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## Supplemental Information

# ULK1/2 Restricts the Formation of Inducible SINT-Speckles, Membraneless Organelles Controlling the Threshold of TBK1 Activation

**Vera Vivian Saul, Markus Seibert, Marcus Krüger, Sylvia Jeratsch, Michael Kracht, and Michael Lienhard Schmitz**

# Supplemental information

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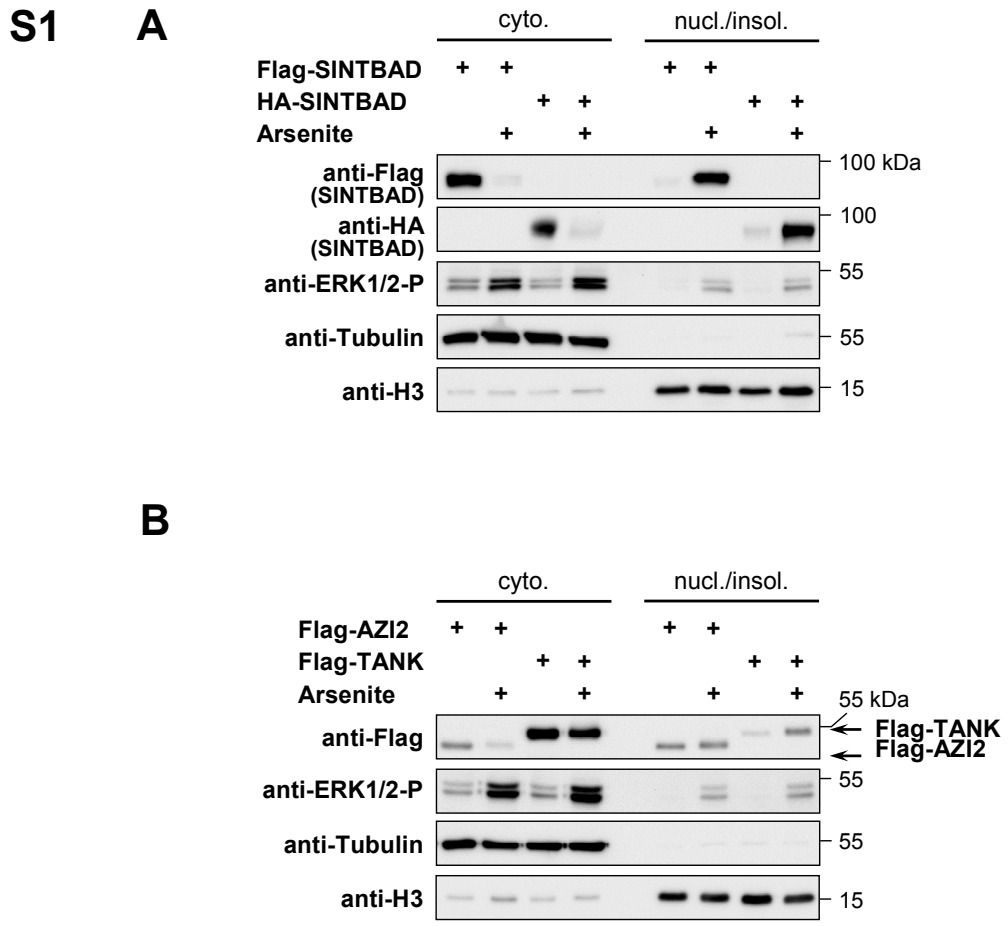
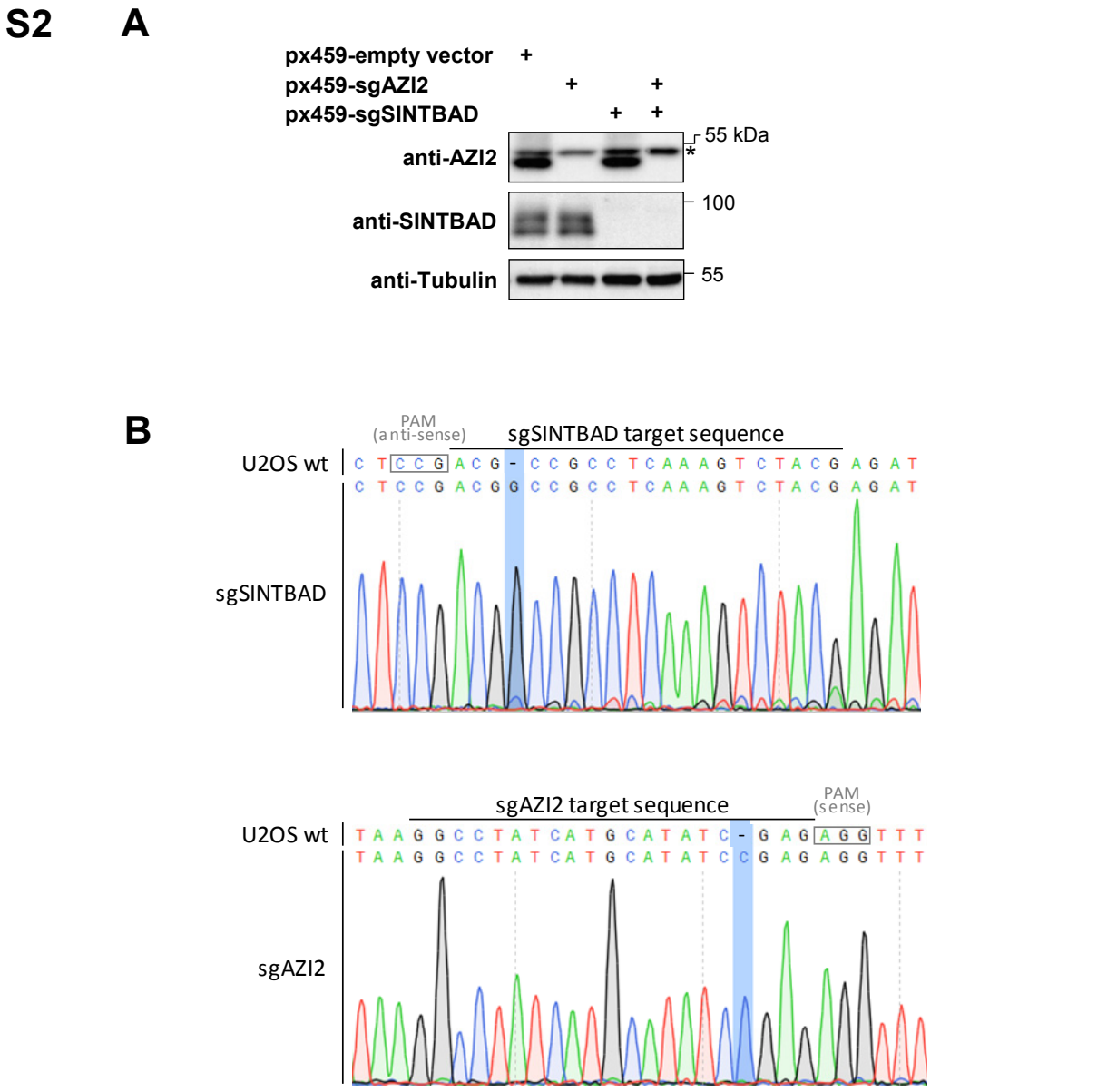
# Supplementary figures




**Suppl. Fig. S1. Relocalization of adapter proteins in response to cell stress, Related to Figure 1.** 293T cells were transfected to express Flag- or HA-tagged SINTBAD **(A)** or AZI2 and TANK **(B)** as shown. Cells were stimulated with 0.5 mM arsenite for 1 h, harvested and fractionated into cytosolic (cyto.) and nuclear/insoluble (nucl./insol.) extracts. Western blotting was performed to detect the adapter proteins, controls for successful cell fractionation (histone H3 and Tubulin) and cell stimulation (ERK1/2-P). The positions of molecular weight markers are indicated.

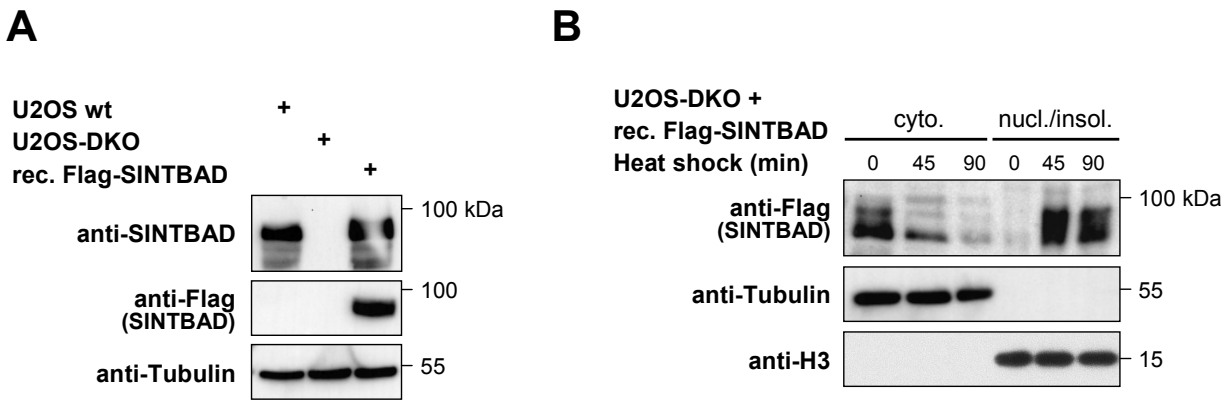
# Supplementary figures




**Suppl. Fig. S2. Generation of SINTBAD- and/or AZI2-deficient U2OS cells, Related to Figure 1. (A)** U2OS cells were transfected with a px459 plasmid encoding a sgRNA targeting the second exon of the AZI2 gene or the first exon of SINTBAD. After selection of non-transfected cells with Puromycin, individual cell clones were grown and analyzed by Western blotting using AZI2-and SINTBAD-specific antibodies. The AZI2 antibody also detects a non-specific band which is indicated by an asterisk. Double-deficient U2OS cells were generated by transfecting the sgRNA against AZI2 into SINTBAD knockout cells. **(B)** Genomic DNA was isolated from SINTBADdeficient cell clones and double-knockout cells. The respective genomic region encompassing the Cas9 cleavage site was amplified by PCR and sequenced as shown. The position of homozygous insertion of a nucleotide base in comparison to the wt sequence is highlighted in blue.

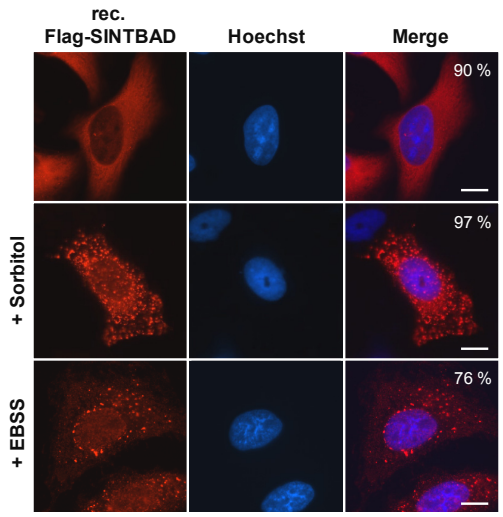
# Supplementary figures

## S3




**Suppl. Fig. S3. Characterization of U2OS-sgAZI2/sgSINTBAD (U2OS-DKO) cells reconstituted to stably express Flag-SINTBAD, Related to Figure 1. (A)** U2OS wt cells, U2OS-sgAZI2/sgSINTBAD double knock-out (DKO) cells and their derivative cell clone reconstituted to stably express Flag-SINTBAD were lyzed and tested by Western blot analysis for expression of Flag-tagged SINTBAD as shown. **(B)** U2OS-sgAZI2/sgSINTBAD cells reconstituted to stably express Flag-SINTBAD were exposed to heat shock for the indicated periods. Cells were harvested and fractionated into cytosolic (cyto.) and nuclear/insoluble (nucl./insol.) extracts. Western blotting was performed to detect the dynamic relocalization of Flag-SINTBAD, the detection of histone H3 and Tubulin show the purity of cell fractions.

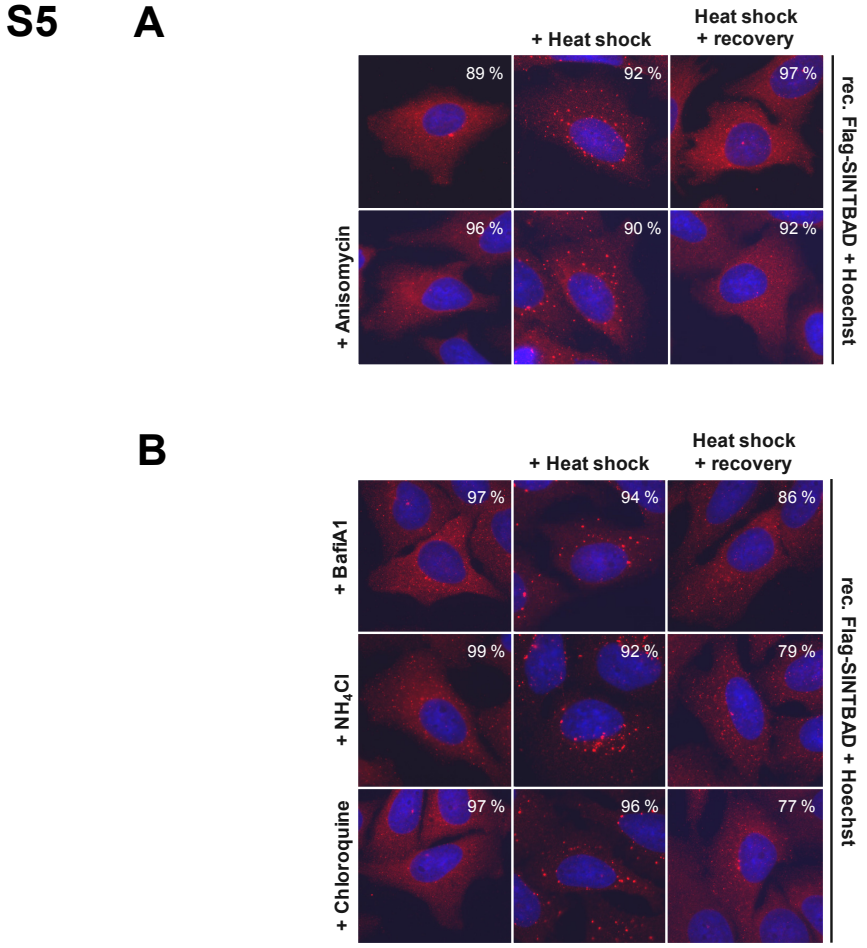
## S4




**Suppl. Fig. S4. Analysis of stress-induced SINTBAD relocalization, Related to Figure 1.** U2OS-sgAZI2/sgSINTBAD cells reconstituted to stably express Flag-SINTBAD were treated with 0.5 M sorbitol for 1 h or the starvation medium EBSS (Axe et al., 2008) for 4 h and analyzed by indirect immunofluorescence for the intracellular distribution of SINTBAD. Scale bar = 10 µm, the percentage of cells showing the displayed phenotype is indicated.

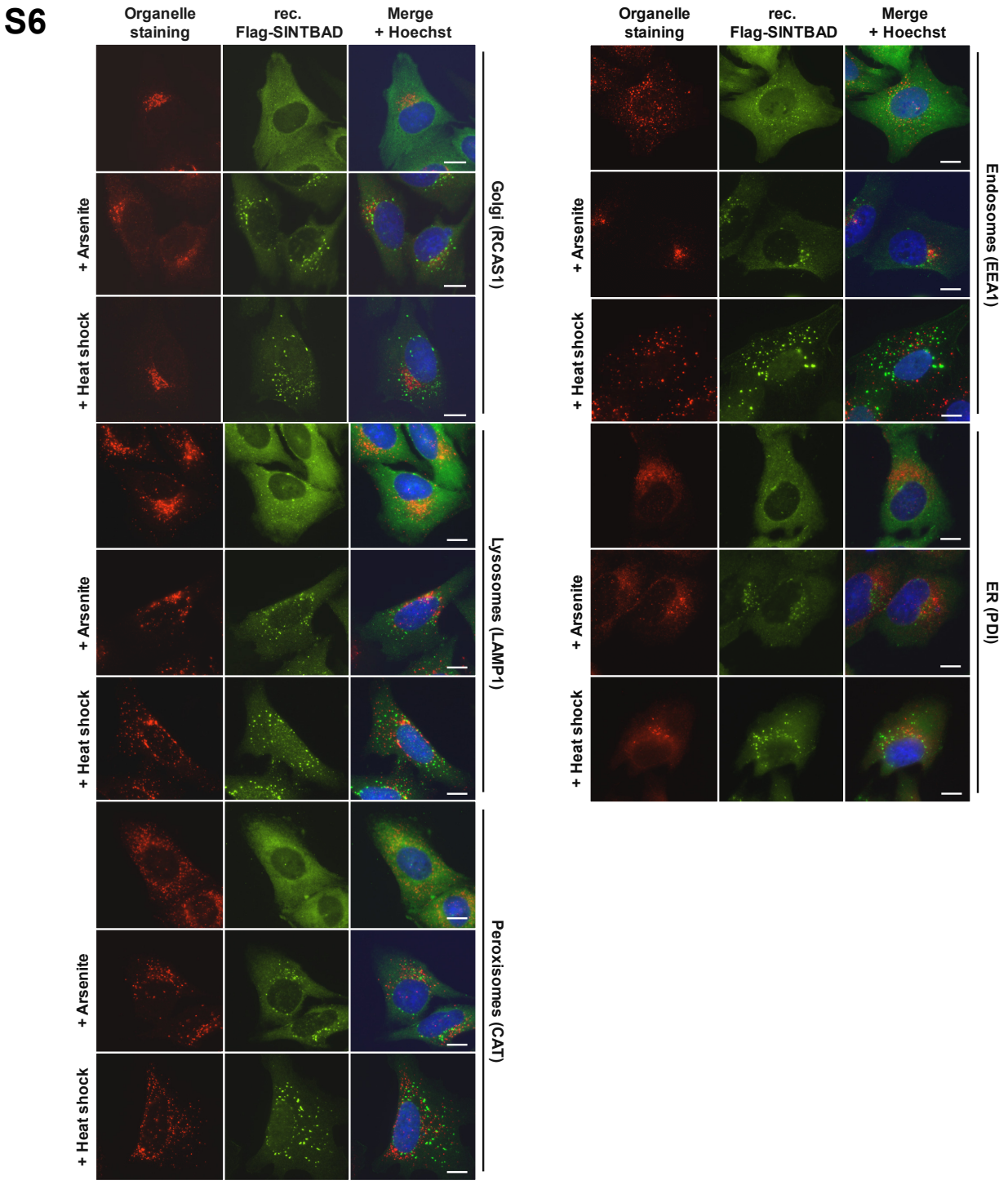
# Supplementary figures

**S5**



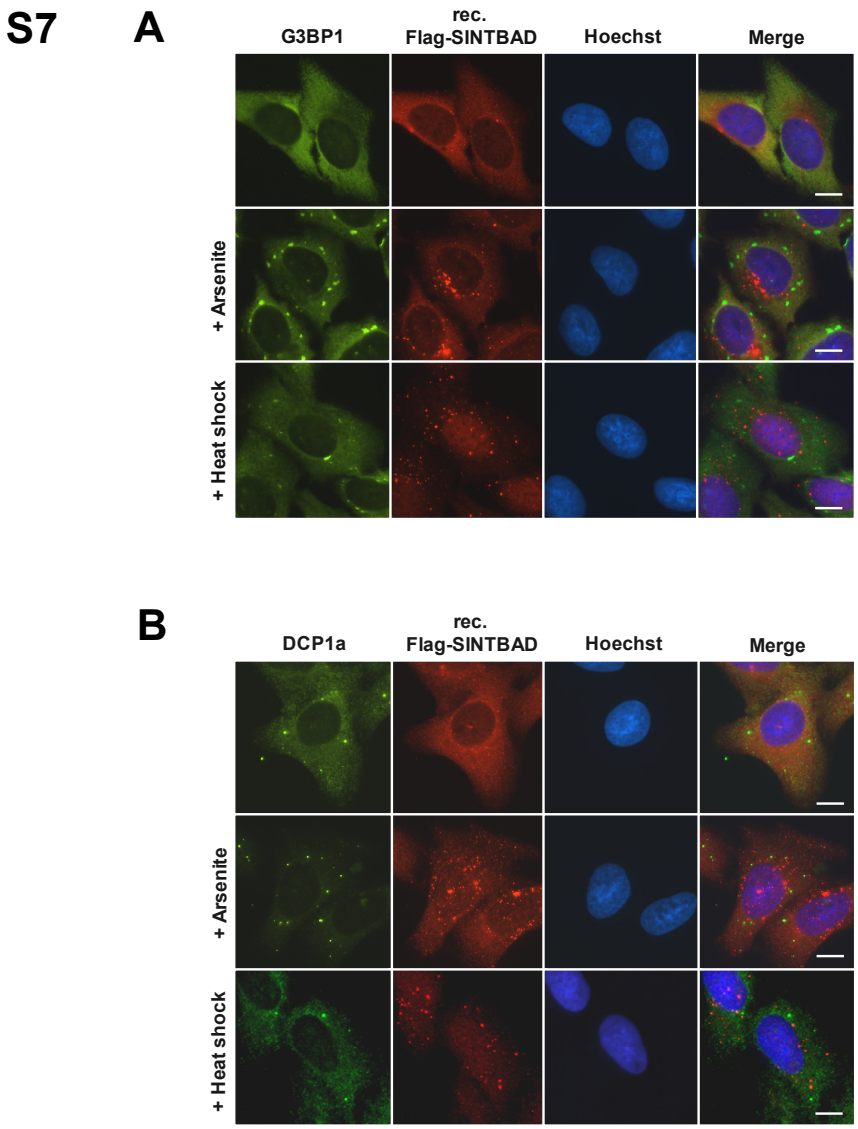

**Suppl. Fig. S5. Disaggregation of heat shock-induced SINT-speckles is independent of de novo protein expression and autophagy, Related to Figure 3. (A)** SINTBAD-deficient U2OS cells reconstituted with Flag-SINTBAD were left untreated or exposed to heat shock. Indicated cells were allowed to recover from heat shock at 37 °C for 3 h in the absence or presence of the translation inhibitor anisomycin (5 µg/ml). **(B)** SINTBAD-deficient U2OS cells stably expressing Flag-SINTBAD were left untreated or exposed to heat shock, followed by a 3 h long recovery period at 37 °C. Treatments and recovery were performed in the presence of the indicated autophagy inhibitors (0.5 µM Bafilomycin A, 20 mM $NH_4Cl$, 20 µM Chloroquine). The percentage of cells showing the displayed phenotype is indicated.

# Supplementary figures




**Suppl. Fig. S6. SINTBAD does not colocalize with membrane-surrounded organelles, Related to Figure 4.** U2OS cells stably expressing Flag-SINTBAD were treated with arsenite, exposed to heat shock or left untreated as shown. Immunofluorescence studies were performed by costaining Flag-SINTBAD together and the indicated marker proteins for Golgi (RCAS1), lysosomes (LAMP1), peroxisomes (CAT), endosomes (EEA1) and endoplasmatic reticulum (PDI). Representative pictures are shown. Scale bars = 10 µm.

# Supplementary figures




**Suppl. Fig. S7. SINTBAD does not colocalize with or influence the formation of other MLOs, Related to Figure 4.** Reconstituted U2OS cells were left untreated or exposed to arsenite or heat shock. Costaining of Flag-SINTBAD with G3BP1 **(A)** or DCP1a **(B)** allowed the analysis of the occurrence of stress granules and P bodies, respectively. Scale bars = 10 µm.

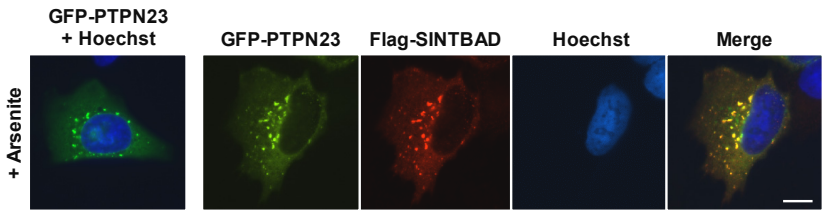
# Supplementary figures

## S8

**A**

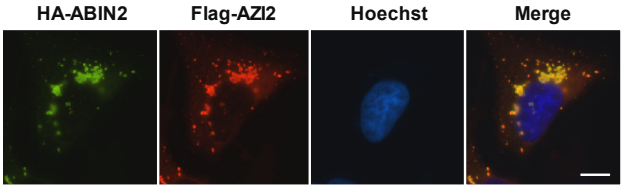
| IP | Group 1 | Group 2a | Group 2b |
|---|---|---|---|
| **control IgG** | no detection in both experiments | detected in both experiments | detected in one experiment |
| **anti-Flag Ab** | detected in both experiments | | |
| Threshold for SINTBAD interactome (+ min. 1 unique peptide in both experiments) | interactors defined by >25.5 log2 intensity | interactors defined by >2 log2 fold-change & >1,3 -log10 p-value | interactors defined by >4 log2 fold-change enrichment in comparison to median of control IgG |

**B**




**Suppl. Fig. S8. Characterization of SINTBAD interactors, Related to Figure 5. (A)** 293T cells were transfected to express Flag-SINTBAD and co-immunoprecipitation experiments were performed. After identification of co-immunoprecipitating proteins by mass spectrometry, the specifically interacting proteins were defined according to the criteria indicated above. **(B)** U2OS cells were transfected to express the SINTBAD interactor PTPN23 either alone or together with Flag-tagged SINTBAD, followed by treatment with arsenite as shown. Immunofluorescence was used to reveal the intracellular localization of the proteins. Scale bar = 10 µm.

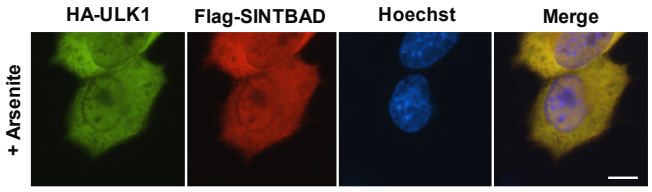
## S9




**Suppl. Fig. S9. Analysis of ABIN2/AZI2 interaction, Related to Figure 6.** U2OS cells were transfected to express HA-ABIN and Flag-AZI2 and analyzed by immunofluorescence. Scale bar = 10 µm.

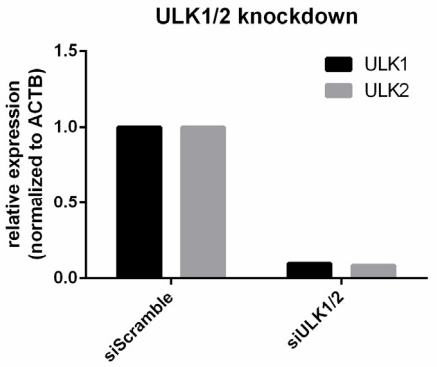
# Supplementary figures

**S10**




**Suppl. Fig. S10. ULK1 restricts SINT-speckle formation, Related to Figure 6.** U2OS cells were transfected to express HA-ULK1 together with Flag-SINTBAD. Cells were treated with arsenite and analyzed by indirect immunofluorescence. A representative experiment is shown, nuclear DNA was stained with Hoechst. Scale bar = 10 µm.

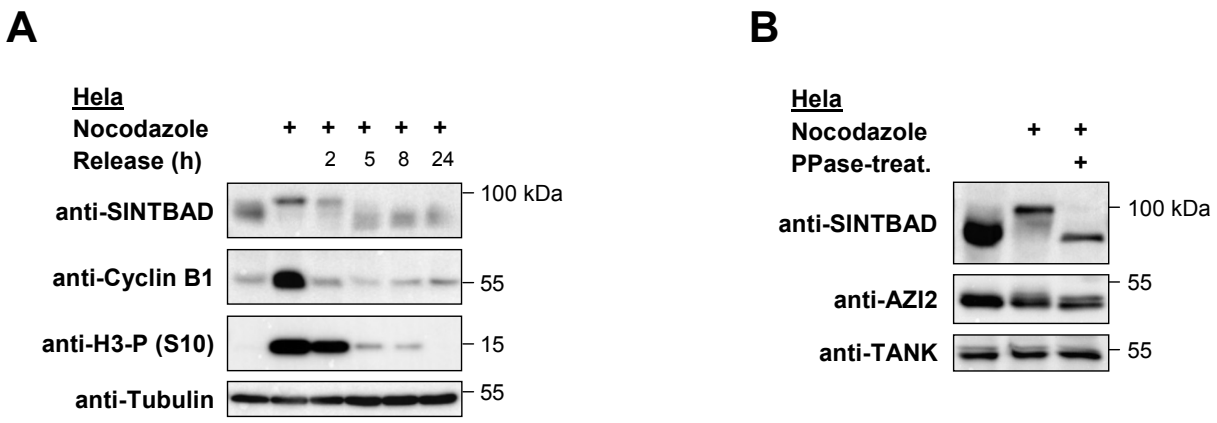
**S11**




**Suppl. Fig. S11. Characterization of ULK1/2 knockdown, Related to Figure 7.** Reconstituted U2OS cells stably expressing Flag-SINTBAD were treated with ULK1- and ULK2-specific Accell siRNAs. Three days after siRNA transfection, one aliquot of the cells was analyzed for efficient mRNA knockdown by qPCR using primers specific for ULK1 and ULK2, respectively. Gene expression values were normalized to transcription of the β-Actin encoding gene (ACTB), ULK1/2 expression in the control cells transfected with scrambled siRNAs was set as 1.

# Supplementary figures

## S12

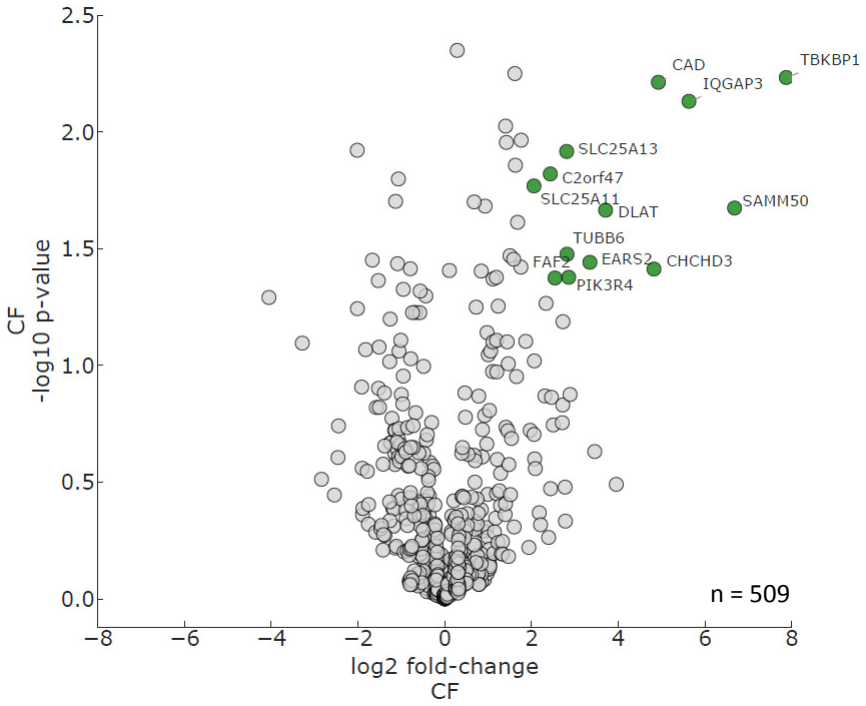



**Suppl. Fig. S12. Characterization of mitotic SINTBAD phosphorylation, Related to Figure 7. (A)** HeLa cells were arrested in prometaphase by adding 100 ng/ml nocodazole for 15 h. Cells were washed with PBS and further grown in DMEM for the indicated time periods. Cell extracts were tested for the electrophoretic behavior of the indicated proteins, the retarded migration of the phosphorylated SINTBAD protein (SINTBAD-P) is shown. Cyclin B1 and phosphorylated histone H3 (Ser10) were detected to ensure successful mitotic synchronization and release of cells. **(B)** To prove that the upshift of the SINTBAD band is caused by phosphorylation, SINTBAD extracted from nocodazole-arrested HeLa cells was incubated with λ phosphatase (PPase, 400 U, 30 min at 30 °C) as indicated and proteins were analyzed for their electrophoretic behavior by Western blotting as shown.

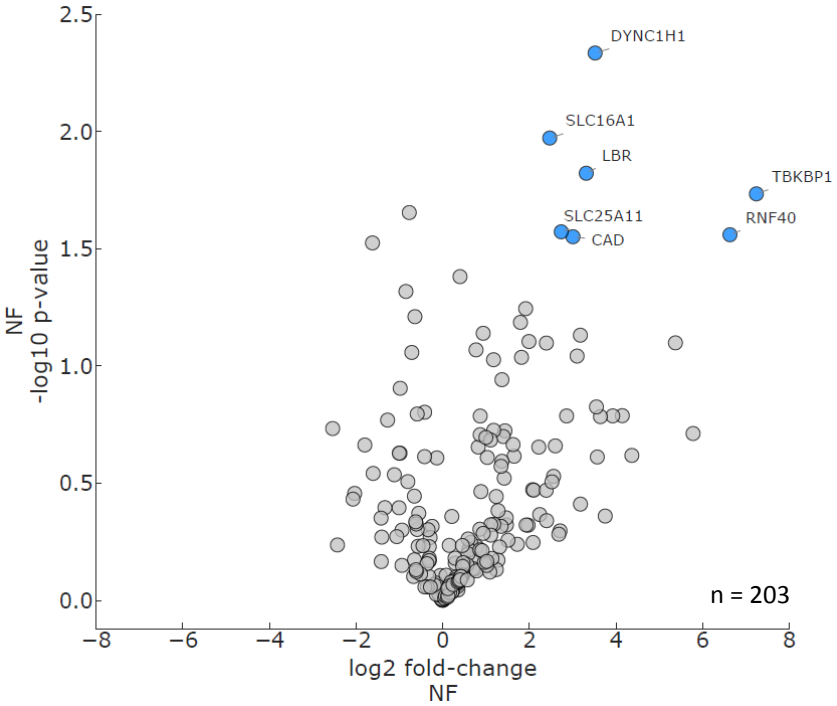
# Supplementary figures

## S13

**A**




**B**




**Suppl. Fig. S13. Analysis of the SINTBAD interactopme, Related to transparent methods.** Volcano plot analysis of the Group 2a interactors according to the definitions given in table 1. Interactors of the cytosolic fraction (CF) (A) or the nuclear/insoluble fraction (NF) (B) were plotted according to their log2 fold-enrichment and –log10 p-values as shown. The selected proteins considered as SINTBAD interactors are named and indicated by colors.

## TRANSPARENT METHODS

### Reagents and primary antibodies

The following reagents were purchased from the indicated companies: Anisomycin (A9789, Sigma-Aldrich), Bafilomycin A (tlrl-baf1, Invivogen), Chloroquine (C6628, Sigma-Aldrich), Geldanamycin (HN71, Carl Roth), IL-1β (gift from M. Kracht), LPS (L4130, Sigma-Aldrich), Nocodazole (M1404, Sigma-Aldrich), Pifithrin-μ (BML-AP503, Enzo Life Sciences), Radicicol (BN0437, Biotrend), Sodium (meta)arsenite (71287, Sigma-Aldrich), Sorbitol (S1876, Sigma-Aldrich), TNFα (11343015, ImmunoTools) Ver155008 (SML0271, Sigma-Aldrich). The following antibodies were used in this study: anti-AZI2 (WB: 1:1000, ab192253, Abcam), anti-CAT (IF: 1:400, #12980, Cell Signaling), anti-c-Myc (IF: 1:1000, WB: 1:2000, sc-40, Santa Cruz Biotechnology), anti-Cyclin B1 (WB: 1:500, GSN11, Thermo Fisher Scientific), anti-DCP1a (IF: 1:200, sc-100706, Santa Cruz Biotechnology), anti-eIF4G (IF: 1:200, sc-133155, Santa Cruz Biotechnology), anti-ERK1/2-P (T202/Y204-P; WB: 1:1000, #9101, Cell Signaling), anti-Flag M2 (IF: 1:2000, WB: 1:5000, F3165, Sigma-Aldrich), anti-G3BP1 (IF: 1:400, sc-81940, Santa Cruz Biotechnology), anti-HA (IF: 1:500, WB: 1:1000, 11867423001, Roche), anti-Histone H3 (WB: 1:5000, ab1791, Abcam), anti-Histone H3-P (S10-P; WB: 1:1000, #9706, Cell Signaling), anti-p38-P (T180/Y182-P; WB: 1:1000, #9211, Cell Signaling), anti-SINTBAD (WB: 1:1000, #8615, Cell Signaling), anti-TBK1 (IF: 1:200, WB: 1:5000, ab40676, Abcam), anti-TBK1-P (S172-P; IF: 1:150, WB: 1:1000, #5483, Cell Signaling), anti-TANK (WB: 1:400, sc-166643, Santa Cruz Biotechnology), anti-Tubulin (WB: 1:1000, E7, DSHB), Organelle Localization IF Antibody Sampler Kit including antibodies detecting AIFM1, EEA1, LAMP1, PDI and RCAS1 (#8653, Cell Signaling).

### Plasmids

SINTBAD was cloned by PCR from a human sequence-verified cDNA clone pBSII-SK(+)-TBKBP1 (BC167150, Biocat) along with an N-terminal epitope tag into the pcDNA3.1 vector (Invitrogen). Flag-SINTBAD truncation mutants were generated by cloning a PCR-amplified fragment into pcDNA3.1 (ΔN1: 106-615 aa, ΔN2: 165-615 aa, ΔC: 1-520 aa). Expression plasmids for Flag-TANK, Flag-AZI2 and Myc-TBK1 were kindly provided by Dr. A. Chariot (University of Liège, Belgium, (Chariot et al., 2002)); plasmids expressing HA-HSP70 and Flag-HSP90 were from Dr. B. Song (Emory University School of Medicine, Atlanta, USA, (Hwang et al., 2010)); pEGFP-C2-PTPN23 was obtained from Dr. C. A. Tanase (University of Bucharest, Romania, (Tanase, 2010)); pEGFP-AMBRA1 was generated by subcloning the

coding sequence of pLPCX-Ambra1-Flag (Dr. F. Cecconi, University of Rome, Italy, (Nazio et al., 2013)) into the pEGFP-C2 vector (Clontech); PCR-amplified ABIN2 from pCAGGS-E-hABIN2, that was kindly provided by Dr. R. Beyaert (VIB-Ghent University, Belgium, (Van et al., 2001)) was subcloned with a N terminal HA-tag into pcDNA6 (Invitrogen); pcDNA3.1-HA-ULK1 was obtained from Dr. S. H. Tooze (The Francis Crick Institute, London, UK, (Joachim et al., 2015)) and its kinase-inactive K46I mutant was generated by site-directed mutagenesis (QuikChange II XL, Agilent, (Chan et al., 2009)). Flag-KAT2A was from Dr. Ezra Burstein (UT Southwestern, Dallas, USA, (Mao et al., 2009)) and pIRES-3x-Flag-NSs (SFTSV) from Dr. J. U. Jung (University of Southern California, Los Angeles, USA, (Choi et al., 2019)).

**Cell culture, transfections and treatments**

293T, HEK-TLR4, HeLa and U2OS cells were cultured in DMEM (Gibco) containing high glucose (4.5 g/l), L-alanyl-glutamine (4 mM) and sodium pyruvate (110 mg/l) supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown in a humidified incubator at 37 $^{o}$C under 5 % $CO_2$. Transient transfections of plasmids were performed using linear polyethylenimine (PEI, Polyscience Inc.) as described previously (Saul et al., 2015). Unless indicated otherwise, cells were treated with 0.5 mM arsenite for 1 h or were exposed to heat shock at 43 °C in a humidified cell culture incubator with a 5 % $CO_2$ atmosphere for 1 h. Inhibitors were incubated 1 h prior cell stimulation. Cell stimulations for the experiments displayed in Fig. 1C were done as follows: HeLa cells were treated with 0.5 M sorbitol (30, 60, 90 min), 0.5 mM arsenite (20, 40, 60 min) or 4 % (v/v) ethanol (15, 30 and 60 min). In addition, HEK-TLR4 cells were treated with 1 µg/ml LPS (1, 2, 4 h), U2OS cells with 10 ng/ml IL-1β (0.5, 1.5, 5 h) and HEK293 cells with 20 ng/ml TNFα (30, 60, 90 min).

**Generation of CRISPR/Cas9-mediated knockout cells and reconstitution of them**

In order to generate SINTBAD- and/or AZI2-deficient U2OS cells, CRISPR/Cas9-mediated genome editing technology was performed as described (Ran et al., 2013). The target site for human SINTBAD was designed as an anti-sense sgRNA (5´-CGTAGACTTTGAGGCGGCGT-3´) within the first exon of the SINTBAD gene. The target site for sgAZI2 was designed within the second exon as 5´-GGCCTATCATGCATATCGAG-3´. Oligos were ligated into px459 V2.0 vector (Addgene plasmid #62988) using standard protocols and verified by sequencing. U2OS cells, seeded in a 6 cm dish, were transfected

with 1 µg of empty px459 or px459-sgRNA vector. One day after transfection, cells were selected for 30 h using 1 µg/ml Puromycin (Invivogen), diluted and further grown to allow the formation of single-cell clones. These clones were picked and analyzed for SINTBAD, AZI2 and Cas9 expression and verified by sequencing of the genomic DNA at the appropriate locus. To obtain SINTBAD/AZI2 double-deficient cells, a SINTBAD-deficient cell clone was transfected with px459-sgAZI2 and selected as described above. To reconstitute SINTBAD-deficient U2OS cells, cells were transfected with an pcDNA3.1/zeo-Flag-SINTBAD expression construct and continuously selected using 400 µg/ml Zeocin (Invivogen). Single-cell clones were picked an analyzed by Western blotting.

**Knockdown of ULK1 and ULK2**

Reconstituted U2OS cells stably expressing Flag-SINTBAD were treated with 1 µM ULK1 and ULK2-specific Accell siRNAs (Dharmacon #A-005049-13-0005 and #A-005396-14-0005) or with a scrambled control RNA (Seibert et al., 2019) according to the protocol using Accell siRNA Delivery Media (Dharmacon #B-005000-500). Three days after siRNA transfection, half of the cells were seeded on coverslips and used for immunofluorescence staining and the remaining cells were analyzed for efficient knockdown by real time qPCR, using the following primers specific for ACTB (5′-CATGTACGTTGCTATCCAGGC-3′, 5′-CTCCTTAATGTCACGCACGAT-3′), ULK1 (5′-ACCCCATTACTGCGAACCTGGA-3′, 5′-GCACGAACAGCAGCGTGAAGC-3′) and ULK2 (5′-TCTGCATCACCATGTGCAAGAA-3′, 5′-AACATCTCATCCAGGGCT-3′).

**Cell lysis protocols and subcellular fractionation**

To prepare cell lysates under native conditions, cells were lysed on ice for 20 min in IGEPAL buffer (20 M Tris/HCl pH 7.5, 150 mM NaCl, 1 % IGEPAL CA-630 (Sigma-Aldrich), 5 % glycerol and freshly added 10 mM NaF, 0.5 mM $Na_3VO_4$, 1 mM PMSF, 5 µg/ml leupeptin and 5 µg/ml aprotinin). The lysates were cleared by centrifugation and the supernatants were transferred into a fresh tube and either used for coimmunoprecipitation studies or mixed with sample buffer for Western blot analysis. To lyse cells under denaturing conditions, the washed cell pellets were resuspended in 1 × SDS sample buffer and sonicated two times for 20 sec with a Branson sonifier to shear the genomic DNA. After boiling the samples for 5 min, the lysates were analyzed by Western blotting. For subcellular fractionation experiments, cells, grown and treated in a 6 cm dish, were lysed in 160 µl low-salt buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM β-mercaptoethanol and 0.5 mM freshly

added PMSF) on ice for 10 min. NP-40 (Roche) was added to a final concentration of 0.25 %, samples were briefly vortexed and centrifuged for 10 sec at 16 000 × g. The supernatants representing the cytoplasmic fractions were collected in fresh tubes and mixed with 5 × SDS sample buffer. The remaining pellets representing the nuclear/insoluble fractions were washed twice in low-salt buffer and then resuspended in 180 µl 1 × SDS sample buffer, boiled and sheared two times for 20 sec with a sonifier. The purity of the cellular fractions was confirmed by Western blotting, detecting Tubulin and histone H3 as markers for the cytosolic or nuclear/insoluble fraction, respectively.

**Coimmunoprecipitation experiments and Western blotting**

For coimmunoprecipitation, cleared cell extracts lysed in IGEPAL lysis buffer were filled up to a volume of 600 µl with lysis buffer and supplemented with 1 µg precipitating antibody or control IgG. After adding 20 µl protein A/G agarose (Millipore), the samples were incubated for 4 h at 4°C on a rotating wheel. Alternatively, covalently antibody-coupled affinity gels were used for coimmunoprecipitation (anti-Flag M2 affinity gel, A2220, Sigma-Aldrich; GFP-Trap_A, Chromotek). Agarose beads were then washed four times with 1 ml cold IGEPAL buffer. Precipitated proteins were eluted by adding 1.5 × SDS sample buffer. Equal amounts of protein were separated by SDS-PAGE, followed by semidry blotting to a PVDF membrane (IPVH00010, Millipore). Further analysis was performed using standard methods. After blocking the membrane, primary antibodies, diluted in 2 % nonfat dry milk or 5 % BSA (Sigma-Aldrich) in TBST, were incubated overnight at 4 °C. Peroxidase-coupled antibodies (Jackson ImmuoResearch) were diluted 1:5000 in 2 % dry milk in TBST and incubated 2 h at room temperature. Immunoreactive bands were detected using the Western Lightning Plus-ECL reagent (Perkin Elmer) and visualized on a ChemiDoc Imaging System (Bio-Rad).

**Immunofluorescence staining**

U2OS cells or their derivatives were grown on coverslips in 12- or 24-well plates. Cells were transfected and/or treated as indicated and described in the figure legend. After washing the cells with PBS, cells were fixed for 1 min with ice-cold methanol/acetone (1:1). After rehydration, the cells were blocked with 5 % BSA in PBS for 1 h at room temperature. Coverslips were subsequently incubated with the indicated primary antibodies, diluted in PBS containing 1 % BSA and 0.1 % Triton X-100, for 90 min at room temperature or at 4 °C overnight. After washing three times with PBS cells were incubated with the appropriate secondary Alexa488- or Cy3-conjugated antibodies (Jackson ImmunoResearch) diluted

1:3000 in 1 % BSA in PBS for 90 min in the dark. After incubation, cells were washed three times in PBS and the nuclear DNA was stained with Hoechst 33324 (Invitrogen). The samples were mounted with Mowiol mounting medium and stored at 4 °C. Analysis of the stained cells was done using an Eclipse TE2000-E microscope (Nikon) and a 63 × oil-immersion lens. For each condition >30 healthy individual cells were analyzed and pictures of one representative cell were taken with an OCRA-spark digital CMOS camera (C11440-36U, Hamamatsu). For the quantification of protein localizations and cellular phenotypes, at least 100 cells for each condition were analyzed.

**Mass spectrometry**

To identify SINTBAD interactors by mass spectrometry, 293T cells were either transfected with the pcDNA3.1-Flag-SINTBAD expression construct or the empty vector. Four 10 cm dishes for each condition were taken for large-scale immunoprecipitation. Washed cells were harvested and lysed in 1 ml low-salt buffer as described above. The received cytosolic fraction was mixed with 1 ml IGEPAL lysis buffer, containing Complete Protease Inhibitor Cocktail and PhosSTOP (Roche). The remaining cell pellet was washed two times in low-salt buffer and then resuspended in 2 ml IGEPAL lysis buffer and sonified two times for 20 sec. The two fractions were precleared by incubating them 1 h with 3 µg control mouse IgG (Santa Cruz Biotechnology) and 40 µl Protein A/G Agarose. Afterwards, immunoprecipitation was performed by incubating the lysates with 6 µg anti-Flag M2 antibodies together with 80 µl Agarose beads for 4 h at 4 °C on a rotating wheel. Beads were washed five times with 2 ml IGEPAL lysis buffer, transferred to a fresh tube and proteins were eluted at 70 °C for 10 min in LDS sample buffer (Invitrogen). Samples were separated on a 4-12 % Bis-Tris gradient-gel (NuPAGE, Invitrogen), stained with colloidal Coomassie (Invitrogen) and cut into small pieces (7 slides each lane). In-gel digestion of the proteins with trypsin and purification of the peptides was performed as described (Seibert et al., 2019). Peptide solutions were desalted by stop and go extraction (STAGE) tips (Rappsilber et al., 2003). The purification and mass spectrometry of two individual experiments was performed with a time lag and therefore analyzed with different instrumental settings. Dissenting setting parameters of analysis 1 and 2 are indicated by a slash. Samples were eluted from STAGE tips with acetonitrile and applied to the UHPLC system (EASY-nLC 1000, Thermo Fisher Scientific) in 0.1 % formic acid. Separation of peptides by hydrophobicity was performed with 50/18 cm in-house packed C18 columns (1.9 µm C18 beads, Dr. Maisch GmbH). Peptide elution was achieved with a binary solvent system (solvent A: 0.1% formic acid; solvent B: 80% acetonitrile, 0.1% formic

acid) by increasing the relative amount of B from 10 % to 38 % in a linear gradient within 35/20 min, followed by 5/3 min up to 60 % and another 5/2 min to 95%. Re-equilibration was done within 5 min at 5 %. The samples were transferred to an in line coupled QExactive orbitrap/QExactive HF mass spectrometer (Thermo Fisher Scientific) using a nano electrospray ionization source. Full MS spectra were acquired with a data-dependent Top10/15 method that comprised a resolution of 70,000/60,000 at 200 m/z and an automatic gain control (AGC) target of 3e6 at a maximum injection time of 20 ms. The 10/15 most intense ions were further fragmented with higher-energy collisional dissociation (HCD) at a normalized collision energy of 25/27 and $MS^2$ spectra were generated at 35,000/30,000 resolution, AGC target of 5e5/1e5 and maximum injection time 120/64 ms.

Data from two biological replicates (2 × 28 raw files of IgG control and anti-Flag-SINITBAD) were analyzed using MaxQuant (v1.5.5.18) (Cox and Mann, 2008) and the implemented Andromeda search engine (Cox et al., 2011). Protein assignment was accomplished with correlation of fragment spectra with the UniProt human database (July 2016). Common contaminants were excluded from the analysis. Data processing was performed with tryptic specifications and default settings for mass tolerance in MS and MS/MS spectra. The minimal peptide length was set to 7 amino acids by default and the false discovery rate on protein and peptide level was set to 1 %. Prior to further processing of the data, contaminants, reverse entries and proteins that were only identified by a modification site were filtered out. In order to define high confidence interactors, identified proteins were classified according to criteria given in suppl. Fig. 8A. Proteins from all three groups were combined and constitute the SINTBAD interactome.

**Bioinformatic analysis**

The volcano plots for proteins of group 2a are displayed in suppl. Fig. S13 and were done using the Instant Clue program (Version 0.5.2) (Nolte et al., 2018). The SINTBAD interactome was analysed by the STRING database version 11.0. Only interactions with a medium confidence score of 0.4 were shown. Line thickness indicates the strength of data support. The assignment of SINTBAD interactors to biological functions and processes was done by combined Genecards, Uniprot and Pubmed searches (www.genecards.org; www.uniprot.org; www.ncbi.nlm.nih.gov/pubmed). The intrinsic disorder was analyzed using the PONDR prediction tool (http://www.pondr.com) (Peng et al., 2005) and Espritz (Walsh et al., 2012).

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