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APPENDIX METHODS:

Plasmids

Flag-FBXW7alpha, Flag-FBXW7beta, Flag-FBXW7gamma, HA-Cullin1, flag-FBXW1, flag-Skp2, flag-FBXW4 plasmids were kind gifts of Markus Welcker and Bruce Clurman (Fred Hutchinson Cancer Research Center, Seattle, USA). Skp1 and myc-Cul1 plasmids were kind gifts of Nikita Popov (University Würzburg, Germany). STYX-pcDNA3, YFP2-STYX, HA-STYX were described before (Reiterer et al, 2013). MCL1 plasmid was obtained from Addgene (25375). STYX_FQQ was generated by replacing 76FQQ78 with alanine residues. Primers used for site directed mutagenesis are the following:

Forward: 5'- TGG ATT ATC TGC AAT ATC CAG GAC TAA ATA TCT AAA TAA CGC CGC AGC GTT TGG TTT AAT AAA GTT TGC TTC AAT ATT TTG TCG TAT G-3'

Reverse: 5'-CAT ACG ACA AAA TAT TGA AGC AAA CTT TAT TAA ACC AAA CGC TGC GGC GTT ATT TAG ATA TTT AGT CCT GGA TAT TGC AGA TAA TCC A -3'.

HA-FBXW7alpha was obtained by subcloning FBXW7 into HA-pCMV. HA-FBXW7 lacking the F-Box was cloned by site directed mutagenesis using the following primers:

Froward: 5'GTGATAGAACCCCAGTTTCAATGCAAAGAAGAGGGGA3'as

Reverse: 5'-TCCCCTCTTCTTTGCATTGAAACTGGGGTTCTATCAC-3'

HA-FBXW7_R465C mutant was cloned with site directed mutagenesis using the following primers:

Forward: 5' CAT GAA GAT GCA TAC AAC ACA CAG TGG AAG TAT GCC C 3'

Reverse: 5'GGG CAT ACT TCC ACT GTG TGT TGT ATG CAT CTT CAT G 3'.

YFP1-FBXW7 was obtained by PCR subcloning FBXW7 into YFP1-pcDNA3 (Reiterer et al 2013). YFP1-FBXW7_lacking the F-box was obtained by site directed mutagenesis using the primers described above. GST-FboxFBXW7 was obtained by PCR subcloning the F-Box of FBXW7 into pGEX-4t-3 vetcor.

Cycloheximide chase

Cells were treated with 50ug/ml cycloheximide for the indicated time periods. Subsequently cells were lysed in buffer (50 mM Tris‑HCl, pH 7.4, 10 mM EDTA, 100 mM NaCl, 0.1% SDS, and 1% NP-40)

supplemented with proteinase inhibitor (cOmplete Mini; Roche) and phosphatase inhibitor (PhosSTOP; Roche).

Real time PCR

Cells were harvested using the RNA extraction kit (RNeasy, Qiagen), according to manufacturer's instructions. Concentration and purity of mRNA was determined using a Spectrophotometer (Nano drop™). 1 µg total RNA was reverse transcribed into cDNA using High Capacity cDNA kit (Applied Bioscience) according to manufacturer's instruction. The following primers were used:

GAPDH-forward: 5′-CGCTCTCTGCTCCTCCTGTT-3′

GAPDH-reverse: 5′-CCATGGTGTCTGAGCGATGT-3′

c-Myc-forward: 5′-TTCTCTCCGTCCTCGGATTC-3′

c-Myc-reverse: 5′-TCATCTTCTTGTTCCTCCTCAGA-3′

STYX: Hs_STYX_1_SG QuantiTect Primer Assay (Qiagen) were used.

Real-time quantitative PCR was performed using FASR-SYBR Green master mix (Applied Biosystems) according to manufacturer's instructions.

Tissue samples, RNA extraction, Affymetrix GeneChip hybridization, and QPCR

Tissue specimens, 12 non-pathologic breast samples and 61 primary breast cancers, were obtained by patients who underwent surgery at the European Institute of Oncology (Milan, Italy). Written informed consent for research use of biological samples was obtained from all patients, and the research project was approved by the Institutional Ethical Committees. Samples were immediately snap-frozen in liquid nitrogen and stored at -800 C. Briefly, total RNA was extracted from 10-20 serial sections (10 µm thick) of snap-frozen specimens using commercial homogenization (QIAshredder) and purification (RNeasy Mini Kit) reagents (Qiagen, Valencia, CA, USA). RNA was analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (500 ng) was amplified using the T7-Polymerase-based double linear amplification protocol. cRNA probes (25 µg) were hybridized onto the Affymetrix (Santa Clara, CA, USA) HG-U133 ChipSet, according to Affymetrix technical protocols. CEL files were analyzed with the Affymetrix's proprietary MAS5 pre-processing algorithm, and the average intensity of each array was scaled to a predefined value (target intensity) of 500, in order to make arrays comparable. Probeset signal values for STYX and FBXW7 were extracted and data analysis was performed using JMP 10.0 statistical software (SAS Institute, Inc).

For STYX and FBXW7 mRNA reverse transcription quantitative real-time PCR (RT-qPCR) analysis, 250 ng of total RNA (RNA concentration measured using the NanoDrop® ND-1000 Spectrophotometer) were reverse transcribed with random primers using the SuperScript® VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific) and 5 ng of cDNA/reaction were analyzed by PCR.

Quantitative PCR was performed with hydrolysis probes (Thermo Fisher Scientific) using the SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories) in 10 µl of final volume in 384-well plates. PCR reaction was run in LightCycler (LC) 480 real-time PCR instruments (Roche) using the following thermal cycling conditions: 1 cycle at 95°C for 30 sec, 45 cycles at 95°C for 5 sec, and 60°C for 30 sec.

TaqMan gene expression assays were as follows: Hs00377042_m1 (human STYX, RefSeq NM_001130701.1, exon boundary 2-3, assay location 115, amplicon length 68 bp), Hs00217794_m1 (human FBXW7, RefSeq NM_001013415.1, exon boundary 3-4, assay location 548, amplicon length 76 bp), Hs03929097_g1 (human GAPDH, RefSeq NM_001256799, exon boundary 8-8, assay location 1250, amplicon length 58 bp), Hs99999908_m1 (human GUSB, RefSeq NM_000181, exon boundary 11-12, assay location 1925, amplicon length 81 bp), Hs02800695_m1 (human HPRT1, RefSeq NM_000194.2, exon boundary 2-3, assay location 297, amplicon length 82 bp) and Hs00427621_m1 (human TBP, RefSeq NM_001172085, exon boundary 3-4, assay location 666, amplicon length 65 bp).

For RPLP0 detection a custom assay (Forward primer: CCATTGAAATCCTGAGTGATGT; Reverse primer: CAAAGTGGGAGCCAGCGA; Probe: GCAGCTGATCAAGACTGGAGA) was designed using the Primer Express Software V3.0 (Thermo Fisher Scientific).

Each target was assayed in triplicate and average Cq values were calculated (the average was calculated from triplicate values when the standard deviation was <0.4, or from the best duplicate values when the standard deviation was ≥0.4). Data (average Cq) were normalized on the average Cq value of five endogenous reference genes (GAPDH, GUSB, HPRT1, TBP and RPLP0) for accurate sample normalization and to the average of normal samples using the comparative Cq (2-ΔΔCq) method. Statistical analysis was performed using JMP 10.0 statistical software (SAS Institute, Inc). Raw Data and statistical analysis for STYX and FBXW7 are presented in Table EV3. The complete Affymetrix dataset is available upon request.

APPENDIX FIGURE LEGENDS

Figure S1. *A*, HeLa cells were transfected with two different siRNAs against STYX obtained from Dharmacon (D) or from Qiagen (Q). After 72 h, cells were lysed and immunoblotted as indicated. *B*, HeLa cells were transfected with a non-targeting siRNA (Co) or with the two different siRNAs targeting STYX (D and Q as in panel A). After 72 h, cells were lysed followed by immunoprecipitation and immunoblotting of FBXW7. *B,* HeLa cells were transfected with plasmids encoding YFP-tagged or HA-tagged FBXW7. In addition cells, were co-transfected with increasing amounts of STYX. After 24 h, cells were lysed and subjected to immunoprecipitation against HA followed by immunoblotting against the indicated proteins.

Figure S2. *A*, GST-tagged F-box of FBXW7 was expressed in and purified from E-coli. The purified protein was incubated with a lysate from cells expressing HA-tagged STYX followed by GST-pulldown and immunoblotting as indicated. *B,* FRET assay in HeLa cells expressing YFP-tagged FBXW7 and CFP-tagged SKP1 alone (orange bar) or together with non-tagged STYX (grey bar). The blue bar represents FRET between CFP-tagged SKP1 and the YFP signal emanating from the complementation of Y1-tagged FBXW7 and Y2-tagged STYX. FRET was determined using the acceptor photobleaching method. *C,* HeLa cells were transfected with an empty vector or with plasmids encoding for HA-tagged STYX, FBXW7 or SKP1. Cells were lysed 24 h after transfection and subjected to anti-HA immunoprecipitation followed by immunoblotting as indicated. *D,* evaluation of the YFP-BiFC experiment from Figure 5A.

Figure S3. *A,* MDA-MB-231 cells were transfected with a non-targeting siRNA (siCo) or with siRNA against STYX (siSTYX). After 72 h, cells were treated with DMSO or Doxorubicin (10 µM) for 48 h followed by lysis and immunoblotting as indicated. *B,* HeLa cells transfected with siRNA against STYX or with non-targeting control (Co) were treated with doxorubicin (10 µM) for 8 hours followed by lysis and immunoblotting as indicated. *C,* MDA-MB-231 cells were transfected with a non-targeting siRNA (siControl) or with siRNA against STYX (siSTYX). After 72 h, cells were treated with DMSO or Doxorubicin (10 µM) for 48 h. Afterwards cells were stained with 6-carboxyfluorescein diacetate to label live cells (green) and with annexin-V Cy3 (red) to label apoptotic cells. Representative images are shown on the left side and an evaluation of three experiments is shown on the right side. Scale bar= 75µm. *D,* MDA-MB-231 cells stably expressing a non-targeting shRNA or an shRNA against STYX were plated on poly-hema treated dishes to prevent attachment (10'000 cells per well in a 96 well plate). Cells were grown for 5 days and were passaged for 10 times through a 20µl pipette tip followed by fixation in 4% PFA and imaging. The number of colonies was determined and an evaluation of 4 experiments is shown on the right side of the panel. Scale bar = 250µm.

Figure S4. *STYX does not modulate the survival of patients with PIK3CA or GOLGA2 alterations.* Kaplan-Meier plot of the relapse-free survival of 1660 breast cancer patients generated through the kmplot database (http://kmplot.com/).

Figure S1

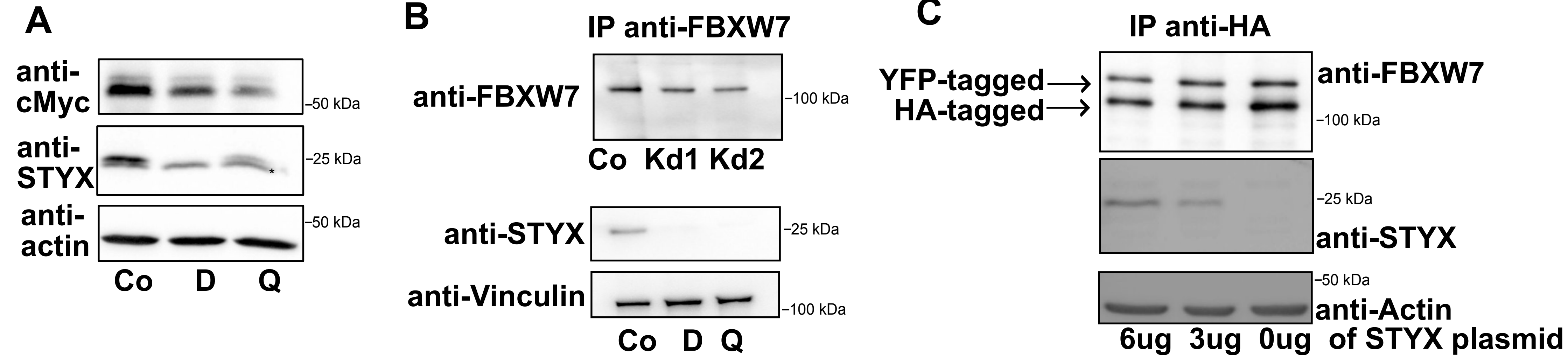
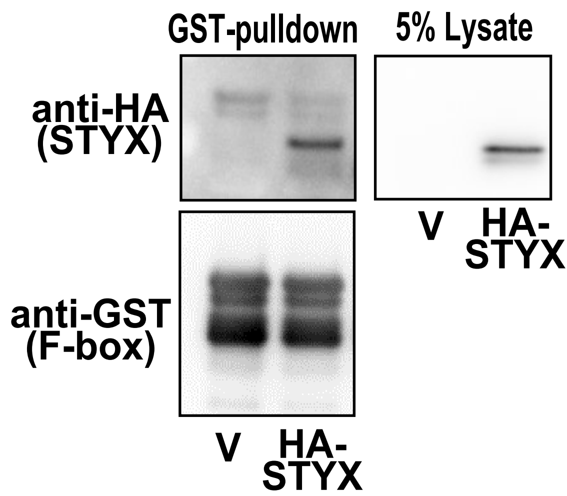
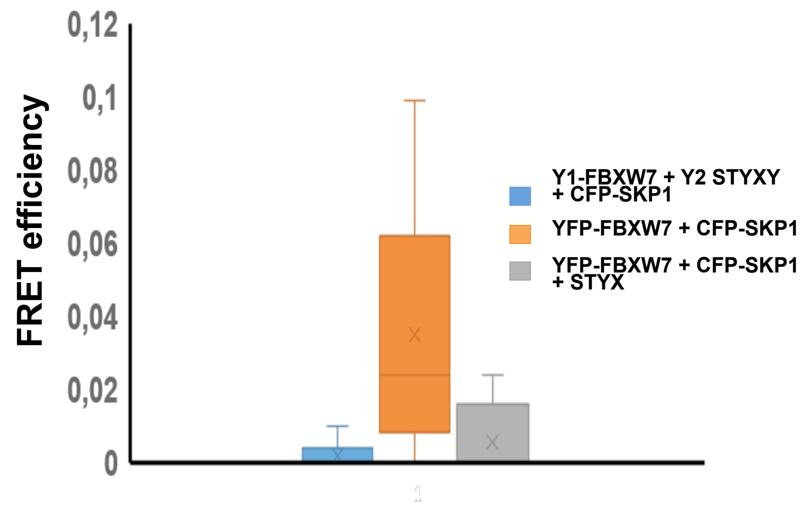
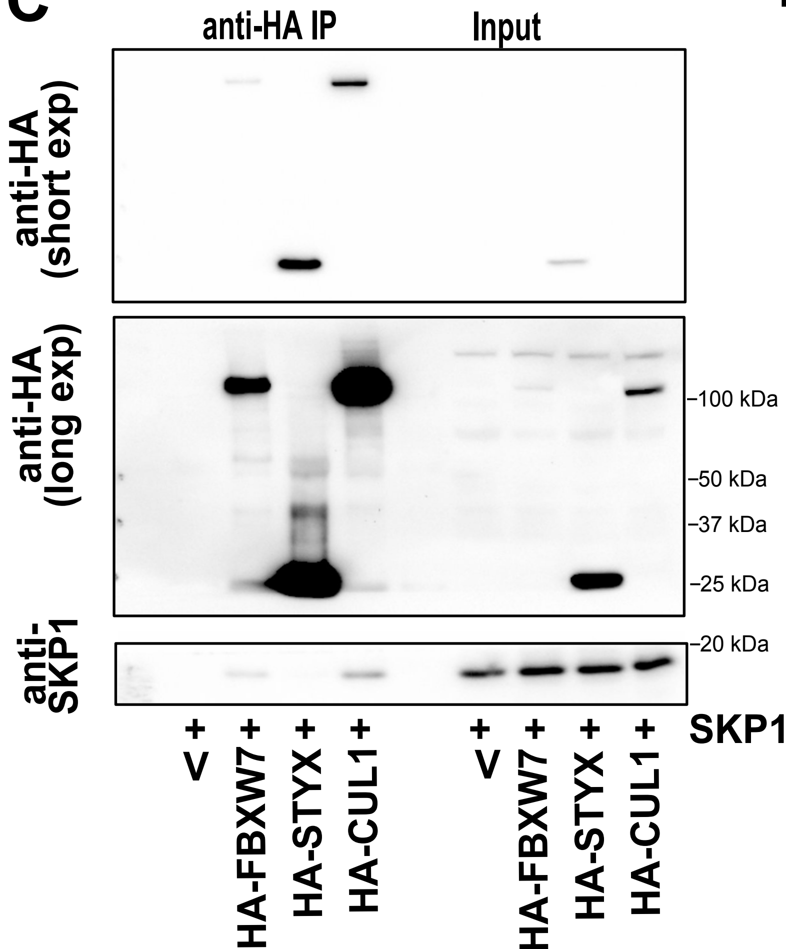


Figure S2







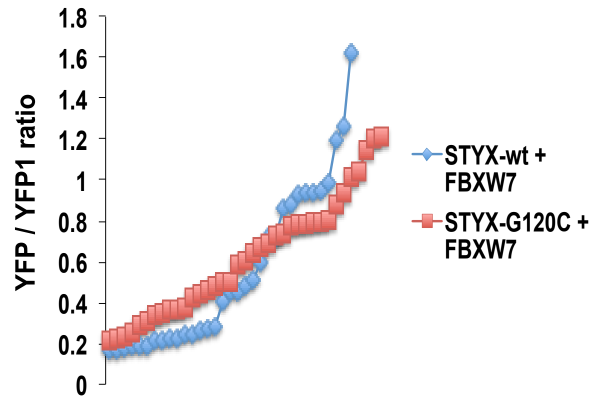
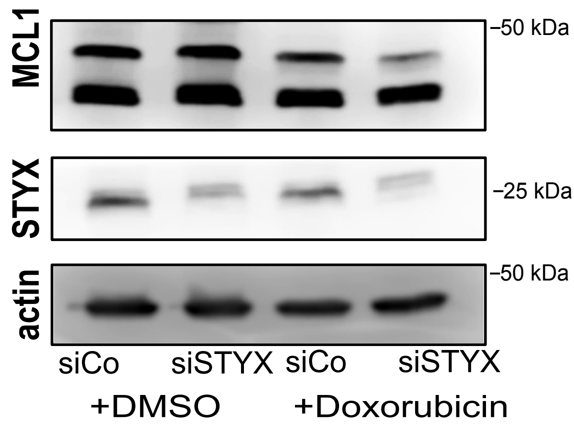
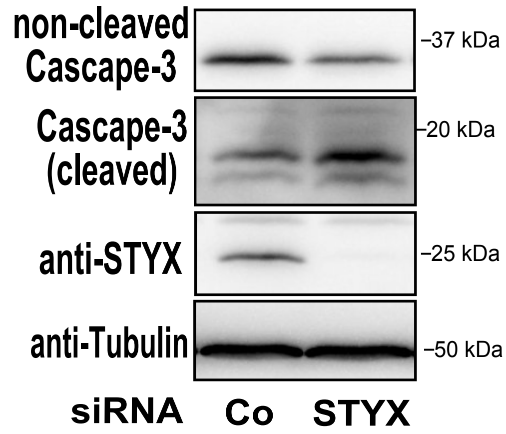
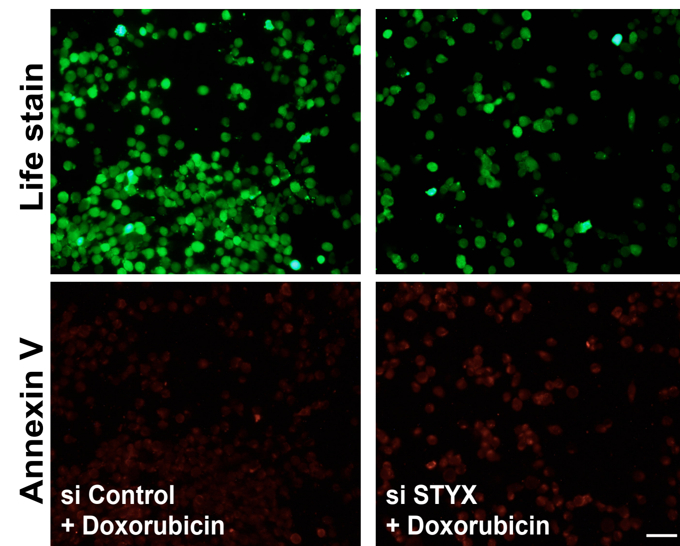
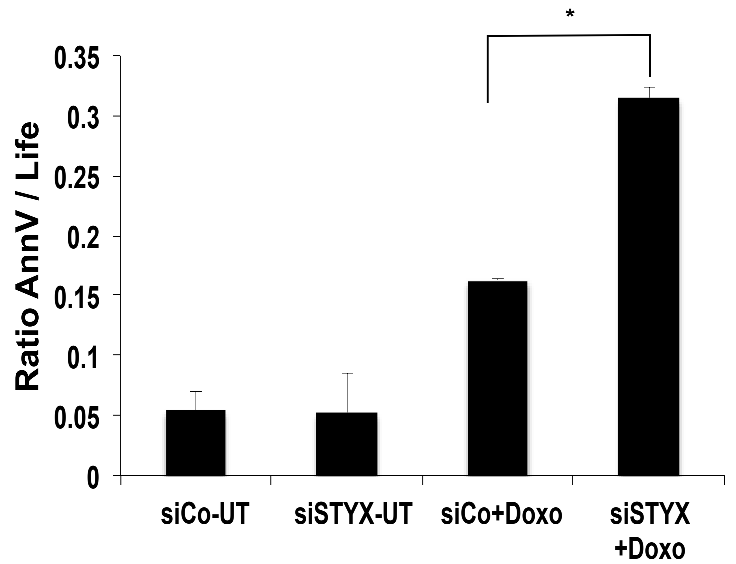


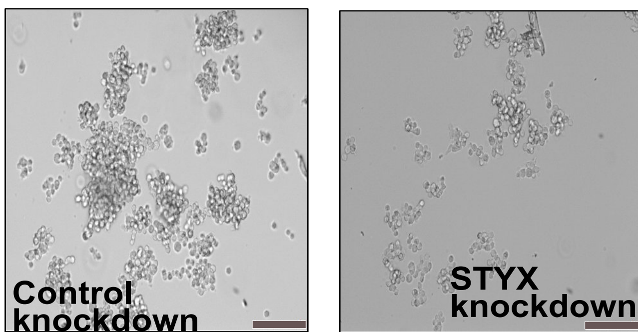
Figure S3











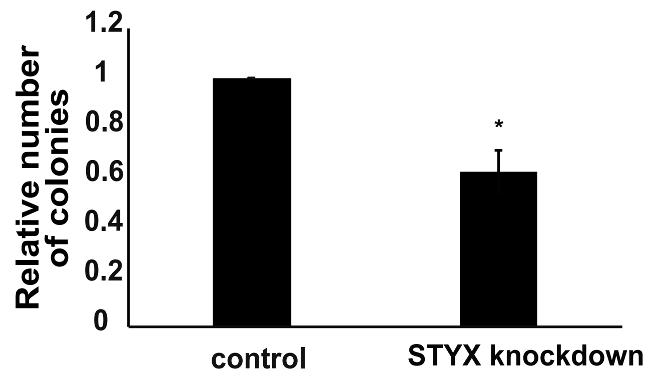
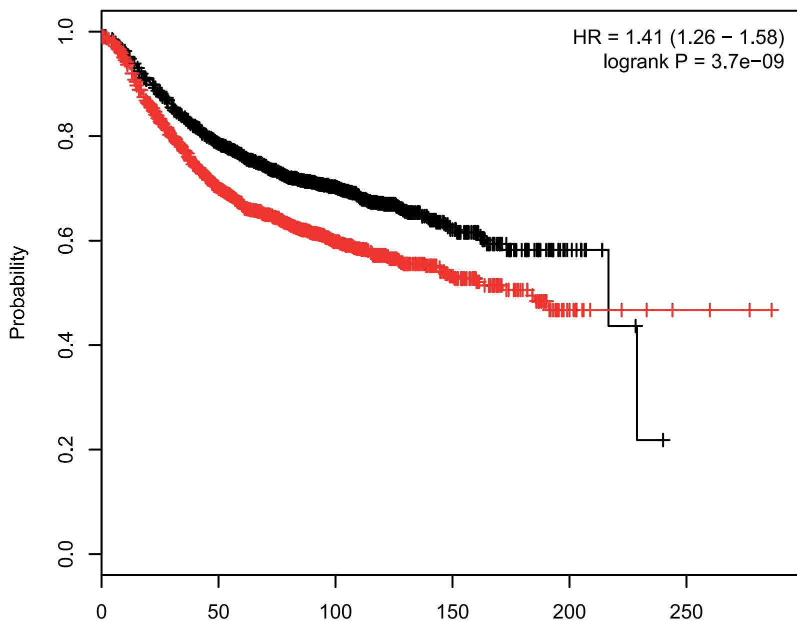
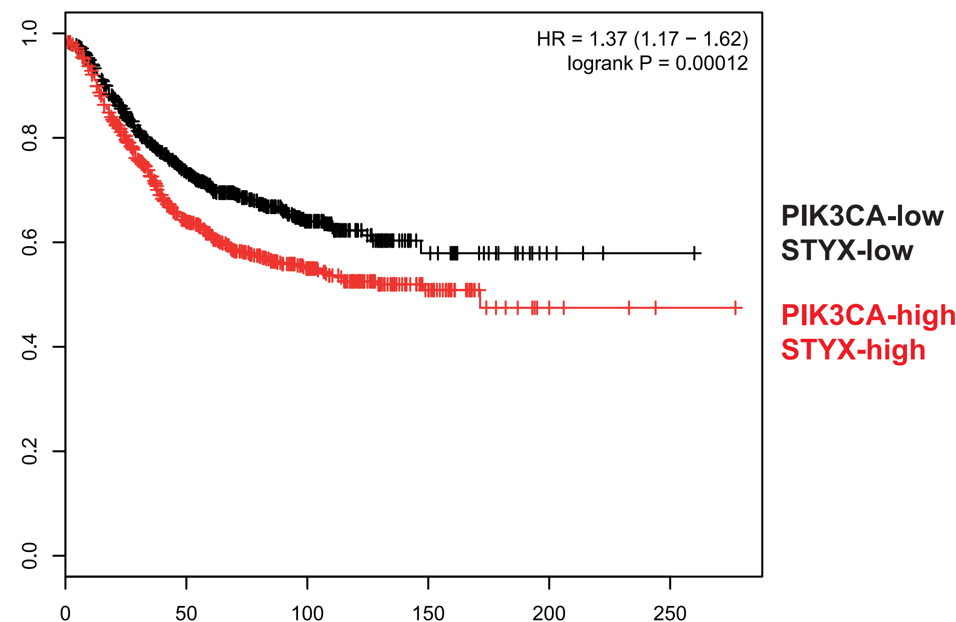
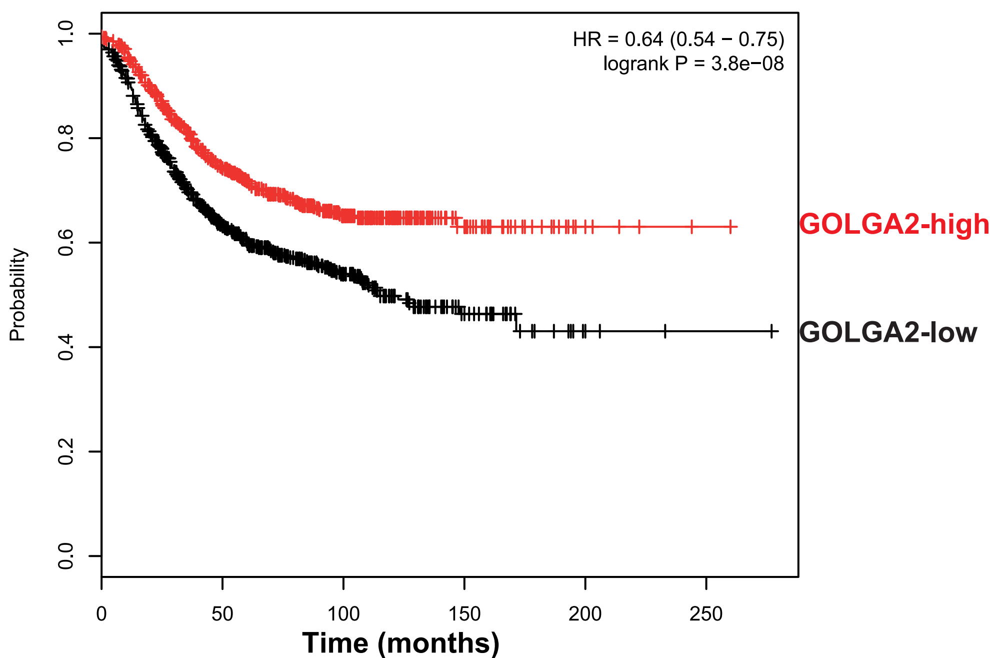
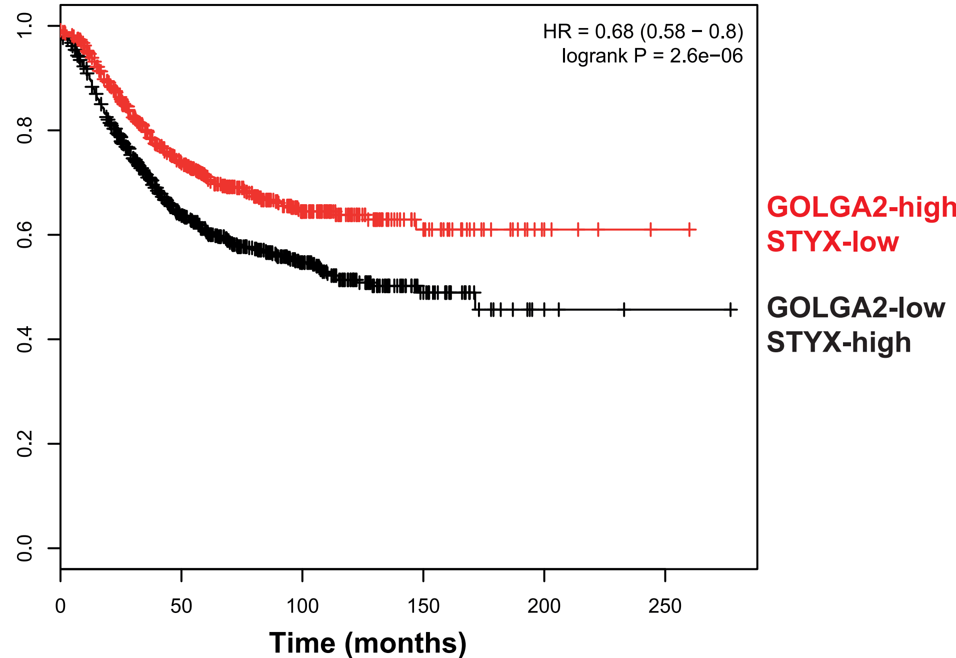


Figure S4









Appendix Table S1- List of antibodies used in this work.

Antibody	nr	company	used
flag-M2	F1804	Sigma Aldrich	IF
FLAG® M2-Peroxidase (HRP) mouse monoclonal antibody	A8592	Sigma Aldrich	WB
Monoclonal Anti-HA−Peroxidase antibody produced in mouse	H6533	Sigma Aldrich	WB
STYX	custom made	Thermo Scientific	WB
c-myc	5605	Cell Signalling	WB
cyclinE	sc-247	Santa Cruz	WB
Mcl-1	sc-819	Santa Cruz	WB
Skp1	sc-7163	Santa Cruz	WB
Cullin 1	sc-12761	Santa Cruz	WB
actin		Abcam	WB
tubulin		kind gift of Karl Matter	WB
Caspase-3	9662	Cell Signalling	WB
GFP	ab32146	Abcam	IF
GST	sc-138	Santa Cruz	WB
GFP	11814460001	Roche	IF
Vinculin		Thermo Fisher	WB
FBxW7	A301-720A	Bethyl	WB