ASAP is a novel substrate of the oncogenic mitotic kinase Aurora-A: phosphorylation on Ser625 is essential to spindle formation and mitosis

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Proper chromosome segregation is required to maintain the appropriate number of chromosomes from one cell generation to another and to prevent aneuploidy, which is mainly found in solid cancers. A correct mitotic spindle is necessary to accomplish such a process. Aurora kinases play critical roles in chromosome segregation and cell division; their deregulation impairs spindle assembly, checkpoint function and cell division causing chromosome mis-segregation. These kinases have been implicated in tumorigenesis. Aurora-A (AurA), in particular has been identified as a cancer-susceptibility gene, is overexpressed in a number of tumors and is required for G2/M transition and spindle assembly. ASAP is a novel spindle-associated protein, the deregulation of which induces severe mitotic defects. We show here that ASAP is a novel substrate of AurA kinase. We have identified serine 625 as the major phosphorylation site for AurA in vivo and localized the phosphorylated form of ASAP to centrosomes from late G2 to telophase, and around the midbody during cytokinesis. AurA depletion induces a proteasome-dependent degradation of ASAP. ASAP depletion induces spindle defects rescued by the expression of the phosphorylation-mimetic mutant ASAP-S625E and not by the non-phosphorylatable mutant ASAP-S625A. Microinjection of mono-specific S625 phospho-antibodies also impaired spindle formation and mitosis. These results strongly indicate that the phosphorylation of ASAP on S625 by AurA is required for bipolar spindle assembly and is essential for a correct mitotic progression. All together, these results suggest that we have identified a novel AurA substrate, pointing out ASAP as a new potential target for antitumoral drugs.

INTRODUCTION

One of the most critical steps during cell cycle progression is the correct segregation of sister chromatids during mitosis. This process depends on the reorganization of the interphase microtubules (MTs) into a highly dynamic bipolar array known as the mitotic spindle. The centrosomes are the primary sites of MT nucleation in animal cells. At the onset of mitosis, segregation of centrosomes to opposite poles of the cell is critical for proper assembly of the bipolar spindle and positioning of the cleavage plane. Various protein kinases and their effector proteins play an essential role in controlling cell cycle progression (1). Aurora kinases, for example, regulate processes in cell division including chromosome condensation and orientation, spindle dynamics, kinetochore–MT interactions, establishment of the metaphase plate and cytokinesis (2–5). Three members of the Aurora family, A, B and C, exist in mammals. They display different subcellular localizations and have specialized functions. Aurora kinases are essential to ensure error-free cell division, and their overexpression appears to be intimately linked to centrosome amplification, tumorigenesis and transformation, explaining their enormous potential as targets in cancer treatment (3,4,6,7). AurA localizes to centrosomes and to MTs at the spindle poles and fits the criteria to be classified as an oncogene (3,4,8). The identification of AurA as a cancer-susceptibility gene provides a compelling link between mitotic aberrations and carcinogenesis. However, AurA contribution to carcinogenesis remains to be elucidated. Indeed, if the Aurora
kinases are bona fide targets for cancer therapy, an integrated molecular and cellular understanding of their upstream regulators and downstream effectors is now required to properly inform drug-screening programs and therapies (9). AurA has been reported to interact with several proteins that could mediate its role in malignant transformation (10). AurA phosphorylates substrates that promote progression through mitosis and include Cdc25B, TPX2, Eg5, Lats2, TACC, Ajuba, BRCA1 and others. Among these substrates are spindle-associated proteins known to be involved in spindle formation and stability or proteins required for commitment of the cells to mitosis (reviewed in 8).

The importance of MTs in mitosis and cell division makes them an important target for anticancer drugs (11). Altering MT stability in cells likely affects spindle assembly and chromosome attachment, processes that need to be carefully controlled to protect cells from genomic instability and transformation. Some MT-associated proteins (MAPs) are either up- or down-regulated in certain cancer cells, whereas others are associated with the development of drug resistance (12). Thus, the identification of new MAPs involved in spindle assembly, mitosis and cytokinesis may shed light on these different mechanisms and could lead to the development of novel diagnostic, prognostic or therapeutic approaches of tumors.

We have recently characterized a novel human spindle MAP named ASAP (ASter-Associated Protein) (13). Overexpression of ASAP induces aberrant multi- or monopolar spindles in mitosis. Depletion of ASAP by RNA interference results in severe mitotic defects; it induces the formation of aberrant mitotic spindle, delays in mitotic progression, defects in chromosome congression and segregation, defective cytokinesis and cell death. These previous results suggest a crucial role for ASAP in organization of the bipolar mitotic spindle, mitotic progression and cytokinesis.

Here, we report that ASAP is a novel substrate of AurA and that this interaction with AurA stabilizes ASAP. Among the particularly rich number of Ser/Thr in ASAP, we have identified the major phosphorylation site for AurA in vivo. The phosphorylated form is detected at centrosomes from the late G2-prophase to telophase. We demonstrate that this AurA-mediated phosphorylation of ASAP is essential to spindle formation. We thus confirm that ASAP is a key component in regulation of the mitotic apparatus. Preliminary data show that ASAP expression is deregulated in different malignant cell lines, which is in line with mitotic defects and aneuploid cells observed in (13) when ASAP is overexpressed or depleted. Its association with and phosphorylation by AurA opens new perspectives for a better understanding of the interplay between AurA kinase, the spindle MTs and the chromosome segregation that might eventually lead to the development of new cancer prognosis and therapy.

RESULTS

ASAP and AurA co-localize and interact in vivo

The phenotypes described for ASAP overexpression and depletion are similar to those reported for overexpression or inhibition of other proteins known to regulate centrosomal maturation, spindle formation and cell-cycle progression, including the AurA kinase (14–17). This similarity and ASAP’s intracellular localization prompted us to investigate ASAP’s spatio-temporal expression relative to AurA in U-2 OS cell line. AurA localizes to the centrosomes of interphase cells, to spindle poles in metaphase cells and to the residual body during cytokinesis (18). We confirmed this localization and showed that ASAP and AurA partially colocalize at spindle poles during mitosis, with ASAP localization extending to the spindle MTs. In telophase, ASAP is also located along the MTs of the central body, whereas AurA is more concentrated at the center region closer to the midbody (Fig. 1A). However, given the background observed with our ASAP antibody, we performed cold-mediated MT depolymerisation, and observed that ASAP and AurA colocalize at centrosomes from interphase to telophase (Fig. 1B), the centrosome localization being confirmed using an anti-γ-tubulin (Supplementary Material, Fig. S1). These data also demonstrate that centrosome localization of endogenous ASAP is independent of MTs because it remained associated with the centrosomes after MT depolymerization.

We therefore assessed whether the interaction between AurA and ASAP occurred in vivo by communoprecipitation assays, either with U-2 OS cells transfected with Flag-ASAP and Myc-AurA proteins (Fig. 1C1) or endogenous proteins (Fig. 1C2). In both cases, lysates of mitotic U-2 OS cells were immunoprecipitated using anti-ASAP antiserum. Immunoblot analysis of the resulting precipitates revealed the presence of AurA.

To determine if ASAP is phosphorylated in vivo, Flag-ASAP constructs were immunoprecipitated with anti-ASAP antiserum from asynchronous, S or M phases and compared before and after λ-phosphatase (λ-PPase) treatment. A modification of the mobility after λ-PPase treatment was observed in both S and M extracts suggesting in vivo phosphorylation of ASAP at different periods of the cell cycle (Fig. 1D). AurA, as an active mitotic kinase, could account (at least partially) for the mitotic phosphorylation of ASAP.

Depletion of AurA destabilizes ASAP

We next asked whether ASAP localization might be controlled by AurA or vice versa. No significant effects on AurA localization were observed in response to ASAP depletion (data not shown). However, when AurA was depleted by siRNA, levels of ASAP decreased significantly, suggesting that phosphorylation of and/or interaction with AurA might be required to stabilize ASAP (Fig. 2). ASAP is a stable protein (half-life ~8 h, data not shown) that is expressed throughout the cell cycle. When AurA-depleted cells were treated with MG132, ASAP degradation was no longer observed, suggesting that ASAP destabilization is proteasome-dependent (Fig. 2). The decreased level of ASAP after AurA depletion was also confirmed by immunofluorescence. In those cells where ASAP was still observed, the knockdown of AurA was incomplete and thus probably sufficient to phosphorylate and stabilize ASAP (data not shown). The suppression efficiency of AurA expression varied from cell to cell but usually impaired G2-M progression. The loss of AurA was accompanied by a significant increase in apoptotic events (19) and was lethal. For this reason a more complete analysis of the significance
of the destabilization of ASAP was not followed up at this stage.

**AurA phosphorylates ASAP in vitro on serine 305 and serine 625**

We next investigated whether ASAP was phosphorylated by AurA in vitro. We performed an in vitro kinase assay with recombinant His<sub>6</sub>-tagged wild-type AurA or a kinase-inactive mutant thereof (K162M, in which Lys 162 in the ATP-binding site was replaced with Met) and with a recombinant ASAP protein fused to glutathione-S-transferase (GST). ASAP underwent marked phosphorylation on incubation with wild-type AurA in the presence of [γ-<sup>32</sup>P], but not when incubated with the kinase-inactive mutant. AurA did not phosphorylate GST alone and histone H3 was used as a positive control for the kinase assay (Fig. 3A). ASAP is especially rich in serine (72 residues) and threonine (34 residues). To verify that the in vitro phosphorylation by AurA was specific, we tried to phosphorylate ASAP with the Ser/Thr kinase CDK5. CDK5 does not phosphorylate ASAP indicating that AurA specifically recognizes consensus sequences in ASAP (Supplementary Material, Fig. S2). The AurA consensus site remains poorly defined as (R/K)(S/T) or (R/K/N)RX(S/T)B where B denotes any hydrophobic residue with the exception of Pro (20) and (RXS/T)(I/L/V) (21). These sequences are
very abundant in ASAP. To delineate ASAP’s domains phosphorylated by AurA, various GST-tagged fragments (G1–G6) of ASAP were used in in vitro kinase assay as described above. As shown in Fig. 3B, G4 (amino acids 296–420) and G6 (amino acids 544–647) were phosphorylated by AurA in vitro. Phosphoamino acid analysis revealed that AurA-dependent phosphorylation of ASAP occurred on Ser residues (Supplementary Material, Fig. S3A). Phosphopeptide mapping of in vitro phosphorylated ASAP using both mass spectrometry (see Supplementary Material) and two-dimensional separation on thin layer cellulose plate (not shown), combined with sequence similarity between consensus sites and/or a conservation in other species, led to the identification of a limited number of potential sites at high confidence. ASAP non-phosphorylatable S-A mutants were generated in the GST-fused full-length construct at S302, S305, S370, S369–S370, S625 and S305–S625 and analyzed by in-vitro kinase assay. The KRHS625F (corresponding to the (R/K/N)RX(S/T)B consensus site that is conserved during evolution of ASAP) located in G6 and the KES305Q located on G4 gave a significant reduction in $^{32}$P incorporation (66 and 80%, respectively) compared with ASAP-WT. The double mutant S305–S625 gave greater decrease in $^{32}$P incorporation (94%) (Fig. 3C). Mass spectrometry analysis of these two sites is shown in Supplementary Material (Supplementary Material, Fig. S3B).

**ASAP is phosphorylated in vivo on serine 625 at the centrosomes during mitosis and at the spindle midzone in cytokinesis**

To explore the functional significance of ASAP phosphorylation by AurA, two affinity-purified polyclonal antibodies (P-S305 and P-S625) were raised against phospho-S305 and phospho-S625, respectively. Anti-P-S305 did not show reactivity suggesting that either this site is not phosphorylated in vivo or its dephosphorylation occurs rapidly or under special physiological conditions beyond our ability to detect. In contrast, anti-P-S625 worked both for in vitro and in vivo detection. As shown by western blot analysis, P-S625 recognized the wild-type YFP-ASAP but not the S625A-YFP mutant protein from U-2 OS transfected cells (Fig. 4A). Western blot immunolabeling was competitively inhibited by the phosphorylated immunogenic peptide but not the unphosphorylated one. This labeling was also abolished by prior dephosphorylation of the immunoprecipitated Flag-ASAP with λ–phosphatase (Supplementary Material, Fig. S4A).

We next investigated the subcellular localization of phospho ASAP in U-2 OS cells. As shown in Figure 4B, P-S625 forms localized specifically to centrosomes from late interphase to telophase. At telophase, additional staining was observed in the spindle midzone. This labelling was also visible with the anti-ASAP in long exposures. The spindle was not labelled as observed with the general population of ASAP detected with the polyclonal antibody against the whole recombinant protein (Fig. 1A). Double staining with AurA confirmed the localization of phospho S625-ASAP at centrosomes during mitosis and at the midbody region during telophase. Again, labelling was effectively competed away by the phosphorylated peptide and not the unphosphorylated peptide (Supplementary Material, Fig. S4B). The centrosomal localization of S625-phosphorylated ASAP in mitotic cells was confirmed using an anti-γ-tubulin monoclonal antibody (Supplementary Material, Fig. S4C). Immunofluorescence on synchronized cells analyzed at S, G2 and M phases showed that S625 was not phosphorylated in S phase and confirmed that interphase staining corresponded to cells in late G2 (Supplementary Material, Fig. S4D, right panel). These data are consistent with previous observations (22) that phosphorylation of AurA on T288 occurred during the late G2-prophase, coincident with catalytic activation and concomitant to phosphorylation of ASAP-S625 (Supplementary Material, Fig. S4D, left panel). Suppression of AurA or ASAP expression by RNA interference abolished the signal for anti-P-S625, confirming that this site corresponds to that phosphorylated by AurA in ASAP (Supplementary Material, Fig. S4E).

**Phosphorylation of ASAP serine 625 affects spindle formation in vivo**

To examine the significance of ASAP S625 phosphorylation in vivo, U-2 OS cells were transiently transfected with YFP-tagged wild-type ASAP (ASAP-WT), YFP-ASAP-S625A (ASAP-A, non-phosphorylatable) or YFP-ASAP-S625E (ASAP-E, phospho-mimetic) mutants. ASAP-WT, ASAP-A and ASAP-E showed identical localization patterns during interphase (cytoplasmic) and mitosis (mitotic spindle) indicating that S625 phosphorylation by AurA is not needed for ASAP localization. The transfection with the double mutants ASAP-S305A-S625A or ASAP-S305E-S625E gave similar results (data not shown). We have previously shown that ASAP-depletion by RNA interference provoked aberrant spindles, which could be efficiently rescued by expression of the wild-type ASAP-SIL that is insensitive to ASAP siRNA. We prepared RNAi-refractory YFP-ASAP mutants (ASAP-A-SIL and ASAP-E-SIL) and transfected ASAP-depleted U-2 OS cells. Expression of mutant proteins in ASAP-depleted cells did not cause obvious defects in interphase cells (not shown). Neither S625A nor S625E affected the centrosomal or spindle localization of ASAP confirming that S625 phosphorylation is not required for localization. However, expression of ASAP-WT and ASAP-E-SIL proteins efficiently rescued the abnormal spindle phenotype (25.7 and 31.4% of abnormal spindles, respectively) caused by the ASAP siRNA (59%). The rescue was not observed with ASAP-A-SIL.
Figure 3. ASAP is phosphorylated by AurA in vitro. (A) Purified GST-ASAP or Histone H3 were incubated with purified His-tagged wild-type (WT) or kinase inactive mutant (K162M) AurA and [γ-32P]ATP. The kinase reaction mixtures were then analyzed by SDS–PAGE and autoradiography (top); the gel was also stained with Coomassie blue (bottom). (B) Purified GST, GST-ASAP or GST-ASAP fragments G1–G6 were used in a kinase assay with wild-type AurA as indicated in (A) and analyzed by SDS–PAGE and autoradiography (top); Coomassie blue-stained gel is shown at the bottom. (C) In vitro kinase assays were performed on GST-ASAP-WT or on GST-ASAP-mutants (SA) as indicated and analyzed by SDS–PAGE and autoradiography (top); Coomassie blue-stained gel is shown at the bottom.
(62.4% of abnormal spindles) (Fig. 5A and B), strongly indicating that S625 phosphorylation is required for bipolar spindle assembly. Transfection of ASAP mutants did not rescue AurA depletion phenotypes as expected from the multiple mitotic substrates identified for AurA.

Microinjection of antibodies to P-S625-ASAP impairs spindle formation and mitosis

To determine the contribution of S625 phosphorylation to mitosis, affinity-purified P-S625 or control antibodies were microinjected in G2 synchronized cells. As observed by immunofluorescence (Fig. 6A), cells microinjected with control IgG presented a normal spindle, whereas cells microinjected with anti-P-S625 were either unable to form a spindle or presented an abnormal spindle with in both cases a chromosome congression failure that resulted in an abnormal metaphase plate. Multipolar spindles were also observed. Estimation of divided cells revealed that compared with control antibodies (30 divided cells for 31 microinjected cells), P-S625 seriously impaired mitotic progression (4 divided cells for 30 microinjected cells) (Fig. 6B). Cells microinjected with P-S625 also underwent cell death (not shown). The phenotypes observed were typical of cells in which spindle formation is disrupted and confirmed that the phosphorylation of S625 is required to form a bipolar spindle and hence ensures a correct mitosis progression.

Cancer cell line expression

In cells, ASAP overexpression or depletion leads to defects that are often seen in tumor cells (multipolar spindles and centrosome amplification or segregation defects) (13). RT–PCR and western blotting were performed to study whether ASAP expression level was altered in tumor cells (Fig. 7A and B). Compared with human fibroblasts, ASAP protein level was very high in a subset of cancer cells such as ovary and breast as described for AurA, high in testis and neuronal-derived cell lines, and hardly detectable in colon-derived cell lines. The absence of ASAP in these proliferating cells may suggest that in the corresponding tissues another protein may take over. One cannot exclude redundancy of function and in vivo studies must be performed to determine the precise role of ASAP. Considering both these mitotic defects and the altered expression of ASAP in several cancer cell lines, it is tempting to speculate that ASAP may be involved in tumorigenesis. A more detailed analysis of tumor samples should be undertaken in the future.
DISCUSSION

During cell division, the mitotic spindle segregates sister chromatids into nascent cells, and errors in spindle formation can result in both chromosome mis-segregation and cytokinesis defects thereby leading to genomic instability. It is well established that protein phosphorylation and dephosphorylation play key roles in the function and regulation of bipolar mitotic spindle (23). Here we report that the mitotic serine/threonine kinase AurA is the first known partner of ASAP, a MAP required for bipolar assembly (13), but for which no mechanism of function has been described to date. Our results confirm that ASAP is a key factor of cell division and leads to the idea that the deregulation of ASAP/AurA interaction might be related to tumorigenesis.

Both the proteins co-localize at spindle poles and centrosomes from late G2 to the end of mitosis. We demonstrate that ASAP is phosphorylated by AurA and we have validated one conserved consensus phosphorylation site in vivo. We have determined that the phosphorylated fraction of ASAP is exclusively localized to the centrosome from late G2-prophase to telophase. This finding reinforces the view of the centrosome as a functional integrator of the control pathway that participate in the triggering of mitosis (24). AurA is a key mitotic regulator involved in centrosomal maturation, G2-M transition and spindle assembly. The timing of the onset of the S625 phosphorylation suggests that it might be required at the beginning of mitosis. Depletion of ASAP did not induce a visible defect in γ-tubulin or pericentrin recruitment (13), ruling out a role in centrosome maturation. However, as microinjected cells progressed through mitosis, they underwent cell death or formed abnormal spindles, which segregated unevenly. These results suggest that phosphorylation of ASAP on S625 by AurA is essential to form a bipolar spindle. This result was confirmed by the fact that the phosphomimetic mutant ASAP-E and not ASAP-S625A is able to rescue the abnormal spindle phenotypes obtained after ASAP depletion. AurA localized to centrosomes and MTs at spindle poles during prometaphase-metaphase and plays an active role in bipolar spindle assembly. Different MAPs have been implicated in these AurA-driven processes such as TPX2, TACC3, ChTog (ortholog of XMAP215), HURP.

Figure 5. Phosphorylation of ASAP-S625 is involved in a correct spindle formation. ASAP-S625E rescues normal spindles. U-2 OS cells were transfected with the control scrambled-ASAP (SC) or ASAP siRNAs (left) or co-transfected with the ASAP siRNA and the different YFP-ASAP (SIL-WT, SIL-A, SIL-E) plasmids (right). (A) U-2 OS cells were fixed with PAF-MTSB and co-stained with ASAP and α-tubulin antibodies (left) or just α-tubulin (right) and Hoechst 33258. (B) Abnormal spindles were quantified 36 h after the double transfection. Data were obtained from three independent experiments (n = 3) (total number of cells scored: SC-siRNA, 258; ASAP-siRNA, 296; ASAP-siRNA+SIL-WT, 99; ASAP-siRNA+SIL-A, 57; ASAP-siRNA+SIL-E, 67).
(8,25,26) together with the plus-end directed kinesin Eg5. We have not observed a delocalization of TPX2, TACC3 or Eg5 when ASAP was depleted (data not shown). ASAP phosphorylated on S625 was observed during metaphase at centrosomes but unlike AurA it was not observed on MTs at spindle poles. This suggests that S625 phosphorylation might be needed to initiate spindle formation.

The phosphorylated form of ASAP is also observed during cytokinesis around the midbody where it colocalizes with AurA. Several studies suggest a role of the centrosome in cytokinesis (27). Surviving ASAP-depleted cells present some cytokinesis-abscission defects that may be a consequence of earlier mitosis defects (13), but we cannot rule out a function of ASAP phospho-S625 at the MTs of the central spindle that could not be observed here because of the dramatic early phenotypes.

Although it is well established that AurA kinase is a crucial regulator of mitosis, the role of ASAP was unclear. Here we not only demonstrated that ASAP is a new in vivo substrate of AurA, but we report also that ASAP is essential for bipolar spindle formation through phosphorylation of a serine at position 625 by AurA. We also observed that in the absence of AurA, ASAP is rapidly degraded in a proteasome-dependent pathway. However, whether ASAP stability directly depends on phosphorylation by AurA remains to be demonstrated. As preliminary results, we did not observe any differences in stability between ASAP-WT, ASAP-E and ASAP-A when overexpressed in U-2 OS cells suggesting that a direct phosphorylation of S625 may not be involved. However, overexpression may have saturated the degradation machinery with the overexpressed proteins.

Our data also indicate that ASAP is essential for mitosis and processes that regulate genome stability, highlighting ASAP as a new attractive target for drug design in cancer therapy. AurA has already been implicated in tumorigenesis and considered as an oncogene (9). The close relationship between AurA and ASAP we show here, combined to the mitotic defects observed when ASAP expression is deregulated, clearly suggest that ASAP defects may contribute to the genetic instability that is common to most cancers. As observed with AurA, ASAP overexpression or depletion leads to defects that are often seen in tumor cells (multipolar spindles and centrosome amplification or segregation defects) and affects dramatically cell division (13). Considering both mitotic defects observed when ASAP is deregulated and very preliminary data suggesting an altered expression of ASAP in several cancer cell lines, it is tempting to speculate that ASAP may be involved in tumorigenesis and that the variations of the molecular ASAP–AurA complex can affect cell division and participate in oncogenesis. A more complete analysis of different tumor samples should be performed.

Taken together, our data indicate that ASAP is essential for mitosis and that its function is regulated by AurA. Finding
more partners of ASAP to clarify further how this MAP controls bipolar spindle formation will be the next step. A better knowledge of the mechanisms controlled by ASAP will undoubtedly help to understand processes that insure chromosome segregation often deregulated in carcinogenesis and to design new approaches to develop antimitotic drugs.

MATERIALS AND METHODS

Plasmids, antibodies and reagents

Clonings were carried out using standard PCR-based techniques. For protein purification from bacterial cells, AurA cDNAs, wild-type and the K162M mutant were subcloned into pET29 vector (Novagen) producing a His
tagged AurA. Full-length and six GST-ASAP constructs (G1: aa 1–108; G2: aa 75–206; G3: aa 187–313; G4: aa 296–420; G5: aa 395–563; G6: aa 544–647) were obtained by cloning the cDNAs in the pGEX-4T-2 vector (Pharmacia). Mutagenesis was carried out using the multisite kit from Stratagene. All constructions were entirely sequenced.

For immunofluorescence studies, the following dilutions were used: for monoclonal antibodies to α-tubulin and γ-tubulin (Sigma): 1/1000; to AurA: 1/100; to ASAP (clone 6G5): 1/1000; for polyclonal antibodies to anti-phosphorylated AurA (Thr288) (Abcam): 1/500, to ASAP: 1/500. Hoehst 33258 was purchased from Sigma.

Polyclonal antibody against phosphorylated S625 (P-S625) was raised against the CEKSKEpSQVTADD peptide then affinity-purified on a phosphorylated peptide column and then on an unphosphorylated peptide column to eliminate antibodies reacting against the unphosphorylated peptide (Eurogentec, Belgium). P-S625 was used at the dilution of 1/500.

Cell culture, transfections, siRNA experiments

All cells were routinely grown at 37°C in a 5% CO₂ atmosphere in DMEM (SIGMA) as described (13) and transiently transfected using JetPEI (Polyplus).

The siRNA targeting AurA, ASAP and the controls scrambled-ASAP (SC-ASAP) or GL2 luciferase were transfected into HeLa cells in a 5% CO₂ atmosphere in DMEM (SIGMA) and transiently transfected using JetPEI 6 h after the siRNA transfection. G2 or M phases. For rescue experiments, plasmids were transfected using JetPEI 6 h after the siRNA transfection.

Immunofluorescence and western blotting

Western blot procedures were carried out as described (13). For immunofluorescence, cells grown on coverslips were fixed either by an incubation in 4% paraformaldehyde in MTSB (MT Stabilization buffer: 100 mM PIPES, 1 mM EGTA, 4% PEG 8000, pH 6.9) 10 min at room temperature followed by 0.5% Triton X-100/MTSB for 5 min (PAF/MTSB fixation) or by formaldehyde 3.6% in PHEM (60 mM Pipes, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 6.9) 10 min followed by methanol 1 min at room temperature (F/PHEM/methanol fixation). Immunodetection was done as described (13).
cellular RNA was extracted using the GenElute Total Mammalian Total RNA kit (Sigma-Aldrich) and treated with DNase (Ambion). cDNA synthesis and PCR amplification were performed with Superscript one-step RT–PCR (Invitrogen), using 200 ng of total RNA. A primer pair was chosen in different exons to discriminate with possible contaminations by genomic DNA (constFIS2F: 5’-ATCATTTGAGGCCGCTG-3’, constFIS1R: 5’-GAACCATGTTTCGCAAAGTGT-3’), and amplification was performed for 30 cycles during which the exponential phase of PCR amplification was maintained. GAPDH cDNA was amplified for 24 cycles with the primers GAPDH1: 5’-GACCAAGTGCCATGGGCT-3’ and GAPDH2: 5’-CTCCACACCTGTGGTCTG-3’. Ten microliters of PCR products were analyzed on a 1% ethidium-bromide-stained agarose gel.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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