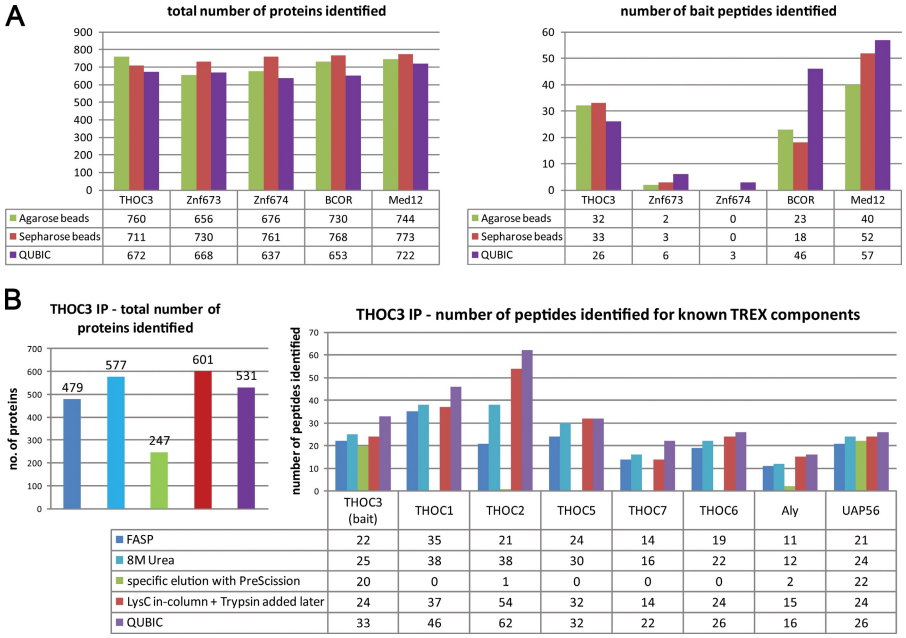
Hubner et al., http://www.jcb.org/cgi/content/full/jcb.200911091/DC1



	THOC3	Znf673	Znf674	BCOR	Med12
Agarose beads	760	656	676	730	744
Sepharose beads	711	730	761	768	773
QUBIC	672	668	637	653	722

	THOC3	Znf673	Znf674	BCOR	Med12
Agarose beads	32	2	0	23	40
Sepharose beads	33	3	0	18	52
QUBIC	26	6	3	46	57

	THOC3 (bait)	THOC1	THOC2	THOC5	THOC7	THOC6	Aly	UAP56
FASP	22	35	21	24	14	19	11	21
8M Urea	25	38	38	30	16	22	12	24
specific elution with PreScission	20	0	1	0	0	0	2	22
LysC in-column + Trypsin added later	24	37	54	32	14	24	15	24
QUBIC	33	46	62	32	22	26	16	26

Figure S1. **Development of the QUBIC technology.** (A) GFP-tagged proteins with different cellular background and localization were immunopurified using anti-GFP antibody coupled to agarose, sepharose, or very small magnetic beads (QUBIC), digested on bead or in column with trypsin, and analyzed in a single run on the mass spectrometer. (right) In all three cases, the QUBIC purification was cleanest in terms of the smallest number of background proteins identified. QUBIC resulted in the best sequence coverage of the bait protein. (B) Different elution methods were tested on GFP-THOC3, including elution with SDS buffer followed by FASP (Wiśniewski et al., 2009), elution with 8 M urea, specific elution involving the PreScission cleavage site in the LAP tag, LysC in-column digest followed by trypsin digestion, and QUBIC (trypsin in-column digestion). (left) As expected, specific cleavage resulted in the highest purity of IP. (right) However, THOC3 is a typical example of a shielded PreScission cleavage site for the complexed bait, as most of the TREX components were not identified. The adequate coverage of the bait protein may result from purified free or UAP56-bound THOC3-GFP in the cell. Overall, QUBIC again resulted in the best sequence coverage of the bait protein.

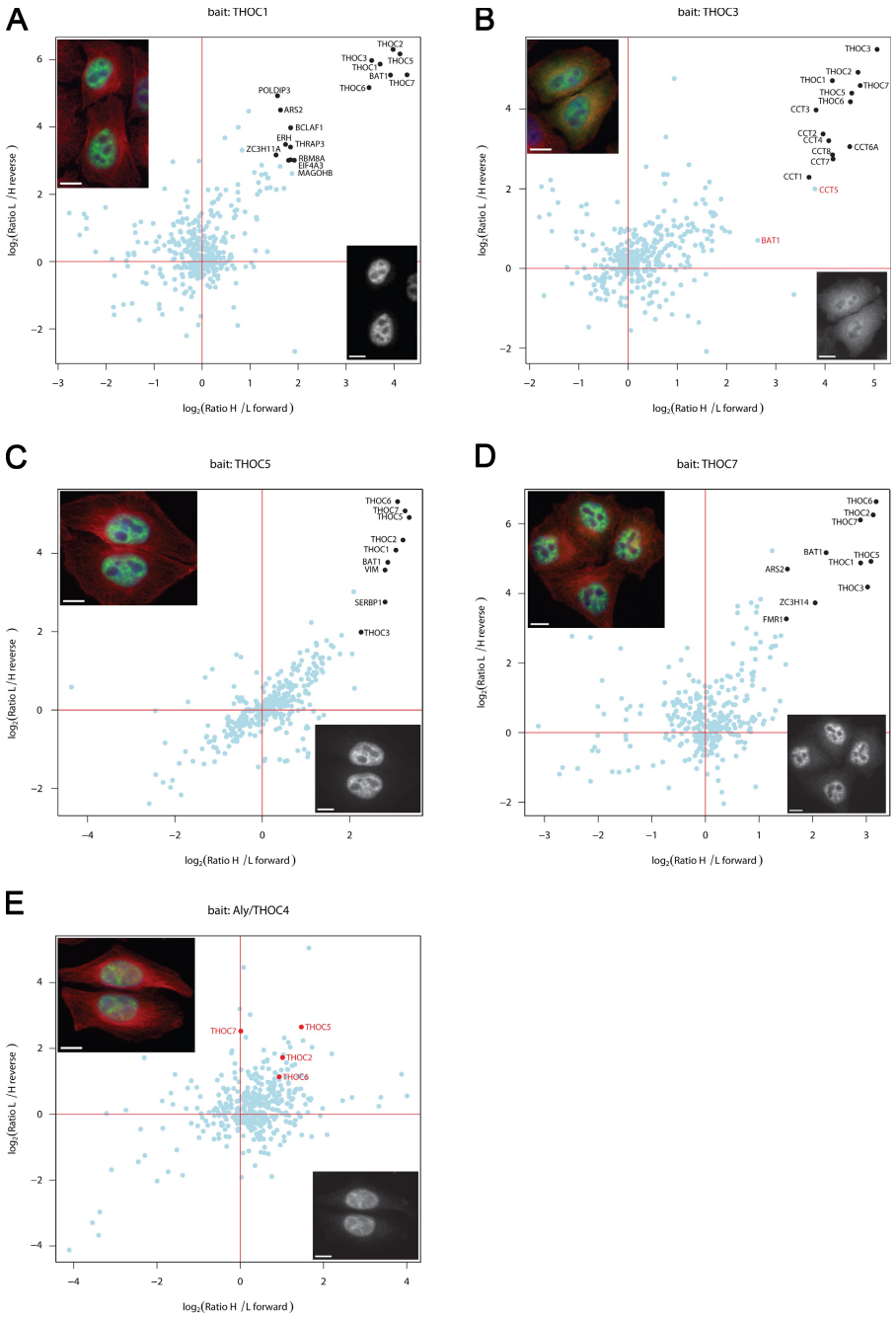


Figure S2. **Additional SILAC pull-downs of the TREX complex components.** (A–E) Forward and reverse pull-down of THOC1, THOC3, THOC5, THOC7, and THOC4/Aly as described in Fig. 2. Specific interaction partners are annotated and marked with black dots. Proteins marked in red were not significant regarding their ratios. Bars, 10 µm.

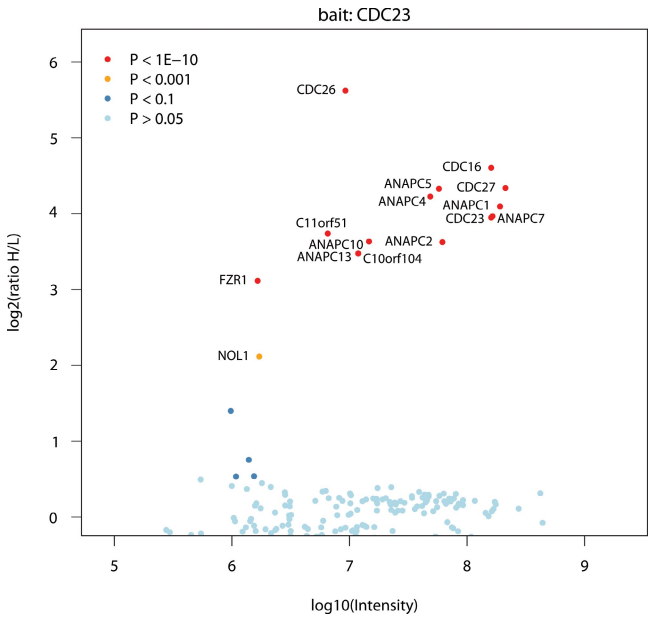


Figure S3. **Additional SILAC pull-down of CDC23.** Single SILAC pull-down of CDC23 with double-SILAC labeling and tryptic digestion of proteins reveals all but one component of the APC and two new interactors, C10orf104/ANAPC16 and C11orf51, with $P < 10^{-10}$.

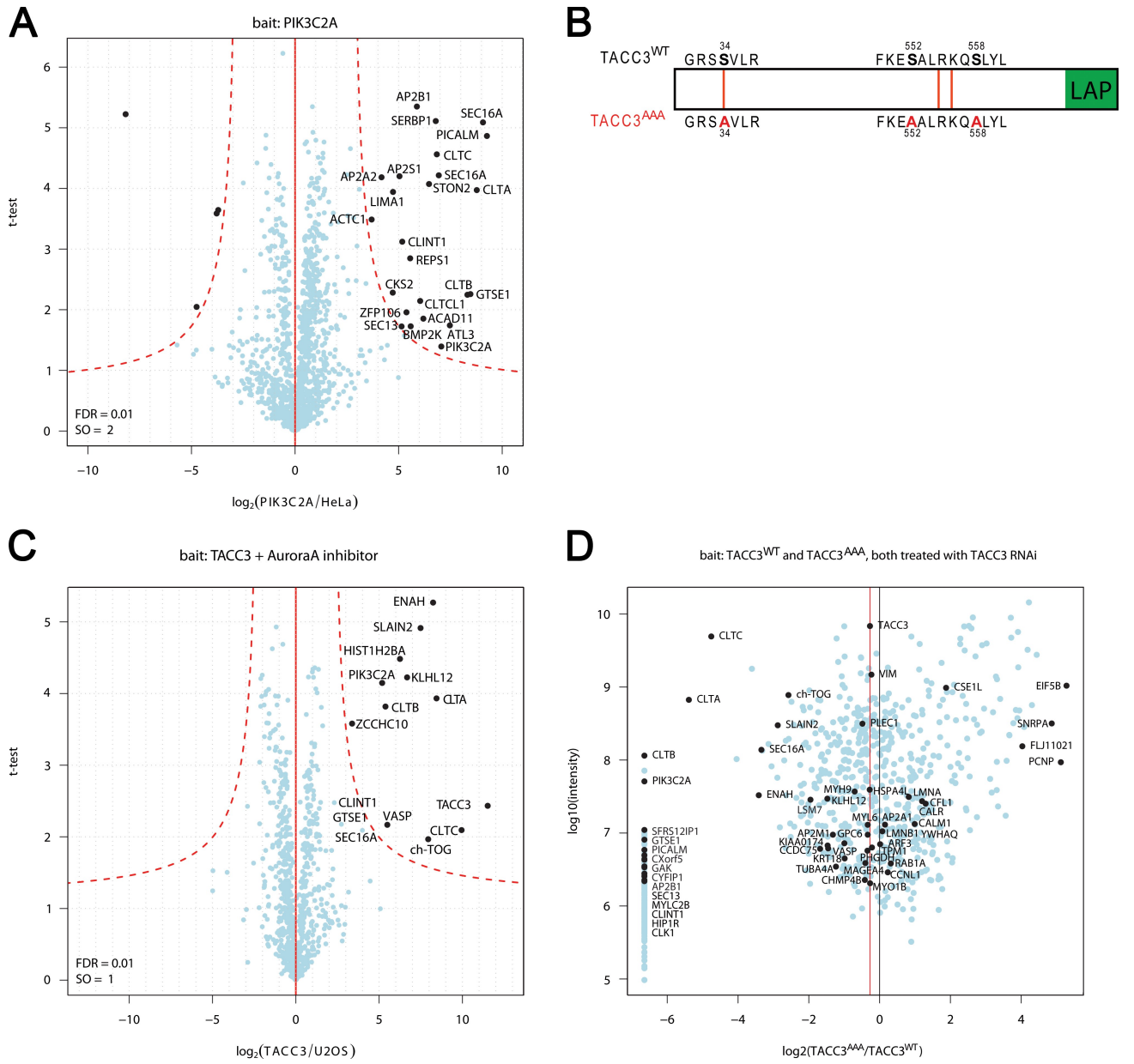


Figure S4. **Additional label-free pull-downs of TACC3.** (A) Volcano plot representing results of the label-free pull-downs of GFP-tagged PIC3C2A. The logarithmic ratio of protein intensities in PIK3C2A^{treated}/HeLa pull-downs was plotted against negative logarithmic p-values of the *t* test performed from triplicates. The hyperbolic curve separates specific PIK3C2A-interacting proteins marked in black (red dotted lines) from background (blue dots). (B) Graphical representation of the TACC3^{WT} and TACC3^{AAA} transgenes used in the experiments. Conserved serines that are known to be a target of aurora A kinase in *X. laevis* were mutated by alanines to create a nonphosphorylatable TACC3 mutant. (C) Volcano plot representing results of the label-free pull-downs of GFP-tagged TACC3 treated with aurora A kinase inhibitor. The logarithmic ratio of protein intensities in TACC3^{treated}/U2OS pull-downs were plotted against negative logarithmic p-values of the *t* test performed from triplicates. The hyperbolic curve separates specific TACC3-interacting proteins marked in black (red dotted lines) from background (blue dots). (D) Label-free pull-down of the TACC3^{WT} versus TACC3^{AAA}, both treated with RNAi against endogenous TACC3. Relative intensities of TACC3^{AAA}/TACC3^{WT} were plotted against relative intensities of the proteins. Annotated proteins are specific interactors of either TACC3^{WT} or TACC3^{AAA}. The red line represents the ratio of intensities of TACC3^{WT} and TACC3^{AAA} and is close to 1. Proteins plotted far from the red line are specific interactors in one of the pull-downs.

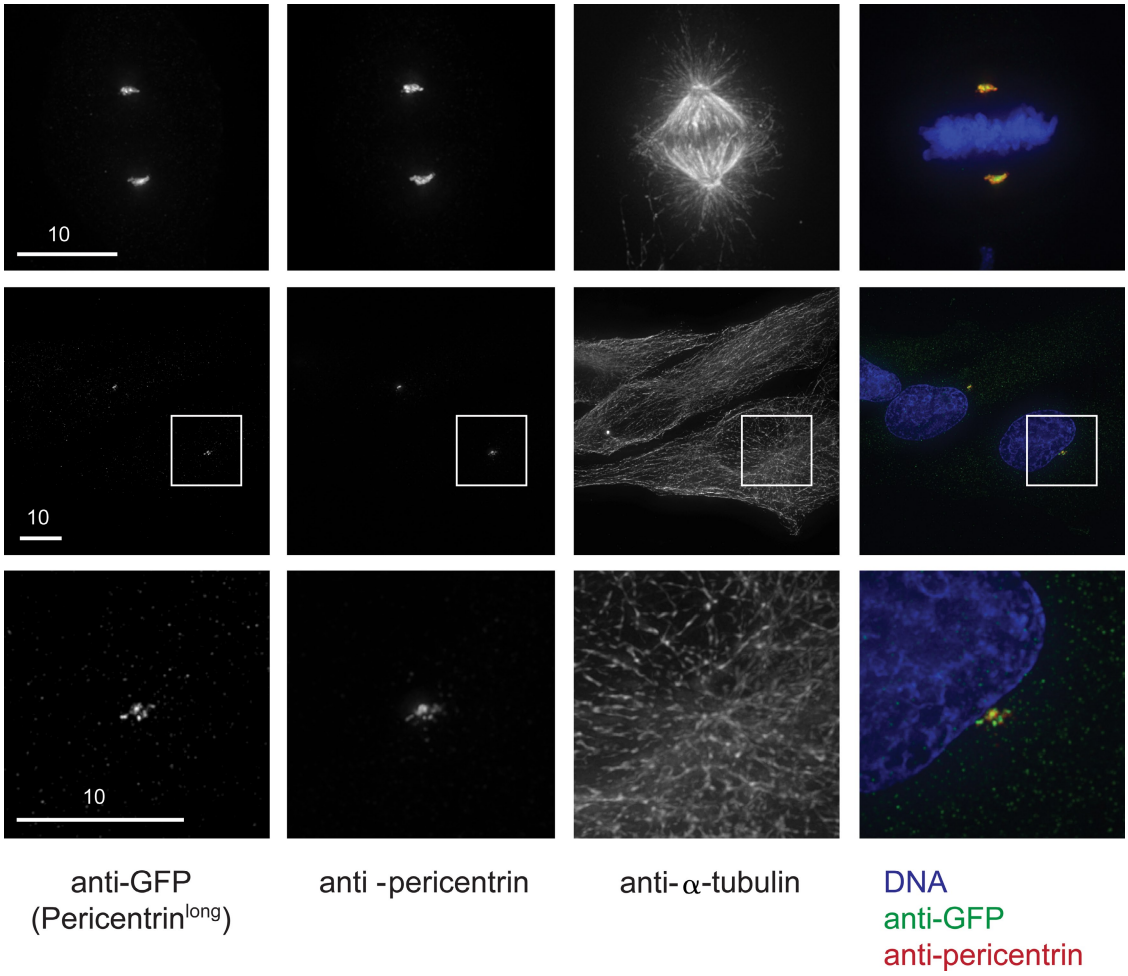
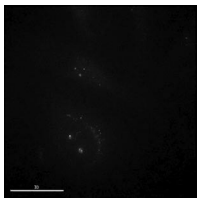
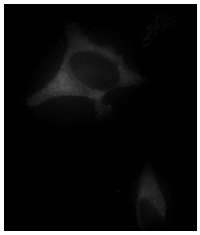


Figure S5. **Pericentrin^{long} GFP colocalizes with anti-pericentrin antibody throughout the cell cycle.** Immunofluorescence showing pericentrin^{long} localization to centrosomes. Anti-GFP (pericentrin^{long}) and anti-pericentrin antibodies label all centrosomes in mitosis (top) and interphase (middle). (bottom) Enlarged images of the above boxed regions are shown. Cells are stained for α-tubulin, GFP (pericentrin^{long}), pericentrin, and DNA. Bars, 10 μm.



Video 1. **Pericentrin^{long} localizes to centrosomes throughout mitosis and the cell cycle.** Pericentrin^{long} cells were imaged at 3-min intervals and displayed at 5 frames/s. Still images are shown in Fig. 6 B. Bar, 30 μm.



Video 2. **Pericentrin^{short} localizes to centrosomes in mitosis but not interphase.** Pericentrin^{short} shows that cytoplasmic localization in interphase rapidly accumulates on centrosomes as cells enter mitosis and quickly returns to cytoplasmic localization with the completion of mitosis. Cells were imaged at 15-min intervals and displayed at 1 frame/s. Still images are shown in Fig. 6 B.

Table S1. **Specific interaction partners of label-free pull-downs of TACC3, CLTC, GTSE1, and PIK3C2A**

Protein IDs	Protein name	Gene name	Uniprot	ENSEMBL	Pept (seq)	Razor pept (seq)	Unique pept (seq)	*t* test	Log2 ratio TACC3/U2OS
IPI00002135; IPI00383480	Transforming acidic coiled-coil–containing protein 3; ERIC-1	TACC3; ERIC1	Q9Y6A5; Q2NKK4	ENSG00000013810	78	78	78	2.418142658	11.54150073
IPI00024067; IPI00455383; IPI00385931	Clathrin heavy chain 1; CLH-17	CLTC; CLH17; CLTCL2; KIAA0034	Q00610-1; Q00610; Q49AL0; Q00610-2	ENSG00000141367	88	88	70	2.189627018	10.72113291
IPI00014587; IPI00790571; IPI00216393	Clathrin light chain A	CLTA	P09496-1; P09496; P09496-3; P09496-2; A8K4W3	ENSG00000122705	9	9	9	4.027574481	9.249117533
IPI00472887; IPI00028275; IPI00749072	Ch-TOG protein; CKAP5 protein; colonic and hepatic tumor– over-expressed protein; cytoskeleton-associated protein 5	CKAP5; KIAA0097	Q2TA89; Q6NSH4; Q14008; Q0VAX7; Q8IYN5	ENSG00000175216	99	99	99	2.040452739	8.434785207
IPI00646954; IPI00374054; IPI00871394; IPI00411623; IPI00749432; IPI00783772; IPI00640474	Protein-enabled homologue; cDNA FLJ44946 fis; clone BRAMY4001863; highly similar to *Mus musculus*– enabled homologue (*Drosophila*; ENAH)	ENAH; MENA	Q8N8S7-2; Q8N8S7; A8K2B4; Q8N8S7-1; Q6ZSB8; Q6ZT55	ENSG00000154380	19	19	19	5.145145617	8.282180786
IPI00853278; IPI00873597; IPI00386228	SLAIN motif–containing protein 2	SLAIN2; KIAA1458	Q9P270	ENSG00000109171	25	25	25	4.398657885	7.590815226
IPI00642182; IPI00646582; IPI00647373	Kelch-like protein 12; CUL3-interacting protein 1; putative uncharacterized protein KLHL12	KLHL12; C3IP1	Q53G59; A6NEN8	ENSG00000117153	15	15	15	3.897240146	6.833664576
IPI00014589; IPI00216472	Clathrin light chain B	CLTB	P09497-1; P09497; P09497-2; Q53Y37	ENSG00000175416	7	7	7	4.051788559	6.156529744
IPI00465363; IPI00889103	Histone H2B type 1-A; histone H2B testis; testis-specific histone H2B	HIST1H2BA; TSH2B	Q96A08; B2R544	ENSG00000146047	9	3	3	2.718854145	6.061641693
IPI00002580	Phosphatidylinositol-4-phosphate-3-kinase C2 domain–containing α polypeptide; phosphoinositide-3-kinase C2-α	PIK3C2A	O00443; B0LPH2	ENSG00000011405	30	30	30	4.011038511	5.756003062
IPI00902584; IPI00301058; IPI00745286	Vasodilator-stimulated phosphoprotein	VASP	P50552; B2RBT9	ENSG00000125753	13	13	13	2.060439467	5.427724202

Table S1. **Specific interaction partners of label-free pull-downs of TACC3, CLTC, GTSE1, and PIK3C2A** *(Continued)*

Protein IDs	Protein name	Gene name	Uniprot	ENSEMBL	Pept (seq)	Razor pept (seq)	Unique pept (seq)	*t* test	Log2 ratio TACC3/U2OS
IPI00160901; IPI00871779	G2 and S phase– expressed protein 1; B99 homologue; putative uncharacterized protein GTSE1	GTSE1	Q9NYZ3; A8MRT0	ENSG00000075218	11	11	11	2.996183171	4.85224851
IPI00641384; IPI00166487; IPI00896436; IPI00219314; IPI00872611; IPI00031242; IPI00167711; IPI00384060	Protein transport; protein Sec16A; protein SEC16 homologue A; KIAA0310	SEC16A; KIAA0310; SEC16; SEC16L; RP11-413M3.10-003	O15027-1; O15027; A0PJ75; O15027-3; A4QN18; A4QN19; O15027-4; O15027-2; Q5SXP2; Q9BV84	ENSG00000148396	32	32	32	2.150942836	4.281937281
IPI00397519; IPI00291930	Clathrin interactor 1; epsin-4; epsin-related protein; enthoprotin; clathrin-interacting protein localized in the trans-Golgi	CLINT1; ENTH; EPN4; EPNR; KIAA0171	Q14677-2; Q14677; Q14677-1	ENSG00000113282	5	5	5	2.834212021	3.284816106

Pept, peptide; seq, sequence.

Table S2. **BACs, BAC length, gene length, number, and name of additional genes used in this study**

Gene	Ensembl ID	BAC ID	BAC length[a]	Gene length[b]	No. add. genes[c]	Add. genes on BAC
			bp	*bp*		
CDC23	ENSMUSG00000024370	RP23-259E2	167,007	17,741	4	4933408B17Rik, Brd8, Kif20a, Gfra3
THOC1	ENSG00000079134	RP11-1150C18	143,619	53,522	0.5	USP14 (incomplete)
THOC2	ENSG00000125676	CTD-3110N20	152,405	86,751	0	ND
THOC3	ENSG00000051596	RP11-959F11	196,718	8,752	1	CPLX2
THOC4	ENSG00000183684	RP11-634L10	172,476	3,741	12	P4hB, ARHGDIA, ANAPC11, NPB, PCYT2, SIRT7, MAFG, LOC92659, PYCR1, MYADML2, NOTUM, ASPSCR1
THOC5	ENSG00000100296	RP11-474I14	162,433	47,868	4	RFPL1S, RFPL1, NEFH, NIPSNAP1
THOC6	ENSG00000131652	RP11-341L6	160,196	3,715	12	FLYWCH1, KREMEN2, PAQR4, PKMYT1, CLDN9, CLDN6, TNFRSF12A, HCFC1R1, CCDC64B, MMP25, IL32, ZSCAN10
THOC7	ENSG00000163634	RP11-615N12	153,849	29,962	1.5	c3orf49, ATXN7 (incomplete)
BAT1	ENSG00000198563	RP11-116B15	164,920	16,363	15	MICB, BCCD1, SNORD117, SNORD84, ATP6V1G2, NFLBILI, LTA, TNF, LTB, LST1, NCR3, AIF1, BAT2, SNORA38, BAT3
TACC3	ENSG00000013810	RP11-42F9	161,517	23,639	3.5	FAM53A (incomplete), SLBP, TMEM129, FGFR3,
PCNT	ENSMUSG00000001151	RP23-156C17	225,536	91,658	4	S100b, Dip2a, 2610028H24Rik, A130042E20Rik
GTSE1	ENSG00000075218	RP11-1152E11	159,897	94,825	6.5	PPARA, c22orf40, PKDREJ, TTC38, CN5H6.4, TRMU, CELSR1 (incomplete)
CLTC	ENSG00000141367	RP11-661B17	204,299	77,267	2	DHX40 (incomplete), PTRH2, TMEM49 (incomplete)
PIK3C2A	ENSG00000011405	CTD-3236F5	212225	80142	0	ND

Add, additional.

[a]Mean, 174,078; SD, 25,053.

[b]Mean, 45,425; SD, 34,778.

[c]Mean, 5; SD, 5.

Supplemental data

A detailed protocol of QUBIC. This step by step protocol makes it easy for any laboratory with access to high resolution MS to identify interaction partners of the protein of interest.

QUBIC*validator*. Software to identify specific interaction partners with label-free QUBIC. This software is necessary to perform *t* test–based validation of label-free pull-downs as described in Materials and methods.

Test dataset. CDC23 experiments in SILAC and label-free format. The SILAC folder contains the proteinGroups.txt and experimentalDesign.txt files used for MaxQuant analysis. The results can be plotted with the provided R script according to the instructions in the supplemental protocol. The label-free folder also contains the proteinGroups.txt and experimentalDesign.txt files. Furthermore, the Groups.txt file that needs to be loaded in QUBIC*validator* in addition to the proteinGroups.txt is also provided. Label-free data can also be analyzed and plotted according to the instructions given in the protocol (see R scripts for download).

R scripts. Several R scripts to visualize QUBIC data. QUBIC-SILAC.R plots forward SILAC against reverse SILAC experiments. QUBIC-LABELFREE.R plots *t* test p-values against ratios (both determined with QUBIC*validator*). QUBIC-LABELFREE_dynamic.R visualizes dynamic interactions. Usage of all scripts and download instructions are described in Materials and methods.

References

Wiśniewski, J.R., A. Zougman, N. Nagaraj, and M. Mann. 2009. Universal sample preparation method for proteome analysis. *Nat. Methods*. 6:359–362. doi:10.1038/nmeth.1322

QUBIC: Detailed protocol

Generating a cell line containing the GFP-tagged protein of interest

Methods for producing BAC-based transgenic cell lines of interest have been previously published (Kittler et al., 2005; Poser et al., 2008):

Kittler, R., Pelletier, L., Ma, C., Poser, I., Fischer, S., Hyman, A.A., and Buchholz, F. (2005). RNA interference rescue by bacterial artificial chromosome transgenesis in mammalian tissue culture cells. Proceedings of the National Academy of Sciences of the United States of America 102, 2396-2401.

Poser, I., Sarov, M., Hutchins, J.R., Heriche, J.K., Toyoda, Y., Pozniakovsky, A., Weigl, D., Nitzsche, A., Hegemann, B., Bird, A.W., et al. (2008). BAC TransgeneOmics: a high-throughput method for exploration of protein function in mammals. Nature methods 5, 409-415.

Affinity purification of GFP-tagged bait and interacting proteins

Cell culture: SILAC (stable isotope labeling by amino acids in cell culture)

Reagents (for HeLa or U2OS cells)

Media:

	company	*order number*
DMEM (4.5g/L glucose, - lysine, + methionine)	Invitrogen	10829018
10 % fetal bovine serum, dialyzed with a cutoff of 10kDa	Invitrogen	26400044
100U/ml Penicillin/Streptomycin	Invitrogen	15140122
49mg/ml lysine light ($C^{12}N^{14}$) or heavy ($C^{13}N^{15}$)	Sigma	616192, 608041
Only transgenic cell lines: 400µg/ml Geneticin	Invitrogen	10131027

Further reagents:
PBS
Trypsin
Liquid nitrogen

Protocol

Transgenic and control cell lines (wild-type cell lines not transfected with a transgene) are each labeled separately with both, the $C^{12}N^{14}$ and $C^{13}N^{15}$ derivates of Lysine to perform forward and reverse experiments:

- Culture cells for at least 5 passages (usually 2 weeks) in heavy and light SILAC media at 37ºC and 5% CO_2
- Expand cells to 2 x 15cm dishes of the heavy labeled transgenic cell line and 2 x 15cm dishes of the light labeled wild-type cell line for forward experiments and vice versa in reverse experiments
- Harvest cells with trypsin, wash once with PBS and snap freeze the pellets in liquid nitrogen
- Store pellet at -80°C

Cell culture: Label-free

Reagents (for HeLa or U2OS cells)

Media:

	company	*number*
DMEM (4.5g/L glucose)	Invitrogen	31966047
10 % fetal bovine serum	Invitrogen	10270106
100U/ml Penicillin/Streptomycin	Invitrogen	15140122
Only transgenic cell lines:		
400µg/ml Geneticin	Invitrogen	10131027

Further reagents:
PBS
Trypsin
Liquid nitrogen

Protocol

- Culture transgenic and control cell lines (wild-type cell lines not transfected with a transgene) in standard cell culture conditions
- Expand each cell line to 3 x 2 x 15cm dishes for triplicate experiments
- Harvest cells with trypsin, wash with once PBS and snap freeze the pellets in liquid nitrogen
- Store pellet at -80°C

QUBIC is also capable of identifying differential interaction partners of proteins present in a different cellular state, e.g. cells treated with and inhibitor against a certain protein, in a certain cell cycle phase or under cellular stress. Therefore pulldowns of each condition and control pulldowns should be done in triplicates.

Anti-GFP co-immunoprecipitation: SILAC

Reagents

Buffers:

Basic buffer (BB)

150mM	NaCl
50mM	Tris-HCl (pH 7.5)
5%	Glycerol

Lysis buffer

in	*BB*
1%	IGPAL-CA-630 (Sigma, #I8896)
1mM	$MgCl_2$
1x	Protease inhibitors, EDTA-free (Roche, #11836153001)
1%	Benzonase (Merck, #70746-3)
1x	Phosphatase inhibitors (Roche, #04906837001); *only for modification dependent interactions*

Equilibration buffer

in	*BB*
1%	IGPAL-CA-630 (Sigma, #I8896)

Wash buffer I

in	*BB*
0.05%	IGPAL-CA-630 (Sigma, #I8896)

Wash buffer II = *BB*

Elution buffer I

2M	Urea *(always prepare fresh!)*
50mM	Tris-HCl (pH 7.5)
1mM	DTT
5µg/ml	Endo-LysC (Wako Chemicals, #129-02541)

Elution buffer II

2M	Urea *(always prepare fresh!)*
50mM	Tris-HCl (pH 7.5)
5mM	Chloroacetamide

Further reagents:
magnetic beads: µMACS anti-GFP (Miltenyi Biotech, #130-091-125)
trifluoroacetic acid
C18-stage tips and solvents as described previously (Rappsilber et al., 2007)

Equipment:
Handmagnet or MultiMACS (Miltenyi Biotech, #130-042-602 or #130-091-937)
µColumns (Miltenyi Biotech, #130-042-701)
Centrifuge at 4°C

Protocol

- Thaw SILAC cell pellets on ice
- Resuspend in 1ml lysis buffer and incubate 30min. at room temperature on a wheel with appropriate inhibitors (i.e. add phosphatase inhibitors when studying phosphorylation dependent interactions)
- Spin lysates at 4.000xg and 4°C for 15min to remove remaining nucleic acids and membrane fractions
- Transfer protein-containing supernatants to new tubes
- Incubate supernatants with 50µl magnetic beads coupled to monoclonal mouse anti-GFP antibody for 15min on ice
- Equilibrate columns with 250µl equilibration buffer
- Add cell lysates to the column
- Wash with 3 x 800µl ice cold wash buffer I and 2 x 500µl of wash buffer II
- Add 25µl Elution buffer I and incubate for 30min. at room temperature
- Elute peptides with 2 x 50µl Elution buffer II and collect them in an Eppendorf tube
- Merge heavy and light eluates of transgenic cell line and the corresponding wild-type cell line immediately after elution from the columns

- Continue to digest over night at room temperature
- Stop digestion by adding 1µl trifluoroacetic acid
- Purify peptides of each experiment on two C18 stage tips and store at 4°C (Rappsilber et al., 2007)

Pulldowns can be done manually on a hand magnet. In our laboratory the complete pulldowns are done on the automated liquid handling platform TECAN freedom EVO 200 in fully automated manner.

Anti-GFP co-immunoprecipitation: Label-free

Reagents

Buffers:

Basic buffer (BB)

150mM	NaCl
50mM	Tris-HCl (pH 7.5)
5%	Glycerol

Lysis buffer

in	*BB*
1%	IGPAL-CA-630 (Sigma, #I8896)
1mM	$MgCl_2$
1x	Protease inhibitors, EDTA-free (Roche, #11836153001)
1%	Benzonase (Merck, #70746-3)
1x	Phosphatase inhibitors (Roche, #04906837001); *only for modification dependent interactions*

Equilibration buffer

in	*BB*
1%	IGPAL-CA-630 (Sigma, #I8896)

Wash buffer I

in	*BB*
0.05%	IGPAL-CA-630 (Sigma, #I8896)

Wash buffer II = *BB*

Elution buffer I	
2M	Urea *(always prepare fresh!)*
50mM	Tris-HCl (pH 7.5)
1mM	DTT
5µg/ml	Trypsin (Promega, #V511C)

Elution buffer II	
2M	Urea *(always prepare fresh!)*
50mM	Tris-HCl (pH 7.5)
5mM	Chloroacetamide

Further reagents:
magnetic beads: µMACS anti-GFP (Miltenyi Biotech, #130-091-125)
trifluoroacetic acid
C18-stage tips and solvents as described previously (Rappsilber et al., 2007)

Equipment:
Handmagnet or MultiMACS (Miltenyi Biotech, #130-042-602 or #130-091-937)
µColumns (Miltenyi Biotech, #130-042-701)
Centrifuge at 4°C

Protocol

- Thaw SILAC cell pellets on ice
- Resuspend in 1ml lysis buffer and incubate 30min. at room temperature on a wheel with appropriate inhibitors (i.e. add phosphatase inhibitors when studying phosphorylation dependent interactions)
- Spin lysates at 4.000xg and 4°C for 15min to remove remaining nucleic acids and membrane fractions
- Transfer protein-containing supernatants to new tubes
- Incubate supernatants with 50µl magnetic beads coupled to monoclonal mouse anti-GFP antibody for 15min on ice

- Equilibrate columns with 250µl equilibration buffer
- Add cell lysates to the column
- Wash with 3 x 800µl ice cold wash buffer I and 2 x 500µl of wash buffer II
- Add 25µl Elution buffer I and incubate for 30min. at room temperature
- Elute peptides with 2 x 50µl Elution buffer II and collect them in an Eppendorf tube
- Continue to digest over night at room temperature
- Stop digestion by adding 1µl trifluoroacetic acid
- Purify peptides of each experiment on two C18 stage tips and store at 4°C (Rappsilber et al., 2007)

Pulldowns can be done manually on a hand magnet. In our laboratory the complete pulldowns are done on the automated liquid handling platform TECAN freedom EVO 200 in fully automated manner.

Mass spectrometric analysis

QUBIC requires access to high resolution mass spectrometry. There are several combinations of nano-LC systems and high resolution hybrid mass spectrometers that can be used. Therefore we cannot provide a general protocol in this case. Information given in this chapter is based on the usage of a Proxeon nano-LC system coupled to an LTQ-Orbitrap (Thermo Fisher Scientific).

Liquid Chromatography

Reagents

Solvents:

Solvent A	
0.5%	0.5% acetic acid

Solvent B	
80%	acetonitrile
0.5%	acetic acid

Solvent A*	
2%	acetonitrile
0.1%	trifluoroacetic acid

Equipment:
Speed Vacuum centrifuge (e.g. Eppendorf)
Nano-LC System (e.g. Proxeon Biosystems)
Column (e.g. fused-silica emitter with an inner diameter of 75mm (Proxeon Biosystems) packed with RP ReproSil-Pur C18-AQ 3 mm resin (Dr. Maisch))

Protocol

- Elute peptides from C18 stage tips with 2 x 20µl of solvent B
- Evaporate acetonitrile in a speed vacuum centrifuge

- Add solvent A* to a total volume of 6µl

Peptides are separated on-line to the mass spectrometer. Sample (2µl) are loaded on the column and are eluted with a constant flow of 250 nL/min and segmented gradient as follows:

Time [min]	solvent B [%]	Flow [nl]
5	8	250
90	30	250
12	60	250
7	80	250
2	95	250
4	95	500
5	8	500
10	8	500

This gradient was optimized for the nano-LC system of Proxeon Biosystems and has to be adjusted for other nano-LC systems such that an equal amount of peptides are eluting over the whole gradient time.

Mass spectrometry

Equipment:
High resolution hybrid mass spectrometer (e.g. LTQ-FT or LTQ-Orbitrap (Thermo Fisher Scientific))

Protocol

The nano-LC system is coupled online to the high-resolution mass spectrometer. In the case of an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) the nanoscale LC interface (Proxeon Biosystems) is used.

Parameters Source

Sprayvoltage	2.1kV
Temperature of heated capillary	180°C

Parameters Orbitrap

Resolution of full scan MS spectra	60,000
Mass range of full scan MS spectra	300-1650 m/z
Target value for full scan MS spectra	1,000,000 ions
Maximum filltime	1,000 ms
Lock mass option for survey scans	enabled

Parameters LTQ

Resolution of MS/ MS spectra	
Mass range of MS/ MS spectra	maximum 2 x m/z of parent ion
Target value for full scan MS spectra	5,000 ions
Collision energy for CID	35%
Maximum filltime	150 ms

Further parameters

The most intense ions (up to ten) from the preview survey scan delivered by the Orbitrap were in the LTQ (TOP10 peptide sequencing). Precursor ion charge state screening was enabled and all unassigned charge states as well as singly charged peptides were rejected. The dynamic

exclusion list was restricted to a maximum of 500 entries with a maximum retention period of 90s and a relative mass window of 5ppm.

Data analysis

Required software

The raw data is processed with **MaxQuant** (Cox et al., 2009b). This software is available for free download at http://www.maxquant.org.

The processed raw data is searched against the human database using **MASCOT** (Matrix Science).

Determination of interaction partners in label-free pulldowns is done using **QUBIC*validator***. This software is available for free and can be found in Supplemental Material.

Results are plotted using the statistical software **R** which is freely available at https://www.R-project.org. **R-scripts** are can be found in Supplemental Material.

Identification of interaction partners with QUBIC-SILAC

In SILAC experiments, raw data is processed and analyzed using MaxQuant. A practical guide for this step is given in the manuscript by Cox and Mann (Cox et al., 2009b):

Cox, J., Matic, I., Hilger, M., Nagaraj, N., Selbach, M., Olsen, J.V., and Mann, M. (2009). A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. Nature protocols 4, 698-705.

General settings in MaxQuant

QUANT.exe

variable modifications	Oxidation (M), Acetyl (Potein N-term)
fixed modifications	Carbamidomethyl
maximum peptide charge	6

SILAC	Doublets → Lys8

IDENTIFY.exe	
Peptide FDR	0.01
Protein FDR	0.01
Maximum peptide PEP	1
Minimum peptide length	6
Min. peptides	1
Min. unique peptides	0
Protein quantification	Use unique and razor peptides
Min. ratio count	1
Re-quantify	yes
Keep low scoring versions of identified peptides	yes

Furthermore load an experimentalDesign.txt file in the following format to make results compatible with the provided R-script QUBIC-SILAC.R:

Name	**Slice**	**Experiment**	**Invert**
'Name of raw file heavy/forward experiment'		QUBICH	No
'Name of raw file light/reverse experiment'		QUBICL	YES

A template for this file can also be found in Supplemental Material (Template_QUBIC-SILAC_experimentalDesign.txt). Alternatively the file experimentalDesignTemplate.txt created by MaxQuant and located in the combined folder can be used.

Analysis of processed data

Plot results using R and the provided script **QUBIC-SILAC.R**:

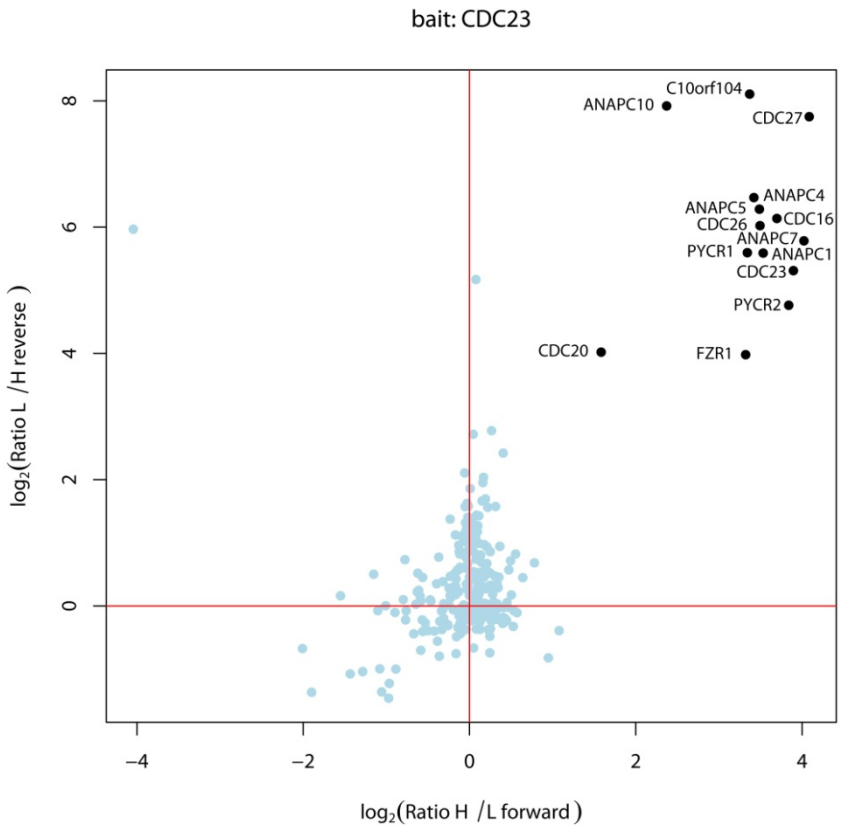
- Set parameters in the beginning of the script:

```
bait <- baitname                    # replace 'baitname' by name of your bait
significance <- 0.01                # required value of significance B (can be changed)
```

- Run the script
- Select working directory: 'Combined' folder created by MaxQuant
- Right click on image and safe

The script filters the proteinGroups.txt file for proteins identified with at least two peptides, thereof one unique and removes all contaminants and reverse hits. The ratios of forward and reverse experiments are plotted against each other. Furthermore it color codes and marks proteins with significant ratios in both experiments. These proteins are considered to be specific interactors.

An exemplary plot of a CDC23 forward and reverse pulldown is shown below:



Identification of interaction partners with QUBIC-LabelFree

Label-free quantification is performed in MaxQuant similar to the QUBIC-SILAC but in addition with the label-free algorithm described in (Cox et al., 2009a):

Cox, J., Luber, C.A., Nagaraj, N., Mann, M. (submitted 2009). Delayed normalization and maximal peptide ratio pairing for proteome-wide label-free.

General settings in MaxQuant

QUANT.exe

variable modifications	Oxidation (M), Acetyl (Potein N-term)
fixed modifications	Carbamidomethyl
maximum peptide charge	6
SILAC	Singlets

IDENTIFY.exe

Peptide FDR	0.01
Protein FDR	0.01
Maximum peptide PEP	1
Minimum peptide length	6
Min. peptides	1
Min. unique peptides	0
Protein quantification	Use unique and razor peptides
Min. ratio count	1
Re-quantify	Yes (does not matter)
Keep low scoring versions of identified peptides	yes
Label-free protein quantitation	yes
Match between runs	yes, time window 2 min.

Furthermore load an experimentalDesign.txt file in the following format to make results compatible to the provided R-script QUBIC-LabelFree.R:

Name	Slice	Experiment	Invert
'Name of raw file control experiment 1'		Control1	no
'Name of raw file control experiment 2'		Control2	no
'Name of raw file control experiment 3'		Control3	no
'Name of raw file GFP-IP experiment 1'		Exp1	no
'Name of raw file GFP-IP experiment 2'		Exp2	no
'Name of raw file GFP-IP experiment 3'		Exp3	no
'Name of raw file different GFP-IP experiment 1'		DiffExp1	no
'Name of raw file different GFP-IP experiment 2'		DiffExp2	no
'Name of raw file different GFP-IP experiment 3'		DiffExp3	no

A template for this file can also be found in Supplemental Material (Template_QUBIC-LabelFree_experimentalDesign.txt). This template can be extended for more replicates or different GFP pulldown experiments that should be compared to the control cell line. Alternatively the file experimentalDesignTemplate.txt created by MaxQuant and located in the combined folder can be used.

Analysis of processed data with QUBIC*validator*

We provide a specific program to facilitate proper statistical analysis of label-free QUBIC data.

- Download, unzip and open QUBIC*validator*
- Load – Generic → proteinGroups.txt
- Processing – Groups – Write group file template → save as Groups.txt
- Open Groups.txt (e.g. with Excel) and name replicates of one experiment with the same name:

Sample	Group
Norm. Intensity Control1	Control
Norm. Intensity Control2	Control
Norm. Intensity Control3	Control
Norm. Intensity Exp1	Exp
Norm. Intensity Exp2	Exp
Norm. Intensity Exp3	Exp
Norm. Intensity DiffExp1	DiffExp
Norm. Intensity DiffExp2	DiffExp
Norm. Intensity DiffExp3	DiffExp

- Processing – Groups – Load groups → load modified Groups.txt
- Processing – Filter – Filter category – Reverse = +
- Processing – Filter – Filter category – Contaminant = +
- Processing – Filter – Filter numerical column – Peptides(seq) < 2
- Processing – Filter – Filter numerical column – unique peptides(seq) < 1
- Processing – Transformation – LOG – Log2
- Processing – Imputation – Replace missing values by normal distribution – width = 0.3, shift = 1.8

T-tests are now performed on basis of the data processed as described above:

- Processing – Testing – Two groups:

Parameter in QUBIC*validator*	
Group1	Select Exp
Group2	Select Control
Test	t-test (equal variance)
Side	both
Use for truncation	Permutation based FDR
Threshold value	**0.1-0.001**
SO (=bend of the curve)	**0.5-2.0**
-LOG10	checked

- Start with a threshold value of 0.05 and an SO of 1
- Plot with R as described below
- Adjust threshold and SO that there are no/few outliers on the control side
- For dynamic comparison: Group1 = Exp, Group2 = DiffExp, Threshold value=1 and SO=1
- Export – Tab separated → choose name and safe (e.g. Exp.txt)

- Select tab “Suppl. Table” – copy whole table – paste in a text editor → safe with same name as above and add “_sup.txt” (e.g. Exp_sup.txt)

Plotting of QUBICvalidator using R

Plot results comparing the *pulldown to a control pulldown* using R and the provided script **QUBIC-LABELFREE.R**:

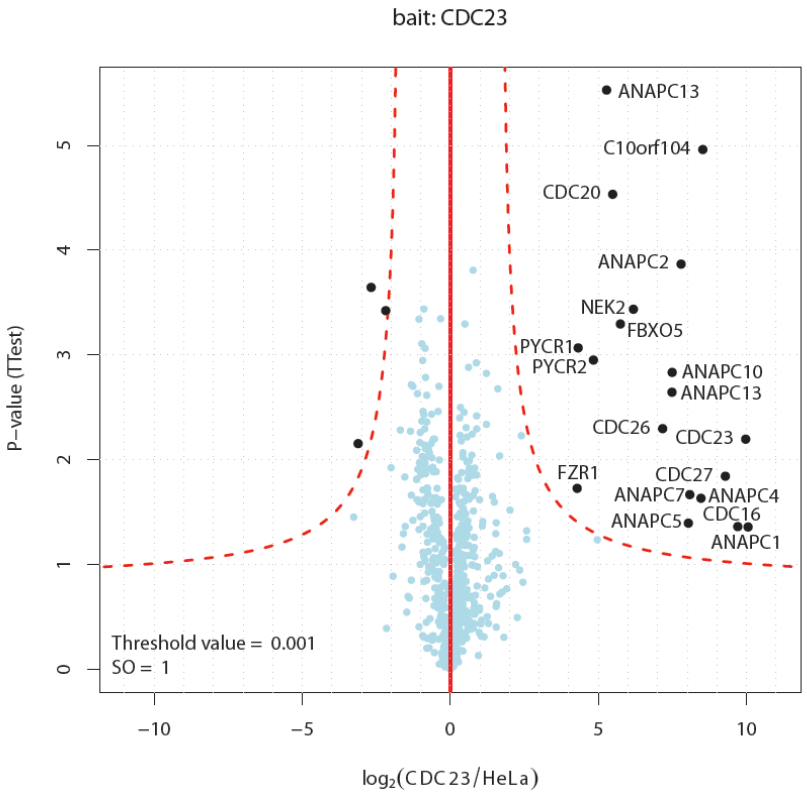
- Set parameters in the beginning of the script:

```
TV <- 0.01                  # set threshold value chosen in QUBICvalidator
SOt <- 1                    # set SO chosen in QUBICvalidator
Exp <- 'Exp.txt'            # set experiment name
Exp_sup <- 'Exp_sup.txt'    # set experiment sup name
bait <- 'baitname'          # replace 'baitname' by name of your bait
```

- Run the script
- Select working directory containing the Exp.txt and Exp_sup.txt file
- Right click on image and safe

The script plots the results generated by QUBIC*validator*. The ratios are plotted against the negative logarithmic P-value of the t-test. Furthermore it adds a curve representing the selected threshold value and SO and colour codes and marks significant binding partners.

An exemplary plot of a CDC23 is shown below:



Plot results comparing the *two pulldowns to each other* using R and the provided script **QUBIC-LABELFREE_dynamic.R**:

- Use QUBIC*validator* to create the Exp.txt file for both pulldowns that should be compared to each other as described above
- Use QUBIC*validator* to create an ExpCompare.txt file using the following paprmeters:

Parameter in QUBIC*validator*	
Group1	Select Exp
Group2	Select DiffExp
Test	t-test (equal variance)
Side	both
Use for truncation	Permutation based FDR
Threshold value	**1**
SO (=bend of the curve)	**1**
-LOG10	checked

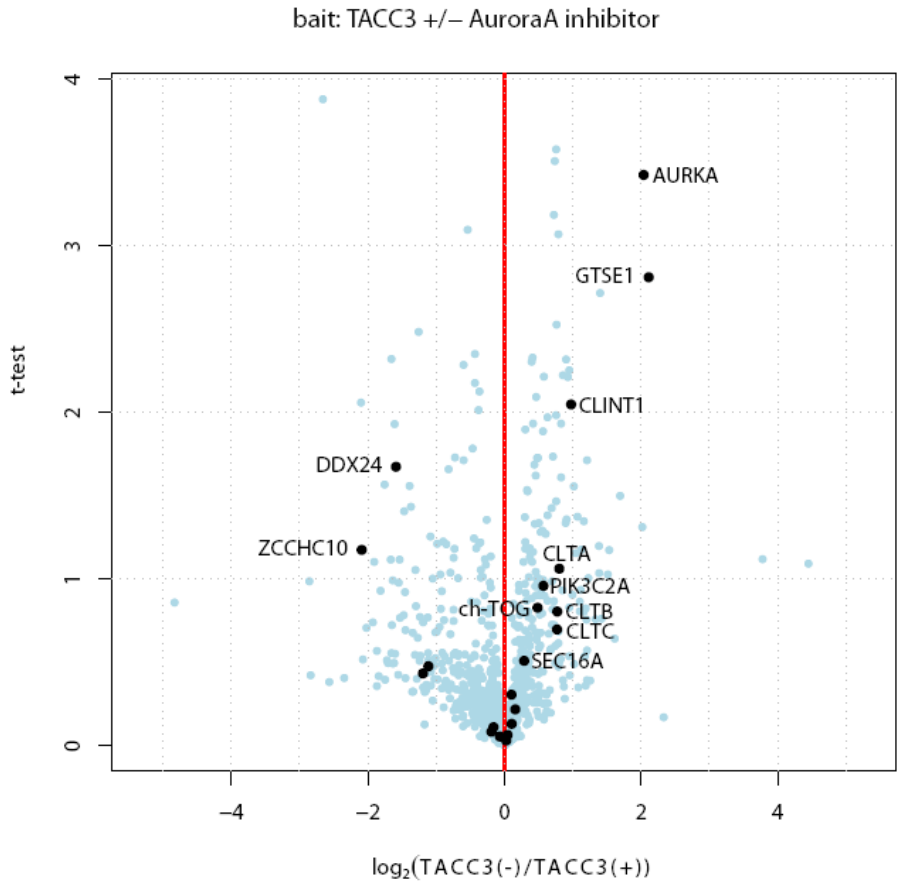
- Set parameters in the beginning of the script:

```
Exp <- 'Exp.txt'                    # set experiment name
ExpDiff <- 'ExpDiff.txt'            # set experiment sup name
ExpCompare <- 'ExpCompare.txt'      # set experiment compare name
tit <- 'title'                      # replace 'title' by title
```

- Run the script
- Select working directory containing the Exp.txt and Exp_sup.txt file
- Right click on image and safe

The script plots the differential binding of interaction partners. The ratios are plotted against the negative logarithmic P-value of the t-test. Proteins significant binders in either condition of the bait are marked in black and annotated. Proteins with a log2 ratio around 0 are not differential binders, proteins with very high or low ratios are differential binders.

An exemplary plot of a two differentially treated forms of TACC3 is shown below:



References

Cox, J., Luber, C.A., Nagaraj, N., and Mann, M. (2009a). Delayed normalization and maximal peptide ratio pairing for proteome-wide label-free quantification. submitted and available upon request.

Cox, J., Matic, I., Hilger, M., Nagaraj, N., Selbach, M., Olsen, J.V., and Mann, M. (2009b). A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. Nature protocols *4*, 698-705.

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