse internal (01-In). The expected size of the PCR amplicon for the *LRP1B* non-deleted allele is 220 bp. Control (+), positive control using genomic DNA from the parental (wild-type) U87 cells and the appropriate primer set for each PCR; Control (-), negative control for each primer set (no DNA template); DNA Ladder, 1 kbp DNA ladder. Schematic representation of the *LRP1B* non-deleted and deleted alleles is depicted on the right side of the panels. Labeled black arrows indicate the positions and orientations of PCR primers. The arrowheads indicate the CRISPR/Cas9 target sites within *LRP1B* exon 1.

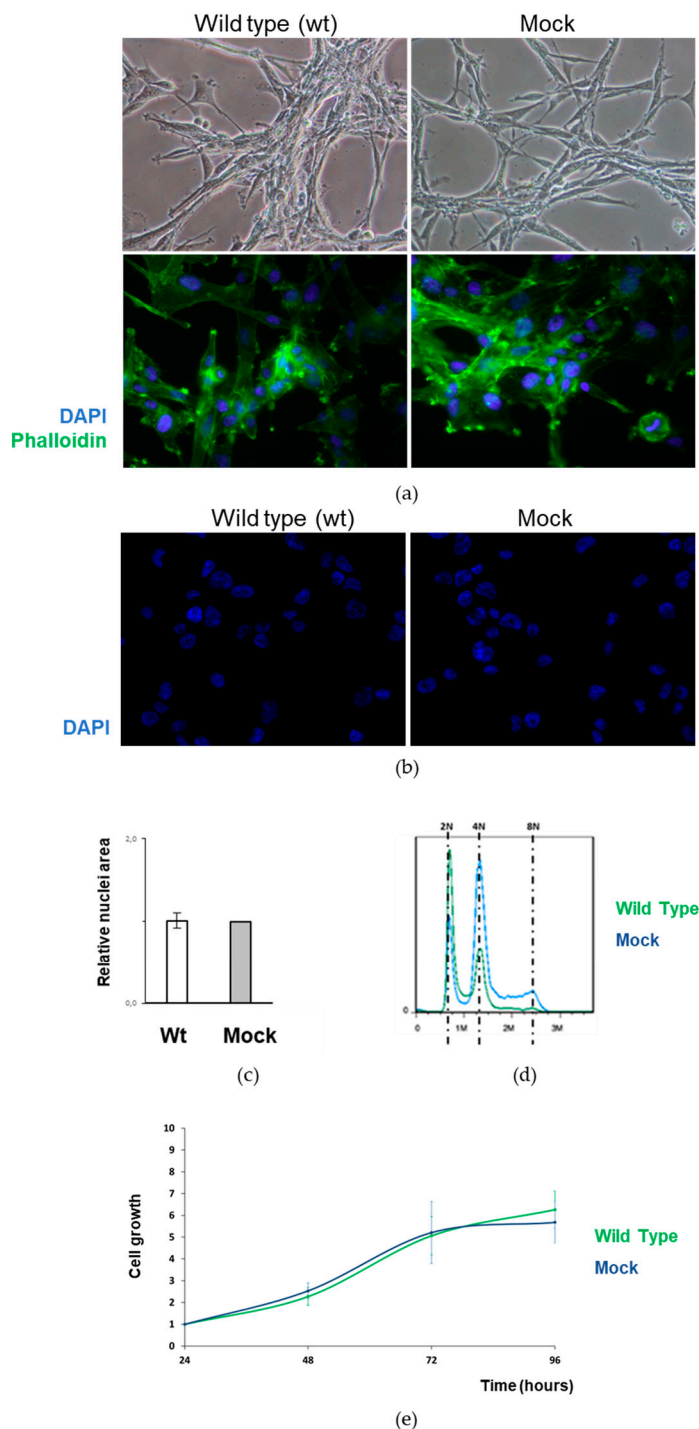


**Figure S3. Representative images of PCR analysis of exon 85 for some clones derived from U87 cells transfected clones with A) PX459-sgRNA3 and PX459-sgRNA4 vectors and B) all PX459-sgRNA vectors (1. -2. -3 and -4 ).** Upper panels, the CRISPR/Cas9-targeted exon 85 was amplified from genomic DNA using the primers forward (85-Fw) and reverse (85-Rv). The expected sizes of the PCR amplicons for *LRP1B* non-deleted and deleted alleles are 682 bp and 632 bp, respectively. Lower panel, the CRISPR/Cas9-targeted exon 85 was amplified from genomic DNA using the primers forward (85-Fw) and reverse internal (01-In). The expected size of the PCR amplicon for the *LRP1B* non-deleted allele is 506 bp. Control (+), positive control using genomic DNA from the parental U87 human glioblastoma cell line and the appropriate primer set for each PCR; Control (-), negative control for each primer set (no DNA template); DNA Ladder, 1 kbp DNA ladder. Schematic representation of the *LRP1B* non-deleted and deleted alleles is depicted on the right side of the panels. Labeled black arrows indicate the positions and orientations of PCR primers. The arrowheads indicate the CRISPR/Cas9 target sites within *LRP1B* exon 85.



**Figure S4. Decreased LRP1B expression in LRP1B-edited clones E6 and H7, compared to mock cells, evaluated by immunofluorescence.** Images are representative of 2 independent experiments.





**Figure S5. U87 wild type (wt; non-transfected) and mock cells.** (a) Cell morphology in adherent cells in culture evaluated by phase contrast microscopy and fluorescence microscopy (b) Representative images of DAPI-stained nuclei from cells after cytopsin. Cell nuclei area (evaluated with ImageJ software with NII plugin) was analyzed in relation to mock cells nuclei area. Results are the mean  $\pm$  SEM of 3 independent experiments. (d) Representative cell cycle profile evaluated by flow cytometry following propidium iodide staining. Images are representative of, at least, 3 independent experiments. (e) Cell growth analysis, with Presto blue viability assay following 24 h, 48, 72, and 96 h in culture. Results, expressed in relation to the 24 h, are the mean  $\pm$  SEM of at six independent experiments.