AKAP95 interacts with nucleoporin TPR in mitosis and is important for the spindle assembly checkpoint

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Abbreviations:

AKAP: A-kinase anchoring protein
CCF: chromatin cytoplasmic fragments
KT: kinetochore
MAD: mitotic arrest deficient
MT: microtubule
NEBD: nuclear envelope breakdown
SAC: spindle assembly checkpoint
ABSTRACT

Faithful chromosome segregation during mitosis relies on a proofreading mechanism that monitors proper kinetochore-microtubule attachments. The spindle assembly checkpoint (SAC) is based on the concerted action of numerous components that maintain a repressive signal inhibiting transition into anaphase until all chromosomes are attached. Here we show that A-Kinase Anchoring Protein 95 (AKAP95) is necessary for proper SAC function. AKAP95-depleted HeLa cells show micronuclei formed from lagging chromosomes at mitosis. Using a BioID proximity-based proteomic screen, we identify the nuclear pore complex protein TPR as a novel AKAP95 binding partner. We show interaction between AKAP95 and TPR in mitosis, and an AKAP95-dependent enrichment of TPR in the spindle microtubule area in metaphase, then later in the spindle midzone area. AKAP95-depleted cells display faster prometaphase to anaphase transition, escape from nocodazole-induced mitotic arrest and show a partial delocalization from kinetochores of the SAC component MAD1. Our results demonstrate an involvement of AKAP95 in proper SAC function likely through its interaction with TPR.
INTRODUCTION

Eukaryotic cell division is a complex and highly ordered process relying on the participation of several multi-protein complexes. Sophisticated surveillance mechanisms operating under cell division ensure proper distribution of DNA into daughter cells. Errors in the faithful partition of chromosomes between daughter cells can result in severe molecular intracellular imbalances, leading to the development of congenital diseases and tumor formation. The spindle assembly checkpoint (SAC) is a regulatory mechanism ensuring that all kinetochore (KT) – spindle microtubules (MTs) attachments are established by delaying anaphase as long as unattached chromosomes remain.

The SAC accomplishes this by monitoring KT – spindle MTs attachments and preventing the degradation of securin and cyclin B1 triggered by the ubiquitinating activity of the APC/C complex (anaphase-promoting complex/cyclosome and its coactivator Cdc20). Two important proteins in the inhibition of APC/C are mitotic arrest deficient 1 and 2 proteins (MAD1 and MAD2). MAD2 together with BUBR1, BUB3 and CDC20, forms the mitotic checkpoint complex, a diffusible macromolecular structure assembled at KTs that inhibits the activation of APC/C, thereby preventing entry into anaphase. MAD1 is believed to form a stable tetramer with active MAD2 at unattached KTs, but the precise mechanism of MAD1/MAD2 complex recruitment to KTs during prometaphase remains unknown.

During interphase, MAD1 and MAD2 are anchored at the nuclear pore complex (NPC) by TPR, a protein residing in the nuclear basket of the NPC. siRNA-mediated depletion of TPR results in loss of MAD1/MAD2 nuclear envelope localization. The role of TPR in recruitment of MAD1 to KTs is the subject of discussion with one report showing reduction and others showing normal or even increased MAD1 levels at KTs following TPR knockdown. However, reduction of TPR levels consistently results in the absence of MAD2 at KTs and in the inability of MAD1 to activate MAD2. This suggests a role of TPR in the recruitment and proper function of central SAC components. Accordingly, siRNA-mediated knock-down of TPR causes mitotic defects such as chromosome lagging and multipolar spindle formation.

AKAP95 is a member of the A-kinase anchoring protein (AKAP) protein family, the members of which distribute and localize the pleiotropic effects of cAMP-dependent protein kinase A (PKA) to distinct subcellular compartments. AKAP95 is mainly found in the nucleus, in a detergent- and DNAse-insoluble fraction. Besides binding to the regulatory
type II (RII) subunit of PKA, AKAP95 associates with several cellular effectors that suggest a role for AKAP95 as transcription regulator scaffold or chromatin organizer 13–16.

At the G1-S phase transition, AKAP95 orchestrates the initiation of DNA replication by recruitment to chromatin of the minichromosome maintenance protein 2 (MCM2), a component of the prereplication complex 17. AKAP95 has also been reported to interact with D-type cyclins to form a PKA(RII)-cyclin D/E1 complex 18,19. Work from our laboratory has shown that AKAP95 is required for chromatin condensation at mitosis onset, as well as for chromatin condensation maintenance in a PKA-dependent manner 12. AKAP95 further facilitates loading of condensin I onto chromosomes after nuclear envelope breakdown (NEBD) 20,21. Furthermore, interaction of AKAP95 with its paralog homologous to AKAP95 (HA95) and with histone deacetylase 3 (HDAC3) has been shown to be required for normal mitotic progression by deacetylating histone H3 22. Finally, AKAP95 enrichment has been observed along with RII at the spindle area in mitosis 23. However, the role of AKAP95 during progression through mitosis has not been explored.

RESULTS AND DISCUSSION

AKAP95 is involved in mitotic cell division 21,22,24, but its mechanism of action remains obscure. Knockdown of AKAP95 in HeLa cells using small interfering RNA (siRNA; supplementary Fig 1A) had modest effects on cell cycle progression (data not shown; 22). However, nearly 50% of AKAP95-depleted cells contained DAPI-stained chromatin cytoplasmic fragments (CCFs; Fig 1A) clearly distinct from the nucleus, compared to less than 10% in control cells (siControl) and in cells siRNA-knocked down for an unrelated protein (siUnrProt). CCFs were often observed in association with aberrant nuclear morphology consisting of misshapen nuclei with protrusions. Similar results were observed with an independent AKAP95 siRNA oligonucleotide (supplementary Fig 1B.).

To characterize CCFs, we stained siAKAP95 knock-down HeLa cells with chromatin and nuclear envelope markers. CCFs displayed a clear staining pattern for nuclear envelope components lamins A/C and B and for active (H3K4me3) and repressive (H3K9me3 and H3K27me3) chromatin associated marks (supplementary Fig 1C). We wondered whether CCFs in AKAP95-depleted cells were targeted for autophagy processes. None of the autophagy (p62, LC3) and lysosome (LAMP-1) markers we used showed an enriched localization at CCFs (supplementary Fig 1D), discarding the possibility that siAKAP95-induced CCFs are chromatin fragments processed via an autophagy-lysosomal pathway 25.
Cell cycle progression of AKAP95-depleted cells was next examined by time-lapse microscopy using stably-expressed histone H2B tagged to EGFP as a chromosome tracker. Strikingly, lagging chromosomes were observed during sister chromatid segregation at anaphase in AKAP95-depleted HeLaH2B-EGFP cells (Fig 1B, lower panel; Fig 1C; supplementary Movie 1), unlike control cells which progressed normally through mitosis (Fig 1B, upper panel; Fig 1C; supplementary Movie 2). After completion of mitosis, the lagging chromosomes gave rise to micronuclei (Fig 1B, lower panel, arrowheads), which resulted in the CCFs observed earlier (Fig 1A). In some cases, micronuclei were detected throughout the following cell cycle until the start of the next mitosis when they underwent chromatin condensation (supplementary Fig 1E). Similar results were observed with an independent AKAP95 siRNA oligonucleotide (supplementary Movie 3). Altogether, our results show a role for AKAP95 in preventing chromosome lagging during mitosis which leads to subsequent micronuclei formation.

To further define the function of AKAP95 in mitotic cell division, we used proximity-dependent labelling of proteins (BioID) to screen for AKAP95 proximate proteins since this assay is well suited for insoluble proteins which are less amenable to biochemical affinity-capture characterization. A modified E.coli biotin ligase (BirA*) is fused to AKAP95 and the resulting fusion protein will biotinylate interacting and vicinal AKAP95 proteins in vivo. In U2OS cell lines containing a BirA*-AKAP95 fusion construct to which a Myc tag was added (Myc-BirA*-AKAP95; Fig 2A), addition of doxycycline resulted in robust expression of Myc-BirA*-AKAP95 (Figs 2B and C) with a nuclear localization and distribution comparable to endogenous AKAP95 (Fig 2C, bottom). Strong biotinylation was detected when expression of Myc-BirA*-AKAP95 was induced in the presence of biotin in the culture medium (Figs 2B and C), showing that Myc-BirA*-AKAP95 is targeted to the same specific location as endogenous AKAP95 where it can biotinylate proximal endogenous proteins.

U2OS cells stably expressing Myc-BirA*-AKAP95 with levels similar to those of endogenous AKAP95 were cultured in the presence of biotin for 24h (supplementary Fig 2A), before recovery of biotinylated proteins using streptavidin-coated paramagnetic beads (supplementary Fig 2B) and identification by liquid chromatography coupled to mass spectrometry (LC-MS). A total of 428 proteins were identified with at least two unique peptides in the Myc-BirA*-AKAP95 sample and no peptides in control cells (supplementary Table 1). Validating our approach, several known AKAP95-binding partners were detected including RII, DPY30, MCM2 and NCoR2/SMRT. Relative abundance of the identified proteins was calculated, corrected for protein size before broad classification.
based on their predominant subcellular localization and function (Fig 2D, left chart). Approximately 60% of the identified proteins were ranked in the nuclear category. The remaining non-nuclear proteins were categorized as cytoplasmic/ER/membranes and mitochondrial proteins, possibly as a result of the discrete cytoplasmic pool of AKAP95 and from limited endogenous biotinylating at mitochondria. Further sub-classification of nuclear proteins (Fig 2D, right chart) revealed that signalling, chromatin and transcriptional regulation, RNA processing and nuclear pore complex/nuclear transport were the most represented groups.

Of all proteins identified in the AKAP95-BioID screen, the nucleoporin translocated promoter region (TPR) was ranked first based on the number of spectral counts (184) and unique peptides (76) which cover 36% of the protein sequence (supplementary Fig 2C). TPR has been involved in a wide variety of nuclear functions including RNA transport, chromatin organization and autophagy, and depletion of TPR results in a chromosome lagging phenotype similar to AKAP95 depletion, suggesting that the two proteins might be functionally related.

To confirm our BioID results and examine a potential interaction between TPR and AKAP95 in regulation of normal chromosome congression, we first performed co-immunoprecipitation experiments. TPR was detected in lysates of Myc-BirA*-AKAP95-expressing U2OS cells by immunoprecipitation of both tagged and endogenous AKAP95. Conversely, myc-BirA*-AKAP95 was detected in endogenous TPR immunoprecipitates (Fig 3A). Furthermore, Western blot analysis on the protein samples that were submitted to BioID analysis revealed TPR among the proteins biotinylated following expression of Myc-BirA*-AKAP95 (Fig 2E). Confocal microscopy analysis in U2OS cells revealed that while TPR displayed a nuclear periphery staining, AKAP95 appeared largely excluded from the nuclear periphery (supplementary Fig 3A). We then investigated AKAP95 and TPR distribution during mitosis. At metaphase, TPR is enriched around the metaphase plate in a zone overlapping with spindle MTs and becomes enriched at the spindle mid-zone after anaphase onset (Fig 3B), consistent with previous observations. Notably, AKAP95 shows the same localization enrichment at the mitotic spindle area during metaphase, then at the spindle mid-zone during anaphase (Fig 3B; supplementary Fig 3B), also consistent with previous observations. The interaction of AKAP95 and TPR in the spindle area was confirmed by in situ proximity ligation assay (PLA) which revealed a large number of fluorescent foci at this localization. AKAP95 depletion by RNAi abolished the formation of fluorescent PLA foci (Figs 3C and 3D). Finally, the interaction between TPR and AKAP95 was confirmed by...
immunoprecipitation experiments in mitotic-arrested HeLa cell lysates, showing recovery of a fraction of TPR with endogenous AKAP95 (Fig 3E).

We next examined whether AKAP95 and TPR localization in mitosis were dependent on each other. In AKAP95-depleted cells, the enriched localization of TPR at the spindle microtubule area in metaphase and spindle mid-zone area in anaphase was lost, and TPR was homogenously distributed throughout the cytoplasm from prometaphase to late anaphase (Fig 3F). Interestingly, the nuclear pore localization of TPR was normal in interphase AKAP95-depleted cells, showing that AKAP95 is not required for TPR localization outside of mitosis (Supplementary Fig 3A). Depleting TPR in HeLa cells did not affect AKAP95 localization at any mitosis stage (Supplementary Fig 3C), and yielded a much lower proportion of cells displaying micronuclei (15%) compared to AKAP95 depletion (65%; Fig 3G). These results demonstrate that AKAP95 and TPR are associated in the mitotic spindle area and that mitotic TPR localization is dependent on AKAP95.

TPR has been reported to be a regulator of mitosis progression by associating with important components of the SAC such as MAD1. Since we show that loss of AKAP95 results in lagging chromosomes (Fig 1) and delocalization of TPR in mitosis (Fig 3F), we reasoned that the SAC might be compromised in AKAP95-depleted cells. Testing this hypothesis, we observed by immunofluorescence a reduction of more than 50% of MAD1 enrichment at KTs in AKAP95-depleted cells compared to control (Figs. 4A and 4B). No AKAP95 accumulation was detected at KTs, similar to what has been reported for TPR (10,32; Schweizer et al., 2013). Furthermore, mitotic progression was evaluated in AKAP95-depleted and control HeLa H2B-EGFP cells by live-cell imaging. The time spent by cells from NEBD to anaphase onset was recorded in each condition. While control (siControl) cells showed a transition time of 30±7 minutes, AKAP95-depleted cells showed a 27% reduction (22±4 minutes) in the time required from NEBD to anaphase (Fig 4C). This shows that similar to TPR, mitosis is shortened in the absence of AKAP95. Finally, we measured the mitotic index in HeLa cells grown in the presence of 100 ng/ml nocodazole. We could observe only 27.4% of AKAP95-depleted cells arrested in mitosis after 12 hours, compared to 42.3% in control cells. This indicates that more than half of the cells normally arresting in mitosis exit from mitotic arrest when AKAP95 is depleted (Figs. 4D and 4E). Altogether, these results show that mitotic checkpoint signaling and recruitment of MAD1 are compromised in the absence of AKAP95, similarly to what has been reported for TPR.

Previous work from our laboratory has uncovered a role of AKAP95 in chromosome condensation during mitosis by targeting subunits of the condensin I complex to mitotic
Depletion of condensin I subunits including hCAP-D2 (the subunit shown to be recruited by AKAP95 to mitotic chromatin) leads to chromosome displacement and failure to align in metaphase; these phenotypes were however not detected in AKAP95-depleted cells. Loss of condensin I subunits also results in chromosome bridging with no detectable chromosome lagging, together with delayed transition from NEBD to anaphase. By contrast, AKAP95-depleted cells show a strong chromosome lagging phenotype and faster mitotic progression, respectively. We propose therefore that the effects on chromosome lagging are independent from the role of AKAP95 in global recruitment of condensin I to mitotic chromosomes, and suggest instead that AKAP95 is implicated in MAD1/MAD2-mediated mitotic checkpoint signaling. Since condensin I is known to localize to KTs, one cannot exclude however that the selective recruitment of condensin I to KTs is dependent on AKAP95 and is required for proper MAD1/MAD2-mediated mitotic checkpoint signaling; the latter seems however unlikely given the low frequency of lagging chromosomes in condensin I-depleted cells.

TPR has been shown to anchor MAD1 and MAD2 at the nuclear periphery in interphase and to unattached KTs in mitosis and its depletion leads to chromosome segregation defects. Also similar to AKAP95, TPR knock-down leads to faster prometaphase-to-anaphase transitions and to reduced association of MAD1 with KTs. Together with the loss of TPR mitotic enrichment in the spindle area upon AKAP95 depletion reported here, this suggests that the MAD1-mediated mitotic checkpoint signalling defect in AKAP95-depleted cells might be at least partially dependent on TPR localization. However and given the important differences in micronuclei formation (Fig 3G) and rates of acceleration of mitosis in AKAP95- and TPR-depleted cells (Fig 4C and ), we suspect that the role of AKAP95 in mitotic checkpoint signalling is not restricted to its role in localizing TPR to the spindle area.

Two TPR phosphorylation sites with roles in mitosis have been described. Interestingly, phosphorylation of TPR at S2094 is mediated by PKA and is considerably increased during mitosis, but its significance remains obscure. It is tempting to speculate that AKAP95 might be coordinating TPR phosphorylation on S2094 by recruiting PKA at the onset of mitosis and that TPR phosphorylation could be important for a balanced SAC response. On the other hand and given that AKAP95 also binds protein phosphatase 1 (PP1; T.K. and PC, unpublished observations), AKAP95 might also regulate TPR phosphorylation through PP1 which has been implicated in SAC silencing. In any case, both hypotheses would support our model showing that AKAP95 and TPR are found within the same molecular pathway assembling the SAC response.
The role of AKAP95 in controlling the MAD1/MAD2-dependent SAC reported here could point to an important role in preventing chromosomal instability and aneuploidy. Interestingly, the AKAP95 gene (AKAP8) has been recently associated with microcephaly, in line with the high number of genes involved in chromosome dynamics and cell cycle progression that have been linked to inherited cases of microcephaly. Further work will be required to delineate the underlying mechanism linking genes such as AKAP8 to unregulated cell proliferation in the central nervous system.

MATERIALS AND METHODS

Cell culture and stable cell line generation
U2OS, HeLa and derived stable cell lines (U2OS Myc-BirA*-AKAP95 and HeLa H2B-EGFP) were cultured in DMEM/F12 - GlutaMAX (Gibco) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. To generate U2OS cells stably expressing Myc-BirA*-AKAP95, a Myc-BirA*-AKAP95 insert containing the modified sequence of E.coli biotin ligase (hBirAR118G, BirA*, 26) and flanking Kozak and PolyA sites was synthetized (Genscript). Next, pRetroX Tet-ON Advanced U2OS cells (Clontech) were transfected using X-tremeGENE HP DNA (Roche) with a pRetroX-Tight-Pur6 vector (Clontech) containing Myc-BirA*-AKAP95. Selection with 0.5 µg/ml puromycin was initiated 48 h after transfection and maintained for 10 days. After colony formation, subclones were grown in 96-well plates before induction with 1 µg/ml doxycycline for 24 hours and screening by immunofluorescence. Clones were selected based on their moderate level of expression of Myc-BirA*-AKAP95, similar to levels of endogenous AKAP95.

BioID protein identification
For affinity capture of biotinylated proteins, 4*10^7 U2OS cells stably expressing Myc-BirA*-AKAP95 (BioID) or pRetroX Tet-ON Advanced U2OS cells (Control) were incubated for 24 hours in complete medium supplemented with 1µg/ml doxycycline and 50µM biotin. Immunoprecipitation experiments and affinity capture of biotinylated proteins in U2OS cells were as described in 26. For protein identification by mass spectrometry, proteins eluted from the streptavidin beads using SDS-sample buffer were separated by SDS-PAGE and visualized with Coomassie blue. Gel lanes containing separated proteins were excised and in-gel digestion was carried out by adding 0.1 µg trypsin (Promega) in 20 µl 25 mM NH₄HCO₃ pH 7.8, 1 mM CaCl₂, 5% acetonitrile for 16 hours at 37°C. The tryptic digest was stopped by adding 10 µl 2.5% trifluoroacetic acid. The peptides were purified with μ-C18 ZipTips.
(Millipore), and dried using a Speed Vac concentrator. The dried peptides were dissolved in 10 μl 1% formic acid in water, and then dissolved in 10 μL of aqueous 2% acetonitrile containing 1% formic acid. 5 μl of sample was injected into a Dionex Ultimate 3000 nano-UHPLC system (Sunnyvale) coupled online to an LTQ-Orbitrap XL mass spectrometer (ThermoScientific) equipped with a nanoelectrospray ion source. For liquid chromatography, aqueous 2% acetonitrile containing 0.1% formic acid was used as solvent A and solvent B contained 0.1% formic acid in 90% acetonitrile. Peptides were separated on an Acclaim PepMap 100 column (C18, 3 μm particle diameter, 100 Å pore size, 75 μm internal diameter, 50 cm length) at a flow rate of 0.3 μL/min using solvent gradients of 7% B to 35% B in 40 min and 35% B to 50% B in 3 min. An ion spray voltage of 1.7 kV, tube lens voltage of 120 V and transfer capillary temperature of 200°C were used. The mass spectrometer was operated in data-dependent acquisition mode and precursor ion survey scans (400 m/z to 2,000 m/z) were acquired in the Orbitrap analyzer with resolution R=60,000 at 400 m/z (after accumulation to a target of 500,000 charges in the LTQ). The fragmentation spectra were acquired in the linear ion trap using collision induced dissociation (CID) at a target value of 10,000 charges (normalized collision energy 35%, activation time 30 ms, activation Q 0.25, isolation width 2) for the top seven most abundant ions with a dynamic exclusion time of 60 s. The MS data were analyzed using Mascot (version 2.3.02; Matrix Science) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Mascot was set up to search the SwissProt_2011_11_ database (selected for Homo sapiens, 20,252 entries) assuming the digestion enzyme trypsin. X! Tandem was set up to search the uniprot_sprot database (535,248 entries) also assuming trypsin. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 ppm. S-carbamoylmethylcysteine cyclization (N-terminus) of the N-terminus, oxidation of methionine, acetylation of the N-terminus and acrylamide adduct of cysteine were specified in Mascot and X! Tandem as variable modifications. Scaffold (version Scaffold_4.4.8, Proteome Software Inc) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. To estimate the amount of proteins from the number of sequenced peptides, we used the emPAI index to take protein size into account by factoring in the number of observable peptides for a given protein. Protein classification for the 428 identified proteins
present exclusively in Myc-BirA*-AKAP95 U2OS cells was performed manually using the Subcellular location section of the corresponding protein entry in Uniprot (www.uniprot.org).

**siRNA transfections**

siRNA transfection of HeLa cells was done using HiPerfect reagent (Qiagen) with two rounds of transfection with a 48-hour interval. Cells were analyzed after 72 hours. AKAP95, TPR and an unrelated protein Stealth siRNAs were from Invitrogen and control siRNA was from GenePharma. siRNAs to human genes: AKAP95 GCCUGUUCUGUAUGCAAGUCCGUA, AKAP95 (oligo02) GGAACAAACUGAUGCAGAGUCUAAA, TPR GCACAACCAGGATAAGGTATG, unrelated protein (siUnrProt) AAUCGUUUGAGGCCAUGUCCG, random sequence UUCUCGAACGUGUCACGU T (siCtrl).

**Antibodies**

The following antibodies were used here: polyclonal anti-AKAP95 (Santa Cruz; sc10766), monoclonal anti-AKAP95 (Abcam; ab140628), polyclonal human anti-centromere HCT-0100 (Immunovision), monoclonal anti-FLAG M2 (Sigma; F1804), polyclonal anti-H3K4me3 (Abcam; ab8580), monoclonal anti-H3K9me3 (Millipore; 05-1250), polyclonal anti-H3K27me3 (Millipore; 07-449), polyclonal anti-H3S10P (Cell Signaling; 9701S), monoclonal anti-lamin A/C (Santa Cruz, sc7292), polyclonal anti-lamin B1 (Santa Cruz, sc6216), monoclonal anti-LAMP1 (Developmental Studies Hybridoma; H4A3), polyclonal anti-LC3 (MBL/Nordic Biosite; PM036), polyclonal anti-p62 (Progen, GP62-C), monoclonal anti-MAD1 9B10 (Santa Cruz; sc47746), monoclonal anti-myc (Invitrogen; R950-CUS), monoclonal anti-TPR (Abcam; ab58344), polyclonal anti-TPR (Abcam; ab70610), polyclonal anti-TPR (Bethyl; A300-826A) and monoclonal anti-α-tubulin (Sigma; T5168). Secondary antibodies were from Jackson Immunoresearch except Anti-Rabbit IgG HRP which was from Rockland.

**Flow cytometry**

For flow cytometry analysis of histone H3 phosphorylation, cells were fixed and prepared using anti-H3S10P following the manufacturer’s instructions (Upstate Biotechnologies; H2A.X Phosphorylation Assay Kit). Cells were analyzed with a FACS Calibur with Cellquest Pro software (Becton Dickinson).
Immunoprecipitation and western blotting

For immunoprecipitation in HeLa cells, cells were lysed in cold IP lysis buffer (50 mM Tris-HCl (pH 8), 120 mM NaCl, 1% NP-40, 1 mMDTT) containing PMSF, protease inhibitor cocktail (Roche) and Phosphatase inhibitors (Roche) at 4C for 10 minutes and briefly sonicated. The lysate was centrifuged at 15,000g for 10 minutes at 4C and the supernatant was pre-cleared with protein A/G Dynabeads (ThermoFisher) for two hours. Equal parts of the lysate were incubated 2 hours on a rotating wheel at 4C with 5 µg of following antibodies: polyclonal anti-AKAP95, polyclonal anti-TPR, anti-myc, anti-FLAG M2 and IgG control. Incubation with Protein A/G Dynabeads (40µl beads) was for 2 hours on a rotating wheel at 4C. Immune complexes were washed five times in ice-cold IP lysis buffer before resuspension in SDS sample buffer and resolution by 4-20% SDS-PAGE. Western blotting was as described earlier with the following antibodies: monoclonal anti-AKAP95 (1:2500), monoclonal anti-TPR (1:2500; Fig 3A), polyclonal anti-TPR (1:500), anti-myc (1:2000), and anti-α-tubulin (1:10000). Biotinylated proteins were detected directly using HRP-Streptavidin conjugate (Invitrogen; 43-4323; 1:5000). Input: 2-5% of total cell lysate.

Immunofluorescence and live-cell imaging

Immunofluorescence was performed as described earlier except for the addition of 0.01% Tween 20 and 2% bovine serum albumin to permeabilization and antibody dilution buffers with following antibodies: anti-AKAP95 (1:250), monoclonal anti-TPR (1:2500), anti-MAD1 (1:50), anti-α-tubulin (1:5000), anti-H3K4me3 (1:100), anti-H3K9me3 (1:100), anti-H3K27me3 (1:100), anti-lamin A/C (1:500), anti-lamin B1 (1:1000), anti-p62 (1:2000), anti-LC3 (1:500) and anti-LAMP1 (1:500). Fixation was carried out using ice-cold methanol for 6 minutes at -20C when anti-MAD1 was used. Biotinylated proteins were detected directly using Streptavidin-DyLight 488 (Thermo Scientific; 21832; 1:500). Images were acquired with a 100X objective (NA 1.4) on a PersonalDV Delta Vision widefield imaging station (Applied Precision) or with an Apochromat 63X objective (NA 1.4) on a LSM710 laser scanning confocal microscope (Zeiss). For live-cell imaging experiments, cells cultured in 35mm glass bottom culture dishes (MatTek) were imaged with the PersonalDV imaging station in a 37°C humid 5% CO2 atmosphere. A z-stack made of 8-12 optical sections with a 300 nm step size was acquired every 4 minutes with a binning of two for 4-12 hours. Signal/noise ratio of the videos was improved using the Smooth and PureDenoise plugins in ImageJ 1.51h. Iterative deconvolution was performed on images captured with the PersonalDV imaging station using the softWoRx software (Applied Precision). For PLA, we
combined primary antibodies raised in rabbit with antibodies raised in mouse. Detection was performed using specific secondary antibodies conjugated with PLA probes according to the manufacturer’s instructions (Duolink, Sigma). Quantification of MAD1 levels at KTs was calculated by generating a maximum projection image from all stacks and measuring the gray intensity values obtained with MAD1 relative to values obtained with anti-centromere HCT-0100.

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REFERENCES


**Figure 1**

(A) Quantification of nuclei with indicated phenotypes in DAPI-stained HeLa cells siRNA-treated with oligonucleotides to AKAP95 (siAKAP95), an unrelated protein (siUnrProt) and with a random sequence (siControl). Data (mean +/- SEM) from 4 independent experiments (N=600) analyzed by Student’s t test. **P<0.01, ***P<0.001 compared with siControl. Corresponding HeLa nuclei images (bottom). Scale bar 5 μm. (B). Time lapse imaging of AKAP95-depleted and control HeLa\textsuperscript{H2B-GFP} cells starting from NEBD and with image acquisition every 5 minutes. Chromosome segregation defects and micronuclei (arrowheads). (C) Quantification of cells as in B with indicated chromosome segregation pattern. Data (mean +/- SEM) from 4 independent experiments (N=28).
Figure 2

(A) Schematic representation of Myc-BirA*-AKAP95. (B) Western blot analysis using anti-myc antibody and HRP-labelled streptavidin of lysates from Myc-BirA*-AKAP95 expressing- and control U2OS cells cultured as indicated. (C) Immunolocalization of Myc-BirA*-AKAP95 (anti-myc; red) and protein biotinylation (FITC-labelled streptavidin; green) in stable Myc-BirA*-AKAP95 U2OS cells cultured as indicated. Immunolocalization of AKAP95 in U2OS cells (bottom). Scale bar, 5 μm. (D) Gene ontology analysis and nuclear component classification of proteins identified by AKAP95 BioID. (E) Western blot analysis using indicated antibodies of the samples from Myc-BirA*-AKAP95-expressing and control U2OS cells that were subjected to BioID.

Fig 2 / Identification of *in vivo* binding partners of AKAP95. (A) Schematic representation of Myc-BirA*-AKAP95. (B) Western blot analysis using anti-myc antibody and HRP-labelled streptavidin of lysates from Myc-BirA*-AKAP95 expressing- and control U2OS cells cultured as indicated. (C) Immunolocalization of Myc-BirA*-AKAP95 (anti-myc; red) and protein biotinylation (FITC-labelled streptavidin; green) in stable Myc-BirA*-AKAP95 U2OS cells cultured as indicated. Immunolocalization of AKAP95 in U2OS cells (bottom). Scale bar, 5 μm. (D) Gene ontology analysis and nuclear component classification of proteins identified by AKAP95 BioID. (E) Western blot analysis using indicated antibodies of the samples from Myc-BirA*-AKAP95-expressing and control U2OS cells that were subjected to BioID.
Fig 3 / **AKAP95 targets TPR to the mitotic spindle area.** (A) Western blot analysis using indicated antibodies of immunoprecipitates from Myc-BirA*-AKAP95 U2OS cells. Note the distinct TPR band in AKAP95- compared to FLAG immunoprecipitates. (B) Immunolocalization of AKAP95 and TPR at indicated mitotic stages in unsynchronized HeLa
cells. Insets: DAPI staining. Plot profiles show for both channels the variation in fluorescence intensity along the indicated line. (C) 3D image projections from PLA experiments between TPR and AKAP95. Positive control: PLA between TPR using two antibodies from different species. (D) Quantification of cells as in C with indicated number of foci per nucleus from 1 representative experiment (N= 36). (E) Western blot analysis using indicated antibodies of immunoprecipitates from mitotic-arrested HeLa cells. (F) Immunolocalization of AKAP95 and TPR at mitosis in HeLa cells as indicated. Insets: DAPI staining. Scale bar= 5 μm. Plot profiles show the variation in TPR fluorescence intensity along the indicated line. (G) Quantification of micronuclei in HeLa cells treated as indicated from 3 independent experiments (mean +/- SEM, N= 83).

**Figure 4**

**A**

![Image of immunofluorescence analysis](image)

**B**

![Image of fluorescence intensity quantification](image)

**C**

![Image of quantification of cells](image)

**D**

![Image of quantification of mid-body](image)

**E**

![Image of Western blot analysis](image)

**Fig 4 / AKAP95-depleted HeLa cells have a compromised SAC.** (A) Immunofluorescence analysis with indicated antibodies of AKAP95-depleted and control HeLa cells treated with nocodazole. Indicated areas are shown after deconvolution, magnification and merge of anticientromere HCT-0100 and MAD1 fluorescence signals. Scale bar 5 μm. (B) Fluorescence intensity quantification of MAD1 relative to HCT-0100 signals at KT from cells in A (mean...
+/- SEM, N=100, analyzed by Student’s $t$ test $***P<0.001$). (C) Time lapse imaging of AKAP95-depleted and control HeLa$^{\text{H2B-GFP}}$ cells. Scale bar 5 μm. Quantification of absolute time from NEBD to anaphase onset (mean +/- SEM, N=20, analyzed by Student’s $t$ test. $**P<0.01$). (D) Flow analysis of AKAP95-depleted and control HeLa cells treated with nocodazole for the indicated period and stained with anti-phospho-Histone H3. (E) Quantification of the mitotic cell populations from D from 1 representative experiment.
Supplementary Fig 1 (A) Western-blot analysis of HeLa cells siRNA-depleted for AKAP95 (siAKAP95) and an unrelated protein (siUnrProt). (B) Quantification of nuclei showing indicated phenotypes in HeLa cells stained with DAPI and siRNA-treated with a second AKAP95 (oligo02) or with a random sequence (siControl) oligonucleotide. Representative images shown on the right (mean, N=140). (C, D) Immunofluorescence analysis in AKAP95-
depleted HeLa cells double stained with DAPI and with indicated nuclear envelope and histone 3 modifications markers (C) or autophagy markers (D). Right panels represent the corresponding deconvoluted images. 5 µm scale bar. (E) Images from time-lapse imaging of cells undergoing mitosis. Chromatin condensation in the micronuclei can be observed at the onset of mitosis (arrowheads).

Supplementary Figure 2

Supplementary Fig 2 (A) Western blot using indicated antibodies of protein lysates from Doxycycline-induced and non-induced Myc-BirA*-AKAP95 U2OS cells (B) Coomassie blue staining after 4-20% SDS-PAGE of proteins purifies by streptavidin beads from lysates of Myc-BirA*-AKAP95 and control U2OS cells. (C) TPR protein sequence showing coverage (yellow) by unique peptides recovered in the AKAP95-BioID screen.
Supplementary Figure 3

Supplementary Fig 3 (A) Confocal microscopy analysis of U2OS interphase cells siRNA-depleted for AKAP95 compared to non-depleted cells. (B) Immunolocalization of AKAP95 and α-Tubulin in mitotic HeLa cells. 5 μm scale bar. (C) Immunolocalization of AKAP95 and TPR in mitotic HeLa cells siRNA-depleted for TPR. Insets: DAPI staining. Scale bar= 5 μm.

Supplementary Movie 1 Time-lapse video imaging of AKAP95-depleted HeLa$^{\text{H2B-EGFP}}$ cells.

Supplementary Movie 2 Time-lapse video imaging of control HeLa$^{\text{H2B-EGFP}}$ cells

Supplementary Movie 3 Time-lapse video imaging of HeLa$^{\text{H2B-EGFP}}$ cells AKAP95-depleted using a second AKAP siRNA (oligo02).

Supplementary Table 1 List of 428 unique proteins identified by Myc-BirA*-AKAP95 ranked by number of unique peptides and corrected for protein size (emPAI). Known interactors of AKAP95 are highlighted.