SUPPLEMENTARY INFORMATION

Bognar et al

Oncogenic CARMA1 couples NF-κB and β-catenin signaling in diffuse large B-cell lymphomas

SUPPLEMENTARY MATERIAL AND METHODS

Mass spectrometry approach

2x 10^8 transduced BJAB cells were lysed in co-IP buffer, 2x freeze-thaw cycles and 3 strokes in a dounce homogenizer. Following StrepTactin pull-down, beads were resuspended in 50 mM ammoniumbicarbonate and associated proteins were digested with 2.5 µg trypsin (Sigma, Germany) overnight at 37 °C. Peptides were measured in label-free LC-MS/MS, using an LTQ Orbitrap XL (Thermo Scientific, Waltham, Massachusetts, USA). Three independent precipitations were prepared for proteomics and significance was determined using ANOVA method as described in detail before.[1] Briefly, relative quantification was performed based on cumulated peptide abundances of all unique peptides per protein. After normalization, statistical analysis was performed using transformed normalized abundances for one-way analysis of variance (ANOVA) calculations of all detected features and protein abundances were compared to protein abundances in precipitation control. Identified proteins were considered as specific interaction partners, if (i) the ratio of cumulated protein abundances in the sample versus mock control was above 2, (ii) the calculated ANOVA p-value was below 0.05 and (iii) the candidate was detected in at least in one sample in all three experiments. The relative mean enrichments were depicted with + = 2-10, ++ = 10-50 and +++ ≥50.

Gene expression profiling and gene set enrichment analysis

RNA was extracted using RNeasy Mini Kit (QIAGEN, Germany). Duplicates of each sample were analyzed in genome wide expression analyses on an Illumina HT12 v4 1-color chip (BIO.LOGIS, Germany) in reference to untransduced BJAB samples. Measured gene expressions were imported on raw bead level. All 10 microarrays were preprocessed and normalized together. Their intensity distributions for all measured beads were equalized by quantile normalization. The background mode was fitted and removed and a spot filter was applied to exclude too dim beads (< mean background intensity). Intensities were log2-transformed and aggregated by measured sequence to form beadsets. Beadsets with more than 50% of their beads excluded by the spot filter were also excluded. Further analysis was performed on gene level using median aggregation and manufacturer's annotations. For genes having multiple beadsets, the one with the highest expression (in average over all arrays) was selected. Moreover, signals from mock, CARMA1 WT, R35A or R35A/L225LI transduced BJAB cells were compared to BJAB CARMA1 L225LI as reference. To analyze the association to NF-κB and WNT, gene set enrichment analyses for corresponding signatures from the Molecular Signature Database v3.1,[2] the GeneSigDB v4,[3] and from the Staudt lab library[4] were performed as previously described.[5] For visualization, heatmaps depict gene expressions according to the color scale shown. To assess the significance of differential expressions, signature averages were compared to zero difference via t-tests; error bars depict s.e.m.s. The gene expression data has been deposited in the Gene Expression Omnibus (GEO) database[6] of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/geo; accession number GSE70025).

Quantitative RT-PCR primer

Following primer sequences were used in qRT-PCR: CCL3 (MIP-1α) for: 5´-GGCTCTCTGCAACCAGTTCT -3´ rev: 5´- TGAAATTCTGTGGAATCTGCC -3´; CCL4

for: 5´- CAGCGCTCTCAGCACCAA -3´ rev: 5´- AGCTTCCTCGCAGTGTAAGAAAA -3; CTNNB1 (β-catenin) for: 5´-GCAGAACTTGCCACACG-3´ rev: 5´-GCGTGTCTGGAAGCTTCC-3´; ICAM1 for: 5´-GGCTGGAGCTGTTTGAGAAC-3´ rev: 5´-ACTGTGGGGTTCAACCTCTG-3´; IL-10 for: 5´-CTGGAGGAGGTGATGCCCCAA-3´ rev: 5`-ACCTGCTCGACGGCCTTGCT-3´; IL-6 for: 5´-GGTACATCCTCGACGGCATCT-3´ rev: 5´-GTGCCTCTTTGCTGCTTTCAC-3´; NFKBIA (IκBα) for: 5´-CCGCACCTCCACTCCATCC-3´ rev: 5´-ACATCAGCACCCAAGGACACC-3´; RPII for: 5´-GCACCACGTCCAATGACAT-3´ rev: 5´-GTGCGGCTGCTTCCATAA-3´; TNFAIP3 (A20) for: 5´-TTTTGTACCCTTGGTGACCCTG-3´ rev: 5´-TTAGCTTCATCCAACTTTGCGG-3´; TNFRSF5 (CD40) for: 5´-GCAGTGGGTGGTTCTGGAT-3´ rev: 5´-CTGGTCTCACCTCGCTATGG-3´; TNFα for: 5´-CCCAGGGACCTCTCTCTAATCA-3´ rev: 5´-GCTACAGGCTTGTCACTCGG-3´; mCherry for: 5´-GCTTCAAGTGGGAGCGCG-3´ rev: 5´-GGAAGTTGGTGCCGCGCAG-3´.

Indirect immunofluorescence staining and analyses

Cells were fixed and permeabilized on PolyD lysine (Sigma, Germany) coated glass bottom ViewPlate-96F (Perkin Elmer, Waltham, Massachusetts, USA). Primary antibody staining was followed by secondary antibody staining with Alexa Fluor488 or Alexa Fluor633-conjugated secondary antibody (Lifetechnologies, Carlsbad, California, USA) and counterstained with Hoechst33342. 60x images were taken with an Operetta system (Perkin Elmer, Waltham, Massachusetts, USA) and analyzed with Harmony 3.5 software. For staining of cell colonies, 1.5x 10^4 cells/ml were seeded in collagen gels using 3D Collagen Cell culture system (Millipore, Germany). After 7 days, gels were fixed with 4% PFA and quenched with 0.15 M glycine. Immunostainings were done according to standard protocols.

Gels were mounted with Aqua-Poly/Mount Coverslipping Medium (Polyscience, Germany). Samples were imaged on an inverted confocal laser scanning microscope (Olympus, Japan) with 60x magnification.

SUPPLEMENTARY REFERENCES

1 Hauck SM, Dietter J, Kramer RL, Hofmaier F, Zipplies JK, Amann B *et al*. Deciphering membrane-associated molecular processes in target tissue of autoimmune uveitis by label-free quantitative mass spectrometry. *Molecular & cellular proteomics : MCP* 2010; **9:** 2292-2305.

2 Liberzon A, Subramanian A, Pinchback R, Thorvaldsdottir H, Tamayo P, Mesirov JP. Molecular signatures database (MSigDB) 3.0. *Bioinformatics (Oxford, England)* 2011; **27:** 1739-1740.

3 Culhane AC, Schroder MS, Sultana R, Picard SC, Martinelli EN, Kelly C *et al*. GeneSigDB: a manually curated database and resource for analysis of gene expression signatures. *Nucleic acids research* 2012; **40:** D1060-1066.

4 Shaffer AL, Wright G, Yang L, Powell J, Ngo V, Lamy L *et al*. A library of gene expression signatures to illuminate normal and pathological lymphoid biology. *Immunological reviews* 2006; **210:** 67-85.

5 Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA *et al*. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America* 2005; **102:** 15545-15550.

6 Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M *et al*. NCBI GEO: archive for functional genomics data sets--update. *Nucleic acids research* 2013; **41:** D991-995.

SUPPLEMENTARY TABLE AND FIGURE LEGENDS

Supplementary Table S1: Mass spectrometry approach identifies a list of putative CARMA1 interaction partners. LC-MS/MS identifies a total of 33 proteins in association to CARMA1. Three independent StrepTactin pull-down experiments were conducted in CARMA1 WT, WT stimulated (30 min P/I) and four different oncogenic CARMA1 mutant transduced BJAB. Solely significant protein interaction partners are listed with the premise that they passed ANOVA test (p-value< 0.05) and were identified in all three precipitation experiments of one sample at least. The ratio of mean peptide abundances of each sample related to the mock precipitation control was calculated and depicted with + = 2-10, ++ = 10-50 and +++ = >50. Infinite stands for incalculable values, because no peptides were detected in mock control. ANOVA p-values are given for each identified protein calculated in relation to mock control. † marks proteins that were identified by one single peptide.

Supplementary Table S2: WNT gene signatures are not significantly changed in BJAB cells expressing CARMA1 mutants. Gene set enrichment analyses of 12 publically available WNT signatures were based on the comparison of BJAB transduced with CARMA1 R35A, R35A/L225LI, WT, mock versus BJAB CARMA1 L225LI. p-values were calculated in permutation test.

Supplementary Figure S1: Transduction of CARMA1 in BJAB cells generates a polyclonal cell pool. Schematic depiction of CARMA1 domain structure and mutants. CARMA1 proteins were co-expressed with the cellular surface marker hΔCD2 (humanΔCD2), which was separated from CARMA1 protein via a ribosomal skip mechanism induced by a T2A sequence. hΔCD2 expression was analyzed by flow cytometry after staining with an APC-coupled antibody.

Supplementary Figure S2: CARMA1 mutants are recruited into insoluble aggregates. (a) CARMA1 constructs are expressed equally in transduced BJAB cells. Transduced BJAB cells were lysed with RIPA or co-IP buffer and protein levels were analyzed in Western blot. CARMA1 levels were quantified using β-Actin as loading control and related to mock transduced BJAB cells. (b and c) CARMA1 mutants are recruited into subcellular aggregates. (b) Representative immunofluorescence images of StrepII-tag stained CARMA1 in transduced BJAB cells. (c) Aggregates were quantified and related to BJAB CARMA1 WT (mean ± s.e.m.; n=3).

Supplementary Figure S3: CK1α acts as bridging factor for the association of the β-catenin destruction complex to oncogenic CARMA1. (a) CK1α co-localizes with oncogenic CARMA1 in the cytoplasm of transduced BJAB cells as shown in parallel immunofluorescence staining of endogenous CK1α and overexpressed StrepII-CARMA1. Cell nuclei were counterstained with Hoechst33342. (b and c) CK1α depletion influences CARMA1 L225LI stability. BJAB cells expressing CARMA1 WT or CARMA1 mutants were transduced with a DOX inducible shRNA system containing non-silencing (ns) or shRNA against β-catenin (shβCat) or CK1α. (b) Cell lysates were analyzed in Western blot. (c) StrepTactin pull-down of CARMA1 L225LI was assessed in Western blot after β-catenin or CK1α depletion.

Supplementary Figure S4: Stable expression of oncogenic CARMA1 to endogenous levels induces association of the β-catenin destruction complex in HBL1 cells. (a) Transduction of CARMA1 in HBL1 cells results in a polyclonal cell pool. hΔCD2 co-expression was determined in flow cytometry after staining with an APC-coupled antibody. (b) CARMA1 expression in transduced HBL1 cells was analyzed by Western blot. (c) CARMA1 L225LI but not CARMA1 Δlinker expression protects HBL1 cells from toxicity induced by BTK inhibition. HBL1 cells were transduced with CARMA1 constructs and

treated with Ibrutinib (2nM) for 4 days. Detection of viable HBL1 cells are given in relation to DMSO treated control (mean ± s.e.m.; n=3). (d) Oncogenic CARMA1 associates with the β-catenin destruction complex in transduced HBL1 cells. Complex formation was monitored in Western blot after StrepTactin pull-down of CARMA1 mutants in transduced BJAB cells.

Supplementary Figure S5: High expression of β-catenin in ABC DLBCL is not connected to tumor cell viability. (a and b) HBL1, OCI-Ly3 and OCI-Ly10 ABC DLBCL cells were transduced with a retroviral shRNA delivery system containing three independent shRNAs directed against βCat. (a) β-Catenin knock-down was verified in Western blot. shRNA directed against MSMO1 served as a control. (b) Cell viability was determined in cell counts with trypan blue exclusion (mean ± s.e.m.; n=3). Positive control shRNA directed against MYC was toxic to all DLBCLs.

Supplementary Figure S6: Stable expression of oncogenic CARMA1 in BJAB cells induces constitutive NF-κB activation. (a) NF-κB target gene expression is significantly enhanced in BJAB expressing CARMA1 L225LI. A representative NF-κB signature is shown for two independent RNA replicates of BJAB CARMA1 L225LI in reference to untransduced BJAB cells. Enrichment score (ES) -0.71, p<=0.001 via permutation test. (b-d) Upregulation of NF-κB signatures depends on active NF-κB signaling. Gene signatures of CARMA1 R35A, R35A/L225LI, WT or mock transduced BJAB sample RNA were compared to CARMA1 L225LI. p-values are based on Student t-tests against untransduced BJAB and error bars depict s.e.m.s. ES 0.56, p<=0.001 (b) and ES 0.75, p<=0.001 (c). (d) Gene set enrichment analyses of NF-κB gene signature shown in (c).

Supplementary Figure S7: β-catenin stabilization does not alter β-catenin dependent processes in BJAB expressing oncogenic CARMA1. (a and b) WNT gene signatures are not significantly regulated in transduced BJAB cells. (a) WNT gene signatures profiles were

compared in R35A, R35A/L225LI, WT and mock transduced BJAB cells in reference to L225LI transduced cells. Left: ES 0.26, p=0.613, Right: ES -0.202, p=0.975. (b) Gene set enrichment analyses of WILLERT WNT SIGNALING gene signature based on comparison of BJAB transduced with CARMA1 R35A, R35A/L225LI, WT, mock versus BJAB CARMA1 L225LI. (c and d) β-catenin stabilization is not affiliated with altered E-Cadherin localization or expression. Representative pictures of indirect immunofluorescence staining of endogenous E-Cadherin in CARMA1 WT or L225LI transduced BJAB grown in liquid culture (c) or in collagen gel (d). Nuclei were counterstained with Hoechst33342.

Supplementary Figure S8: TCF/LEF reporter is equally transduced into BJAB cells. (a) Schematic depiction of TCF/LEF reporter construct. 7xTcf-Luciferase reporter cassette is followed by an SV40-mCherry cassette. (b) Lentiviral TCF/LEF reporter transduction results in equal integration into BJAB cells expressing CARMA1. Integration levels of TCF/LEF reporter were determined using genomic DNA extractions of transduced BJAB cells. qRT-PCR were performed using primers directed against mCherry and values were related to RPII (mean ± s.e.m.; n=3). (c) Transduction of TCF/LEF reporter in BJAB cells results in a polyclonal cell pool. mCherry expression was determined in flow cytometry.

Supplementary Figure S9: β-catenin influences the expression of distinct NF-κB target genes. (a) β-catenin does not influence NF-κB DNA binding. BJAB expressing CARMA1 L225LI were transduced with a DOX inducible shRNA system containing shRNA against shβCat and analyzed by EMSA after DOX treatment. Supershift EMSA was performed using antibodies specific for p50, p65 and cRel and shifted complexes are marked with an asterisk (*). Positions of p50/p50, p50/cRel and p50/p65 NF-κB complexes are indicated by arrows. Oct1 EMSA was performed for control. Protein expression was assessed in Western blot analyses. (b) Expression of distinct NF-κB target genes is partially influenced by β-catenin. Left: mRNA levels of NF-κB target genes in untransduced BJAB or BJAB expressing

CARMA1 WT, L225LI or R35A/L225LI were measured in qRT-PCR. Values were normalized to CARMA1 WT (mean ± s.e.m.; n=4). Right: BJAB cells expressing CARMA1 WT or L225LI were transduced with a DOX inducible shRNA system containing ns or shβCat (mean ± s.e.m.; n=3). qRT-PCR was normalized to RPII. (c) β-catenin depletion was verified in RT-qPCR (mean ± s.e.m.; n=3). (d) BJAB cells expressing CARMA1 were transduced with a DOX inducible shRNA system containing ns or shβCat. β-catenin knock down was assessed in Western blot. (e) IL-10 expression is enhanced by β-catenin in CARMA1 L244P, S243P or F123I/K208M mutant BJAB. BJAB cells were transduced with a DOX inducible shRNA system containing ns or shβCat. Left: IL-10 mRNA expression was determined in qRT-PCR (normalized to RPII) and values were related to CARMA1 WT (mean ± s.e.m.; n=3). Right: Secreted IL-10 was measured in supernatants using ELISA (mean ± s.e.m.; n=3).

Mass spectrometry approach identifies a list of putative CARMA1 interaction partners

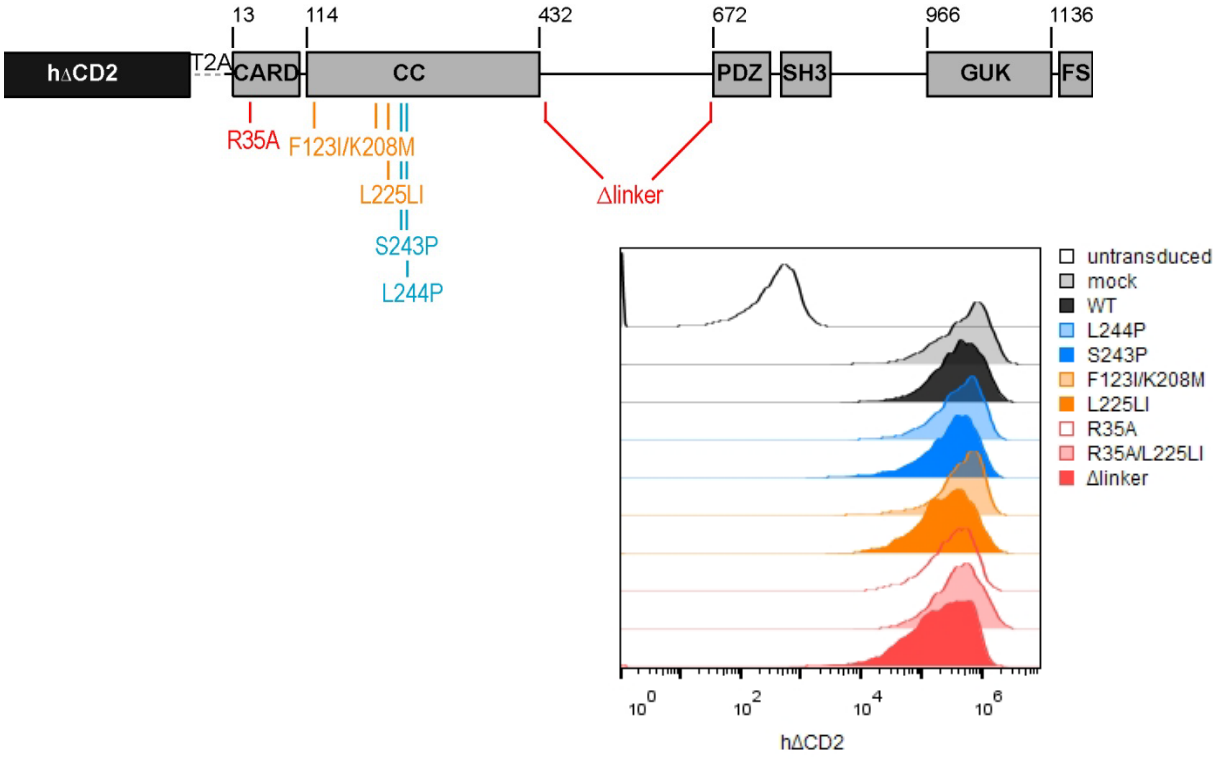
		WT		WT P/I		L244P		S243P		F123I/K208M		L225LI	
CARD11	ENSG00000198286	**+++**	1,6E-06	**+++**	1,6E-06	**+++**	1,7E-06	**+++**	6,7E-06	**++**	0,0001	**+++**	2,7E-06
TRIM14 †	ENSG00000106785											**+++**	5,0E-06
CSNK2A2	ENSG00000070770			**+++**	0,0427			**+++**	0,0465			**+++**	0,0176
GSK3A	ENSG00000105723											**+++**	0,0001
CSNK2B	ENSG00000204435	**++**	0,0087	**++**	0,0048			**++**	0,0071	**+**	0,0313	**+++**	0,0016
FAM83G	ENSG00000188522	**+**	0,0002	**+**	0,0029	**+++**	0,0012	**++**	0,0001	**+**	0,0433	**+++**	2,7E-06
FAM83D	ENSG00000101447			**+**	0,0313	**++**	0,0015	**+**	0,0053			**+++**	0,0006
APC	ENSG00000134982					**+**	0,0059	**+**	0,0220			**+++**	0,0001
CEP170	ENSG00000143702					**+**	0,0080	**+**	0,0122	**+**	0,0489	**+++**	5,1E-06
ZNF318	ENSG00000171467					**++**	0,0011	**+**	0,0015	**+**	0,0035	**+++**	2,7E-05
JUP	ENSG00000173801			**+**		**+**	0,0048					**+++**	0,0006
HMMR	ENSG00000072571					**++**	0,0100	**+**	0,0037			**++**	0,0006
PGAM5	ENSG00000247077	**+**	0,0187	**+**	0,0255			**+**	0,0126	**+**	0,0477	**++**	0,0001
GSK3B	ENSG00000082701											**++**	0,0082
AXIN1	ENSG00000103126					**+**	0,0161	**+**	0,0212			**++**	0,0007
GAPVD1	ENSG00000165219					**+**	0,0107	**+**	0,0028			**++**	0,0005
HSPD1	ENSG00000144381	**++**	0,0001	**++**	0,0001	**++**	0,0001	**++**	0,0001	**++**	0,0002	**++**	0,0001
FAM83H †	ENSG00000180921											**++**	0,0256
CTNNB1	ENSG00000168036											**++**	0,0073
WDR62	ENSG00000075702											**++**	0,0372
FAM91A1	ENSG00000176853	**+**	0,0015	**+**	0,0307	**+**	0,0208	**+**	0,0195			**++**	0,0048
USP9X	ENSG00000124486	**+++**	0,0060	**+++**	0,0040			**++**	0,0263			**++**	0,0230
WDR11	ENSG00000120008	**+**	0,0126	**+**	0,0305	**+**	0,0259					**++**	0,0121
DYNLL1 †	ENSG00000088986											**+**	0,0080
CSNK2A1	ENSG00000101266					**+**	0,0014	**+**	0,0147			**+**	0,0009
USP7	ENSG00000187555			**+**				**+**	0,0005				
MAP4K1 †	ENSG00000104814			**++**	0,0010								
BCL6 †	ENSG00000113916	Infinite	0,0315	Infinite	0,0300	Infinite	0,0169	Infinite	0,0194			Infinite	0,0075
CSNK1A1 †	ENSG00000113712	Infinite	0,0002	Infinite	3,3E-05	Infinite	1,5E-05	Infinite	6,6E-10			Infinite	2,7E-06
KLHL7 †	ENSG00000122550	Infinite	0,0243	Infinite	0,0329								
PLG †	ENSG00000122194	Infinite	0,0228	Infinite	0,0361					Infinite	0,0423	Infinite	0,0088
PSMD4 †	ENSG00000159352			Infinite	3,0E-07								
RLTPR †	ENSG00000159753			Infinite	0,0315	Infinite	0,0349						
SNAPIN †	ENSG00000143553											Infinite	2,7E-07

Supplementary Table S1

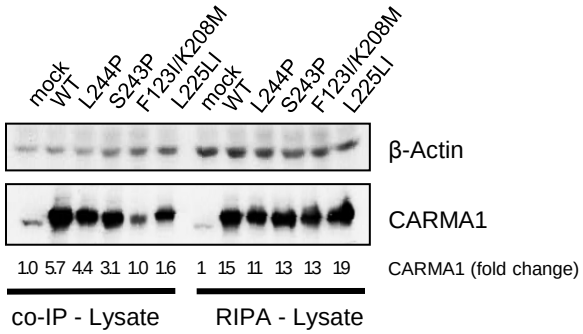
Gene set enrichment analyses for all available WNT signatures

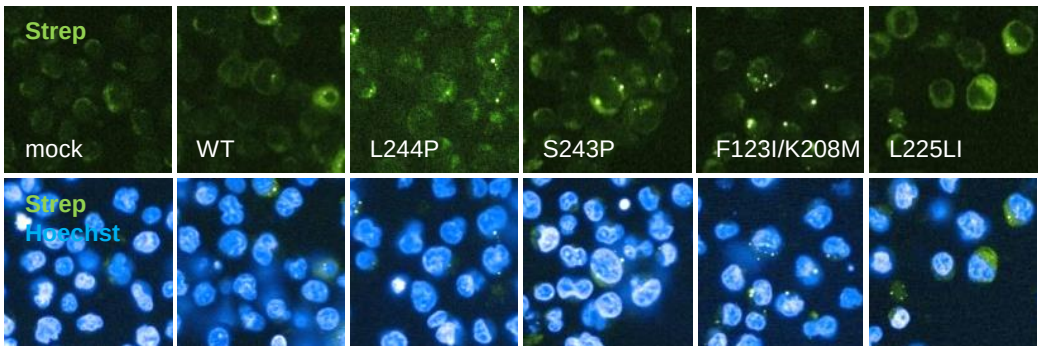
Signatures DB	Signature Name	Interpretation Card	Measured members	enrichment score	permutations	p[GSEA]
MolSigDBv4_0_dMay2014	REACTOME_SIGNALING_BY_WNT	http://www.broadinstitute.org/gsea/msigdb/cards/REACTOME_SIGNALING_BY_WNT	64	0,338	819	0,2210
MolSigDBv4_0_dMay2014	LABBE_TARGETS_OF_TGFB1_AND_WNT3A_DN	http://www.broadinstitute.org/gsea/msigdb/cards/LABBE_TARGETS_OF_TGFB1_AND_WNT3A_DN	108	0,288	352	0,3580
MolSigDBv4_0_dMay2014	WILLERT_WNT_SIGNALING	http://www.broadinstitute.org/gsea/msigdb/cards/WILLERT_WNT_SIGNALING	23	0,332	354	0,6271
MolSigDBv4_0_dMay2014	LABBE_WNT3A_TARGETS_UP	http://www.broadinstitute.org/gsea/msigdb/cards/LABBE_WNT3A_TARGETS_UP	112	0,257	369	0,6125
MolSigDBv4_0_dMay2014	BIOCARTA_WNT_PATHWAY	http://www.broadinstitute.org/gsea/msigdb/cards/BIOCARTA_WNT_PATHWAY	26	0,231	186	0,9624
MolSigDBv4_0_dMay2014	MORF_WNT1	http://www.broadinstitute.org/gsea/msigdb/cards/MORF_WNT1	107	0,206	268	0,9552
MolSigDBv4_0_dMay2014	KEGG_WNT_SIGNALING_PATHWAY	http://www.broadinstitute.org/gsea/msigdb/cards/KEGG_WNT_SIGNALING_PATHWAY	151	0,204	266	0,9925
MolSigDBv4_0_dMay2014	WNT_UP.V1_UP	http://www.broadinstitute.org/gsea/msigdb/cards/WNT_UP.V1_UP	178	-0,188	184	1,0000
MolSigDBv4_0_dMay2014	SANSOM_WNT_PATHWAY_REQUIRE_MYC	http://www.broadinstitute.org/gsea/msigdb/cards/SANSOM_WNT_PATHWAY_REQUIRE_MYC	58	-0,206	70	0,9857
MolSigDBv4_0_dMay2014	LABBE_WNT3A_TARGETS_DN	http://www.broadinstitute.org/gsea/msigdb/cards/LABBE_WNT3A_TARGETS_DN	96	-0,202	79	0,9747
MolSigDBv4_0_dMay2014	WNT_UP.V1_DN	http://www.broadinstitute.org/gsea/msigdb/cards/WNT_UP.V1_DN	170	-0,231	186	0,7204
MolSigDBv4_0_dMay2014	LABBE_TARGETS_OF_TGFB1_AND_WNT3A_UP	http://www.broadinstitute.org/gsea/msigdb/cards/LABBE_TARGETS_OF_TGFB1_AND_WNT3A_UP	111	-0,226	73	0,8493

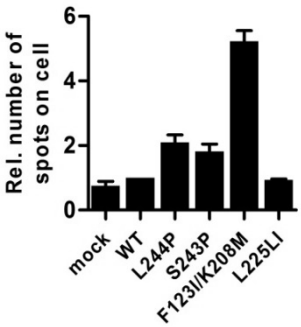
Supplementary Table S2



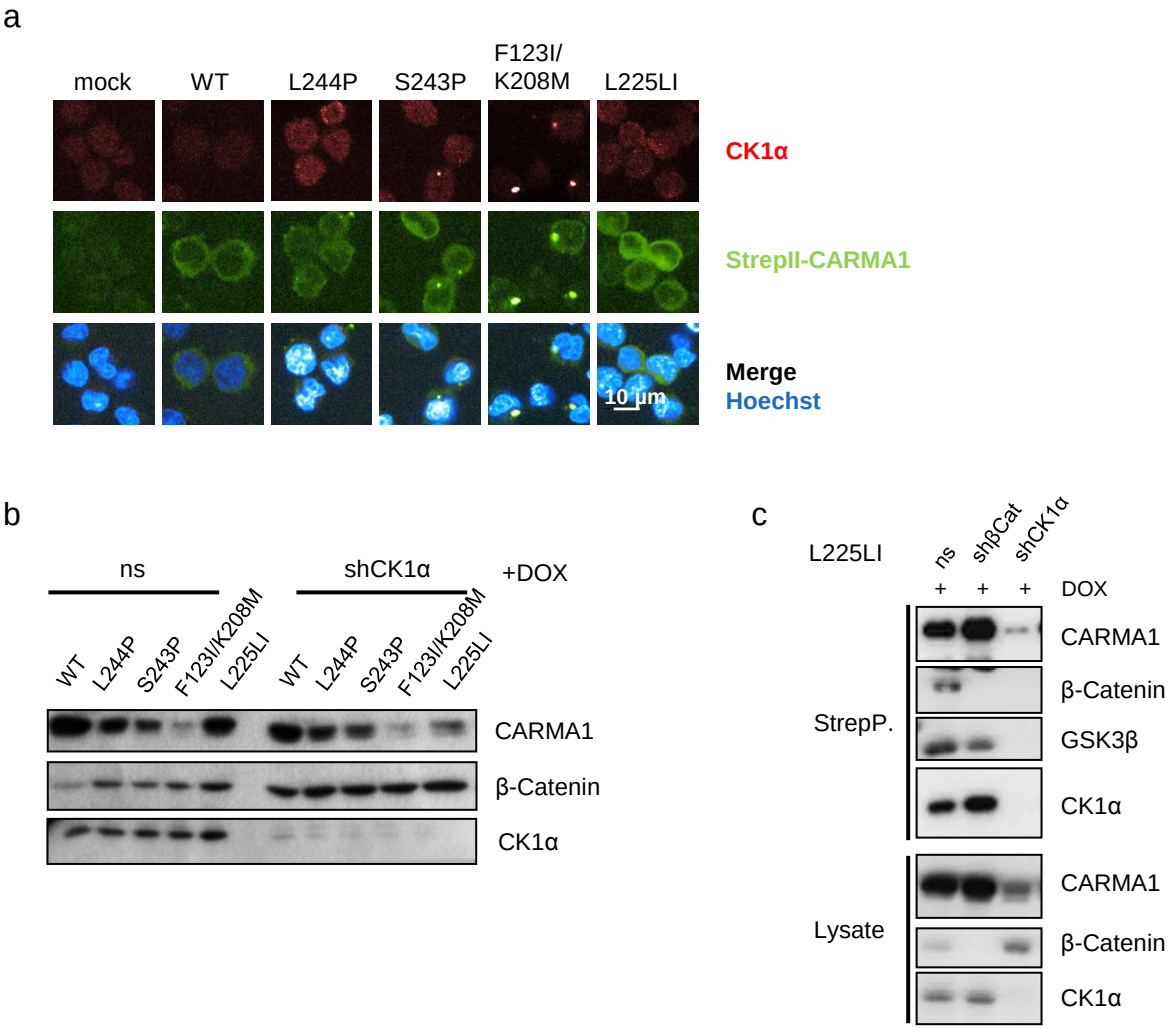
Supplementary Figure S1





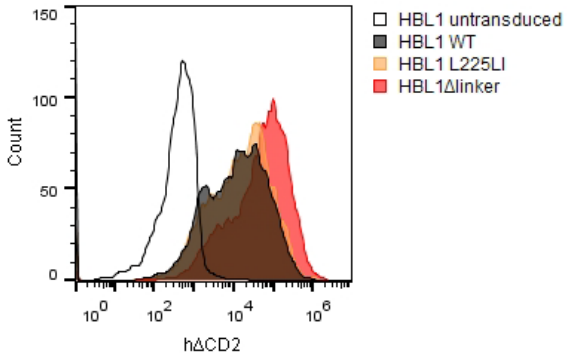


Supplementary Figure S2

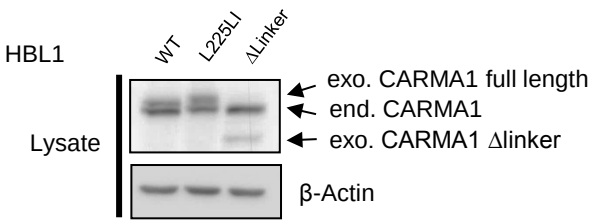


Supplementary Figure S3

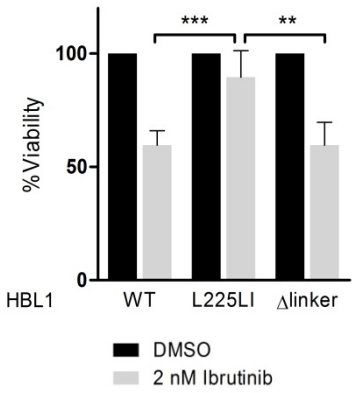
a



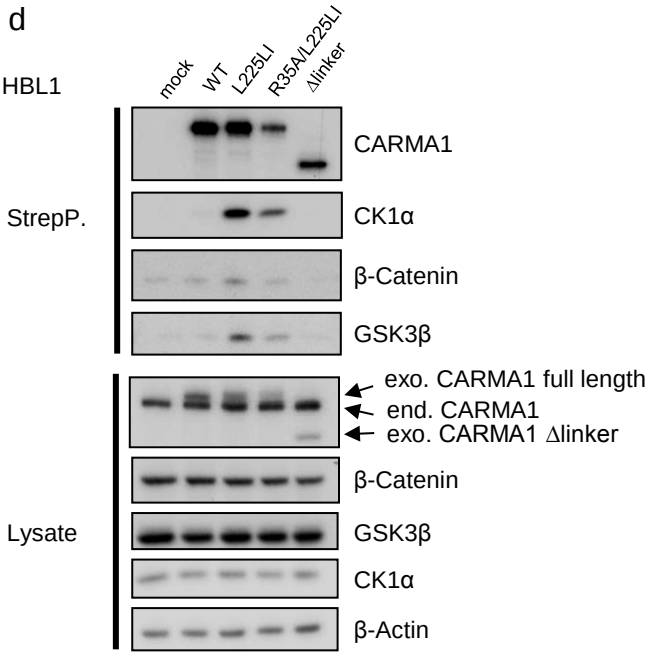
b



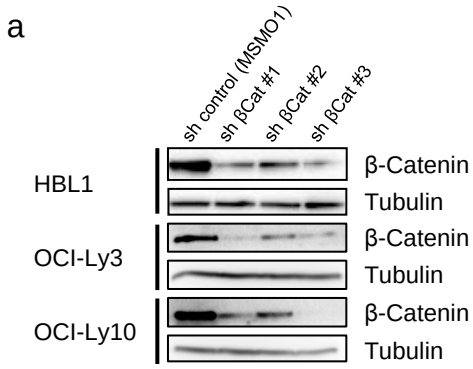
c

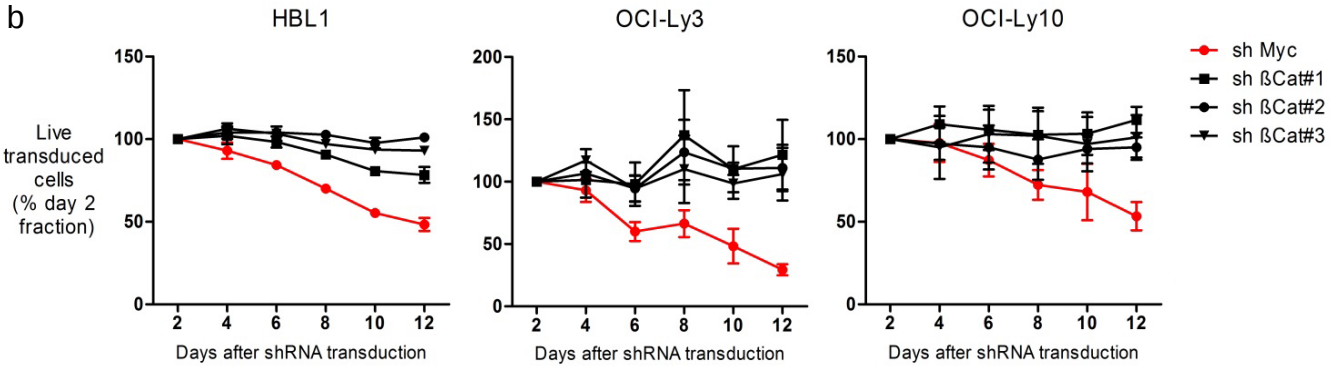


d

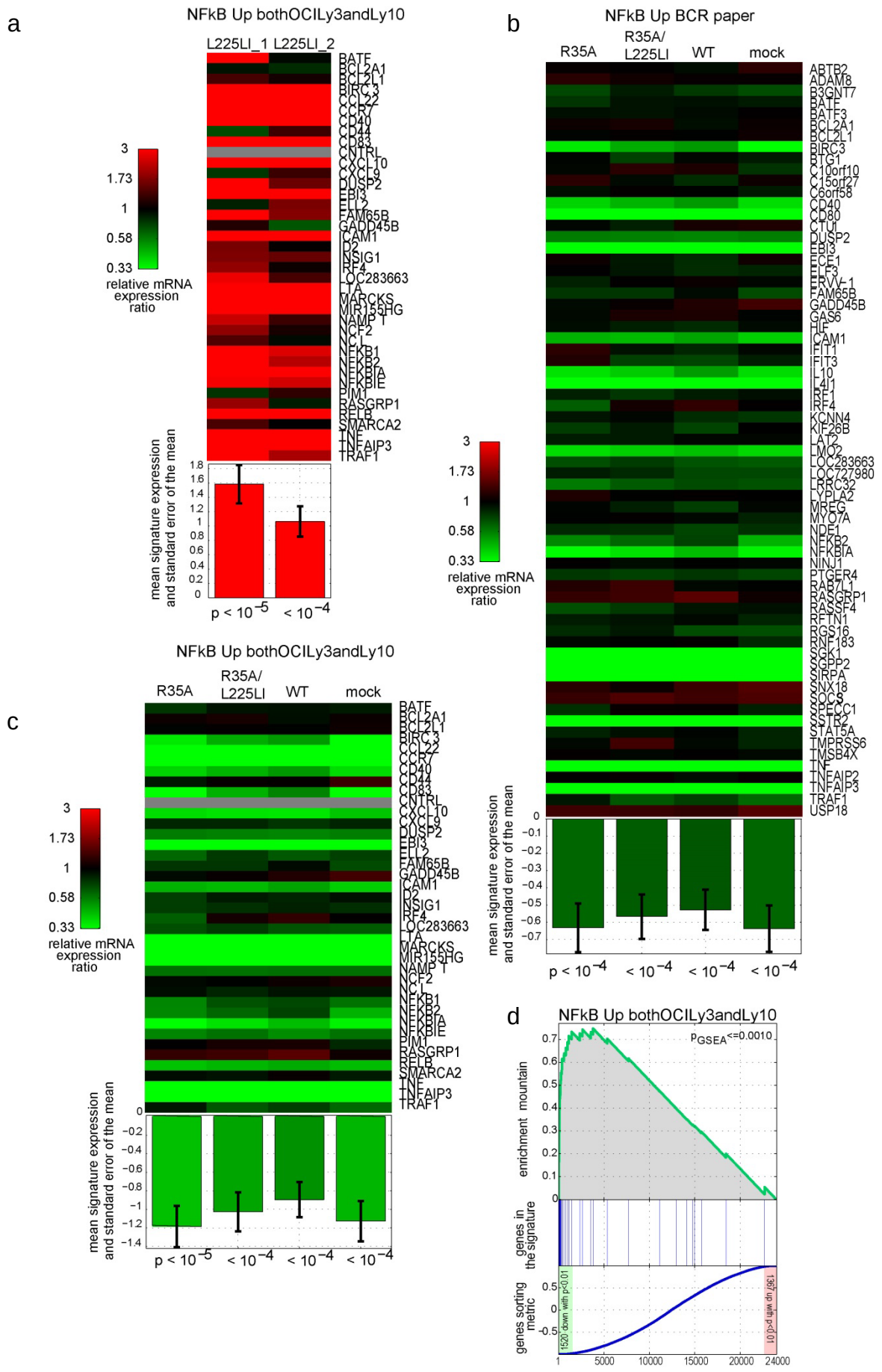


Supplementary Figure S4

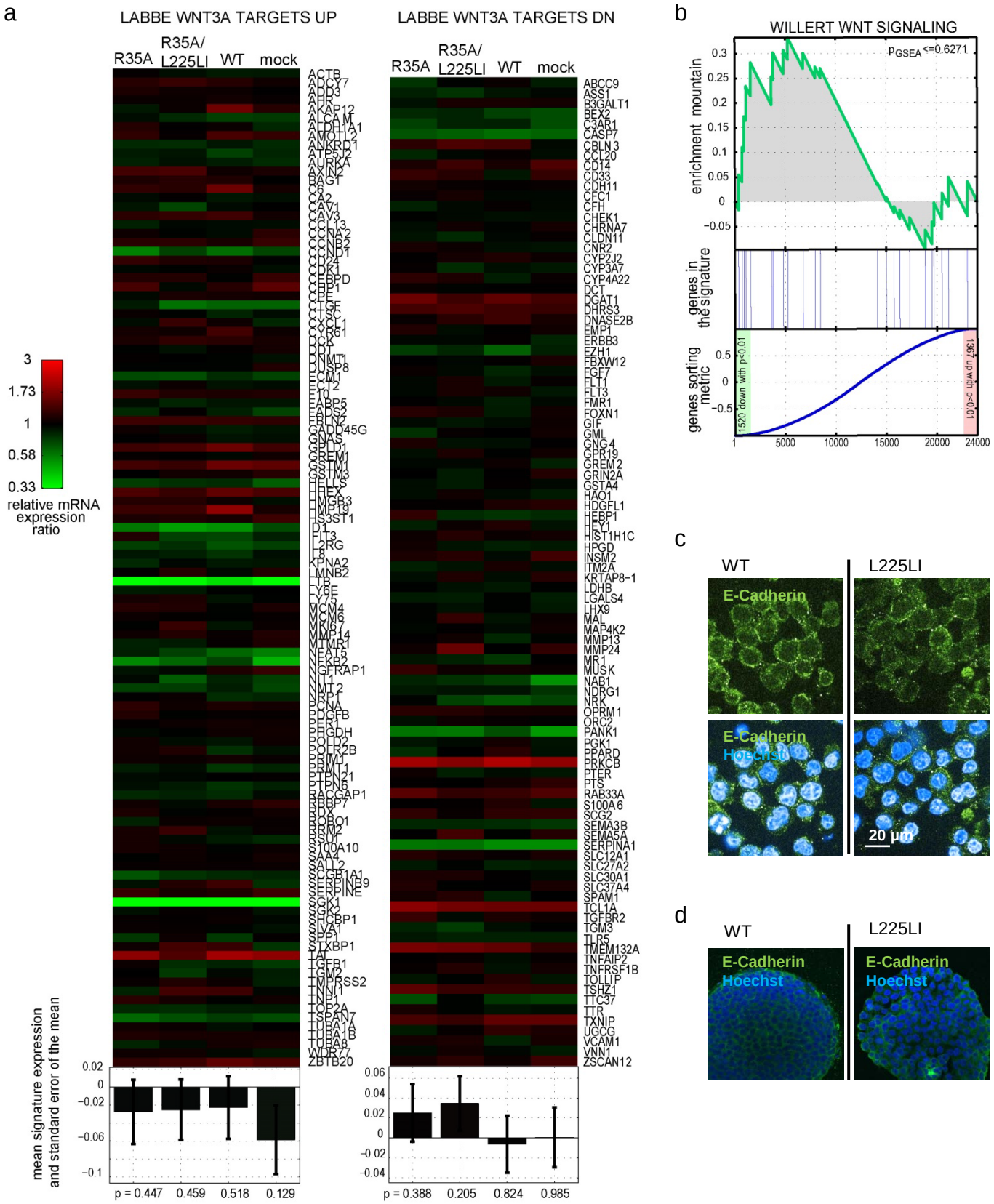




Supplementary Figure S5

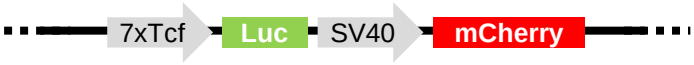


Supplementary Figure S6

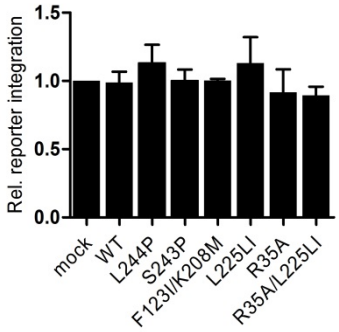


Supplementary Figure S7

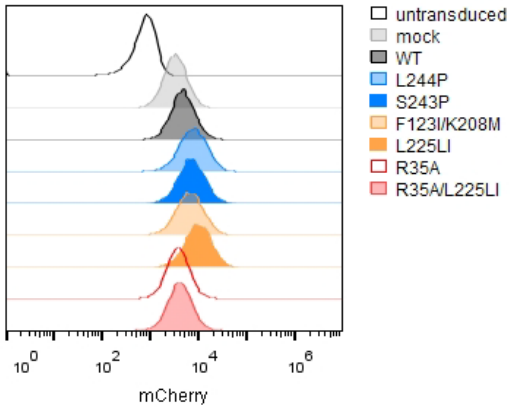
a



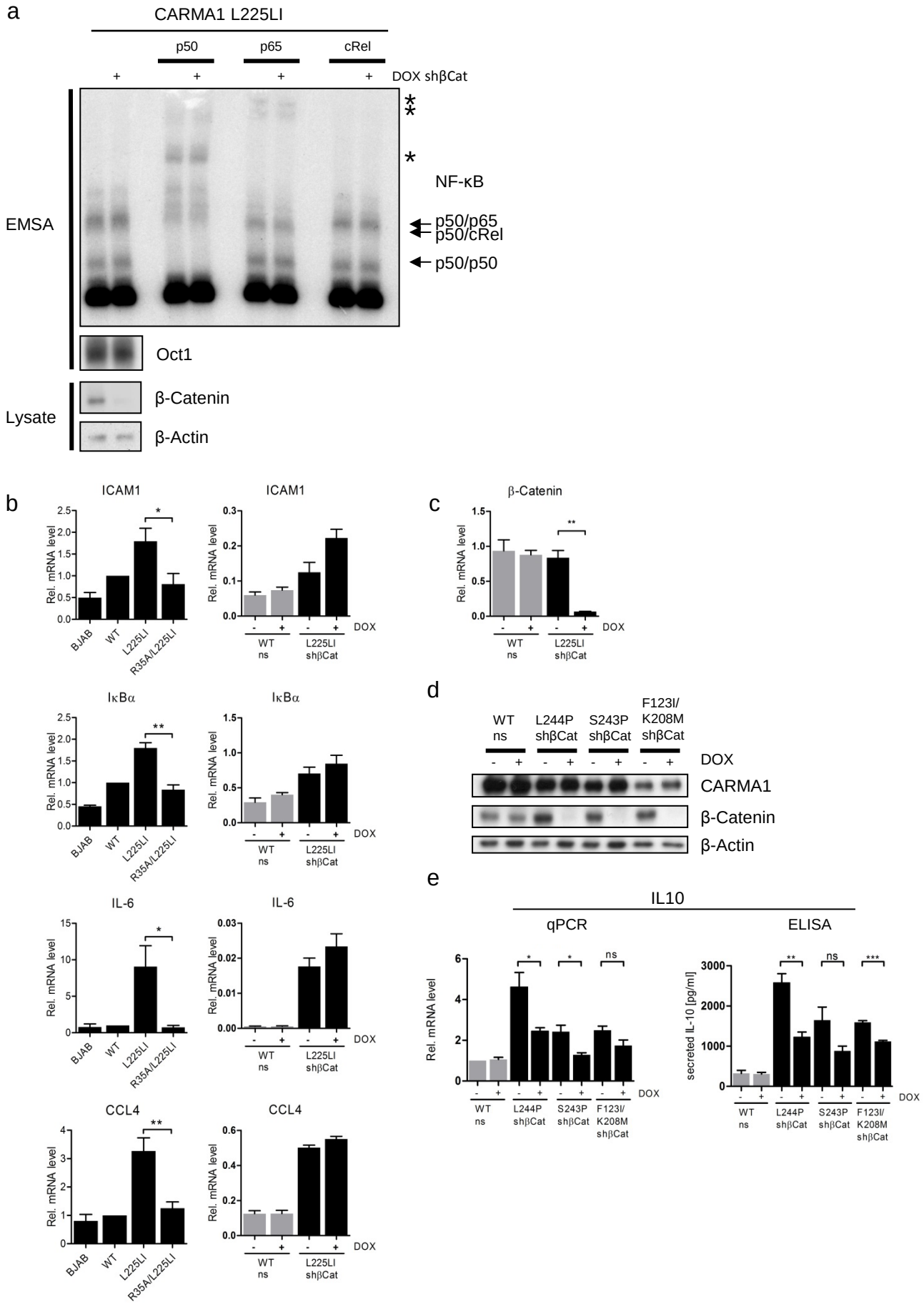
b



c



Supplementary Figure S8



Supplementary Figure S9