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Identification of Ski as a target for Aurora A kinase

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Abstract

Ski is a negative regulator of the transforming growth factor-β and other signalling pathways. The absence of *SKI* in mouse fibroblasts leads to chromosome segregation defects and genomic instability, suggesting a role for Ski during mitosis. At this stage, Ski is phosphorylated but to date little is known about the kinases involved in this process. Here, we show that Aurora A kinase is able to phosphorylate Ski *in vitro*. *In vivo*, Aurora A and Ski co-localized at the centrosomes and co-immunoprecipitated. Conversely, a C-terminal truncation mutant of Ski (SkiΔ491–728) lacking a coiled-coil domain, displayed decreased centrosomal localization. This mutant no longer co-immunoprecipitated with Aurora-A *in vivo*, but was still phosphorylated *in vitro*, indicating that the Ski-Aurora A interaction takes place at the centrosomes. These data identify Ski as a novel target of Aurora A and contribute to an understanding of the role of these proteins in the mitotic process.

Keywords

Ski; Ski phosphorylation; Aurora A

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Conflict of Interest Statement

There are no financial or non-financial competing interests in relation to the work described.

Introduction

The Ski protein is involved in transcriptional regulation and repression of retinoic acid and transforming growth factor-β (TGF-β) signalling, as well as several other pathways [1]. This protein was first identified in a series of avian retroviruses isolated at the Sloan Kettering Institute by virtue of its ability to cause *in vitro* morphological transformation of avian embryo fibroblasts [2,3]. Given these original observations, Ski has been considered an oncoprotein. Consistent with this, a number of studies have described increased expression of Ski in several types of tumors including melanoma [4,5], esophageal squamous cell carcinoma [6], colorectal carcinoma [7], leukemia [8] and gastric cancer [9]. However, participation of Ski in tumorigenesis is more complex as emerging evidence indicates that Ski could also act as a tumor suppressor protein in certain type of cancers [10,11,12,13]. An observation which is consistent with the increased tumour susceptibility observed in *Ski* deficient (*Ski*+/−) mice [14] and the high chromosomal instability found in the *Ski* knockout (−/−) mouse embryonic fibroblasts (MEFs) [15].

The levels of Ski are regulated during the cell cycle by ubiquitylation and subsequent proteasomal degradation [16,17]. Interestingly, the highest levels of Ski are found during mitosis and at this stage, this protein becomes phosphorylated, potentially by the cdk1($p34^{cdc2}$)/cyclin B kinase complex. However, inhibition of cdks did not completely abolish the phosphorylation of Ski during mitosis, suggesting the existence of additional kinase(s) involved in this process [17]. Since phosphorylation/dephosphorylation of Ski could be important for its specific function in mitosis, its localization or even for its recognition by the ubiquitin-proteasome system, we were interested in identifying the additional kinase(s) responsible for this phosphorylation. Here, we showed that Aurora A, an oncogenic mitotic kinase, is involved in the phosphorylation of this protein.

Material and Methods

Tissue culture and Transfection

HEK293T and U2OS cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Expression plasmids were introduced into the cells using FuGENE 6 (Roche Applied Science). For Aurora kinase inhibition, U2OS cells were incubated in 4-(4′-Benzamidoanilino)-6,7-dimethoxyquinazoline [18], also known as aurora kinase inhibitor II (Calbiochem), for six hours.

Plasmids

T7-Ski, GST-Ski, Flag-Ski, EGFP-Ski and HA-Aurora A were described previously [17,19,20]. EGFP-SkiΔ491–728, T7-SkiΔ491–728 and GST-SkiΔ491–728 were constructed by digesting EGFP-Ski, T7-Ski and GST-Ski at an internal *Eco*RI site (nucleotide 490). GST fusion proteins were produced in BL21 cells and recovered on glutathione-Sepharose 4B beads (Amersham BD Biosciences).

In vitro Aurora A kinase assay

For the *in vitro* phosphorylation assay, 1 μg GST, GST-Ski and GST- SkiΔ491–728 proteins were incubated for 15 to 30 min at 30°C in reaction buffer (8 mM MOPS/NaOH pH 7.0, 0.2 mM EDTA) supplemented with 0.5 – 1 μCi [γ^{32}P]ATP, 100 μM unlabeled ATP/10mM MgAc, and 200 ng active Aurora A (Specific Activity: 1,789U/mg, Millipore). Kinase reactions were stopped by boiling the samples in SDS-Lammeli buffer.

Immunofluorescence and Microscopy

U2OS cells were grown on glass coverslips and transfected with EGFP-Ski or EGFP-SkiΔ491–728 and HA-Aurora A expression plasmids. For HA-Aurora A detection, immunofluorescence was performed as described previously [21]. Primary antibodies used were: anti-HA (1/300, Sigma), anti-γTubulin (1/500, Sigma) and anti-αTubulin (1/500, Sigma). Confocal microscopy was performed in a LSM Meta 510 (Carl Zeiss Inc.), using a x100/1.4 oil objective. Image editing was performed in Adobe Photoshop 6.0. For Ski-γTubulin and Ski-Aurora A co-localization analyses, at least 200 transfected cells from 3 independent experiments were scored. Significance was determined by using Student's *t* test.

Immunoprecipitation and Western blotting

Cells lysis and co-immunoprecipitation were performed as described previously [22]. Immunocomplexes were recovered using protein A-Sepharose, and separated on sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) gels and transferred onto nitrocellulose membranes (Protoran, Schleicher & Schüll, Keene). Antibodies used for immunoprecipitation were rabbit anti-HA (Sigma) and normal rabbit immunoglobulin G (IgG, Santa Cruz Biotechnology). For western blotting, the antibodies used were mouse anti-T7 (Novagen) and rat anti-HA (Roche).

Results and Discussion

Identification of Aurora A as a kinase for Ski

Phosphorylation of Ski is clearly increased during mitosis and this could to be related with the increased stability of this protein during this phase of the cell cycle [16,17]. *In vitro* phosphorylation of Ski by the main mitotic kinase cdk1/cyclinB indicated that Ski is a target for this kinase. However, inhibition of cdk1 in mitotic cells did not completely abolish the phosphorylation of Ski [17] and, since phosphorylation of Ski is clearly increased during mitosis, this indicates that an additional mitotic kinase is able to phosphorylate Ski. Therefore, we started our screening by using mitotic kinases inhibitors. One family of mitotic kinases targeted in this screen was the serine/threonine Aurora kinases. Human osteosarcoma U2OS cells expressing the Ski protein fused to Enhanced Green Fluorescence Protein (EGFP) were treated with nocodazole in order to enrich for mitotic cells and incubated with the Aurora kinase Inhibitor II (10 μM). In the cells incubated with the inhibitor, there was a significant decrease in the level of the higher molecular weight band which has been previously shown to correspond to phosphorylated Ski [17] (Figure 1A). This suggested that Ski may be a target for Aurora kinases during mitosis.

Considering the spatial-temporal activity of the Aurora kinase family members during the cell cycle [23,24], Aurora A was the strongest candidate as a kinase for Ski. Expression of the Aurora A protein and its kinase activity are at highest levels during the G2/M phase of the cell cycle [25,26]. Moreover, this kinase localizes at the centrosomes and each spindle pole during mitosis [25,27], a localization pattern that resembles Ski's localization during mitosis [17]. Thus, we investigated whether Ski was a direct target for Aurora A kinase activity. *In vitro* assays showed that Aurora A kinase can phosphorylate a GST-Ski fusion protein in a very efficient and specific manner (Figure 1B). The two phosphorylated bands seen in the GST-Ski lane correspond to full length GST-Ski and a proteolytic fragment-containing Ski (indicated by the asterisk), as confirmed by western blot analysis (Figure 1C). There was no phosphorylation of GST alone in this assay, neither was there phosphorylation of contaminating bacterial proteins present in this preparation of GST-Ski, indicating the specificity of the *in vitro* reaction. In fact as can be seen from the coomassie blue staining (figure 1B), the GST-Ski and its breakdown product were not the major proteins in this

partially purified preparation yet they were clearly the main proteins phosphorylated by Aurora A. Thus, we identified Ski as a novel *in vitro* and potentially *in vivo* target for Aurora A kinase, although we can not rule out the possibility that Ski might also be a substrate for other Aurora kinases.

Aurora A and Ski interact in vivo at the centrosomes

As stated above, Ski and Aurora A have a similar subcellular localization pattern during mitosis, raising the possibility that these proteins may interact with each other *in vivo*. To assess this possibility, we checked the localization of EGFP-Ski and HA-tagged Aurora A (HA-Aurora A) in human osteosarcoma U2OS cells. We found that both proteins co-localized, mainly at the centrosomal level (Figure 2). Next, we evaluated whether centrosomal localization of Ski was required for its interaction with this kinase. The Ski protein has several domains for protein-protein interaction. In its C-terminal region, Ski has a α-helix coiled-coil domain (aa536–710) that has been shown to be important for either homo-dimerization or dimerization with SnoN [28,29]. Since most proteins associated with centrosomes and mitotic spindle have a coiled-coil domain [30], we generated an EGFP-Ski mutant which lack the C-terminal portion of Ski (EGFP-SkiΔ491–728). We found that this mutant had a reduced localization to centrosomes, as judged by co-localization with γ-Tubulin (Figure 3). As expected, the SkiΔ491–728 mutant had also a decreased co-localization with Aurora A (Figures 4A and Supplementary Figure 1). Furthermore, in HEK293T cells co-transfected with T7-Ski and HA-Aurora A, an anti-HA antibody was able to co-immunoprecipitate T7-Ski WT with HA-Aurora A, but not the SkiΔ491–728 mutant (Figure 4B). Therefore, the C-terminal region of Ski –including the coiled-coil domain– appears to be required for Ski's localization to the centrosomes and for Ski's interaction with Aurora A. Finally, to further explore if Ski's phosphorylation by Aurora A was dependent on its C-terminal region or on its cellular localization, we performed and *in vitro* Aurora A kinase assay using a GST-SkiΔ491–728 protein as a substrate. We found that this protein was still phosphorylated *in vitro* by Aurora A, in a similar magnitude as the Ski wild type protein (Figure 4C). A contaminant protein (indicated by the arrowhead) was not phosphorylated by Aurora A, indicating the specificity of the assay. Thus, the C-terminal region of Ski is not required for phosphorylation by Aurora A. Taken together, these results indicate that phosphorylation of Ski by Aurora A depend on the interaction of both proteins at the centrosomes *in vivo*. Moreover, phosphorylation of Ski by Aurora A possibly takes place at Ser/Thr residue(s) localized between aminoacids 1 to 490.

Serine phosphorylation of chicken c-Ski was reported previously [31]. In that study, the phosphorylation sites in c-Ski were thought to be limited to the C-terminus domain of the protein [31]. In agreement with this data, recently Nagata and co-workers have shown that phosphorylation of Ski at S515 was responsible for the electrophoretic mobility shift displayed by this protein [32]. We found that SkiΔ491–728 was still phosphorylated by Aurora A. Thus, phosphorylation events do not seem to be restricted to the C-terminal domain in human Ski. In fact, a recent study identified phosphorylation of Ski at threonine 458 by Akt following activation of this pathway by specific growth factors including insulin, insulin-like growth factor-1, and hepatocyte growth factor [33]. Moreover, two independent phosphoproteome screens, identified S432 and S480 as phosphorylated residues in Ski from HEK293 and human Jurkat T-cell leukemia cell lines, respectively [34,35].

The serine/threonine kinase Aurora A (also known as STK15, BTA K and AIKI), has been shown to play an important role in chromosome segregation and centrosome functions [36]. Aurora A gene amplification and overexpression has been found in a number of tumors, including breast, colorectal and bladder cancer [23,37]. Increased Aurora A kinase activity is related with centrosome dysfunction and aneuploidy and it has been associated with aggressive clinical progression in several tumors including breast cancer [38,39,40]. Thus,

much effort has been made to identify inhibitors of these kinases. Currently, several Aurora A inhibitors are in Phase I and II clinical trials, with promising results on tumor growth and radio-sensitivity in several tumor types [41,42]. The identification of the transcriptional co-repressor Ski as a target for Aurora A kinase will improve our understanding of the role and regulation of Ski during mitosis, as well as the oncogenic mechanism mediated by Aurora A. Further studies need to be conducted to identify the specific target residues for Aurora A *in vivo*. This knowledge might contribute to widen drug screening programmes in the search for more specific therapies based on Aurora A pathway inhibition.

Highlights

- It has been suggested that the transcriptional co-regulator Ski plays a role during mitosis. At this stage, Ski is phosphorylated.
- Here, we show that Aurora A kinase is able to phosphorylate Ski *in vitro*.
- Immunofluorescence, co-immunoprecipitation and *in vitro* kinase assays showed that Aurora A-Ski interaction takes place at the centrosomes.
- These data identify Ski as a novel target of Aurora A and contribute to understand the role of these proteins in the mitotic process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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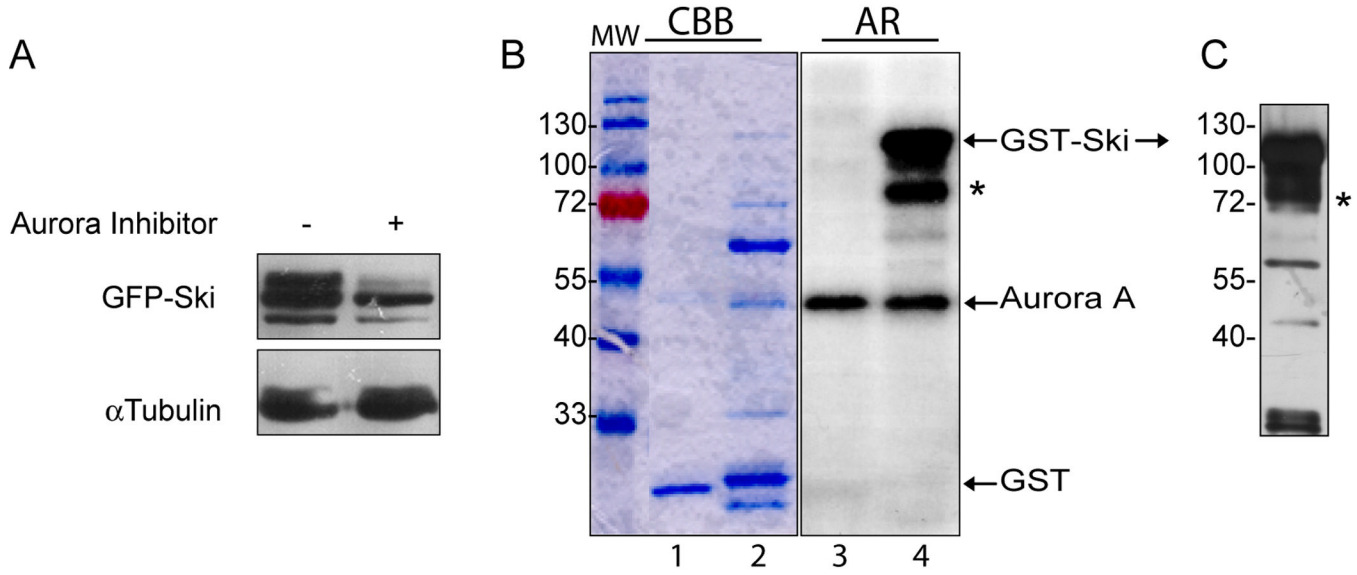

Figure 1. Ski is a target for Aurora A kinase

(A) U2OS cells expressing an EGFP-Ski fusion protein were treated with nocodazole to enrich the mitotic population. During the last 6 hours of incubation, an Aurora kinase inhibitor was added to the culture and cells processed for western blotting to detect Ski. A decrease in the upper band of Ski can be seen. Anti α-tubulin was used as a loading control. (B) *In vitro* Aurora A kinase assay. GST (negative control) and GST-Ski were used as substrates for purified active Aurora A. Coomassie bright blue staining of the purified proteins is shown (CBB, lanes 1 and 2). Autoradiography (AR, lanes 3 and 4) shows the incorporation of [γ^{32}P] in Aurora A (autophosphorylation) and GST-Ski. Arrows point to purified proteins. (*) indicates a breakdown product of GST-Ski. (C) GST-Ski protein was detected by western blotting, using a monoclonal antibody anti-Ski. (*) indicates the breakdown product of GST-Ski shown to be phosphorylated in B.

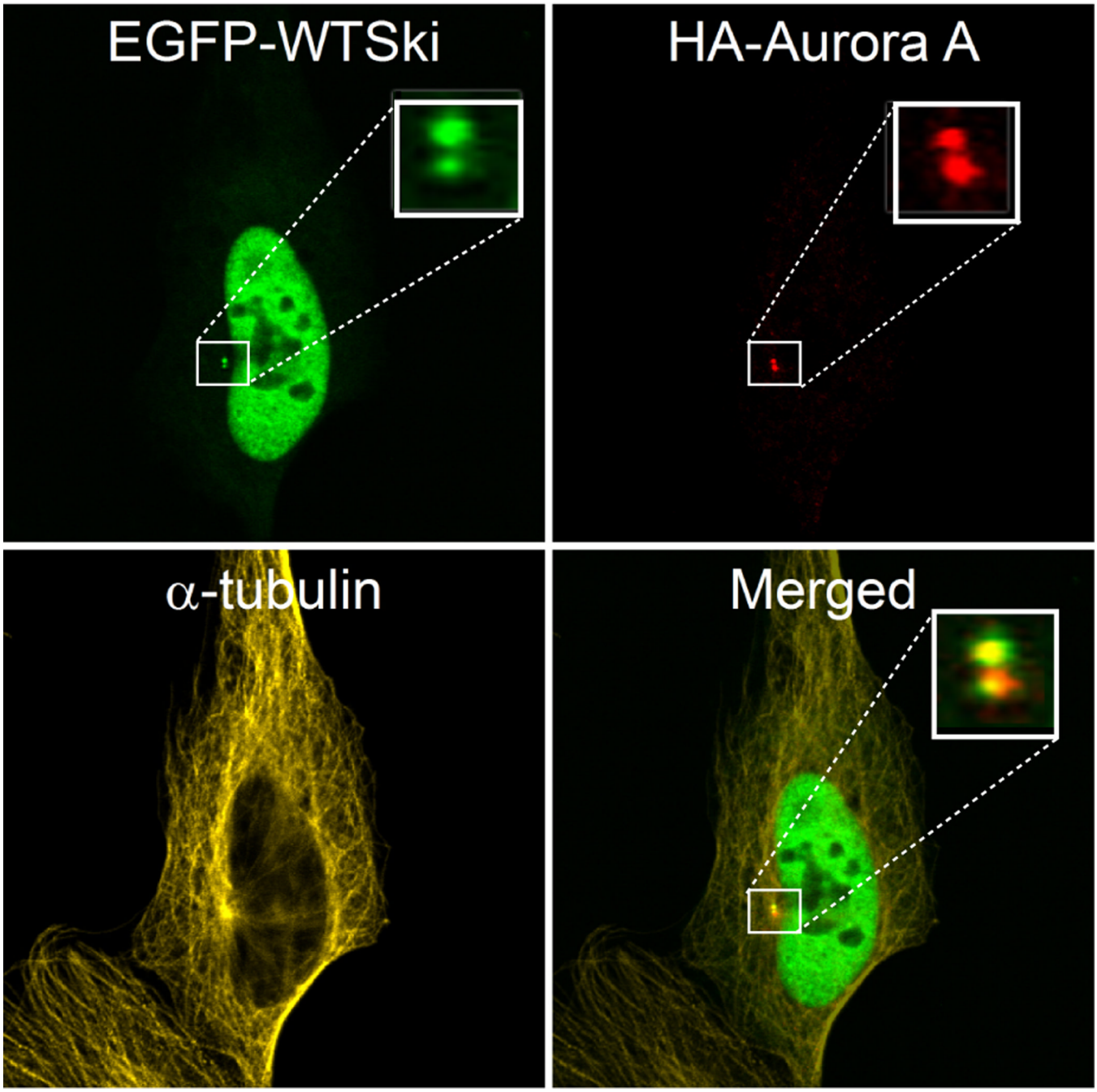

Figure 2. Ski and Aurora A colocalization at the centrosomes

U2OS cells were co-transfected with plasmids coding for full length EGFP-Ski(WT) and HA-Aurora A. 24h after transfection, cells were subjected to immunofluorescence using anti HA and anti α-tubulin and visualized by confocal microscopy. *Insets* show magnification of centrosomes.

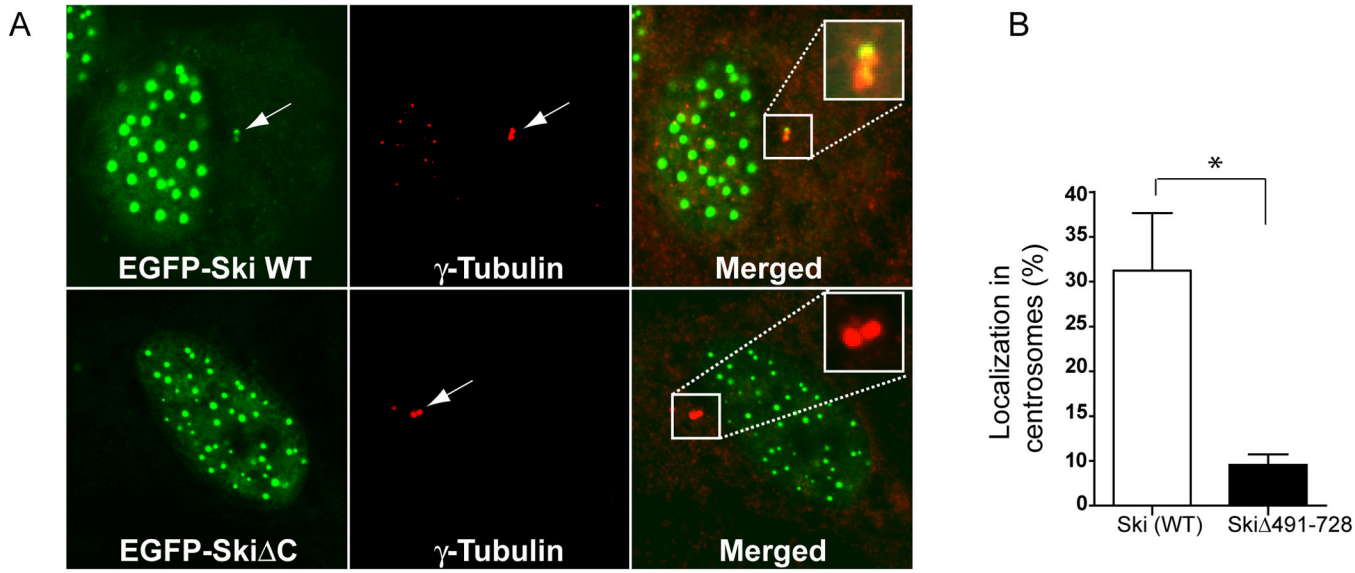

Figure 3. C-terminal portion of Ski is required for centrosomal localization

(A) U2OS cells were co-transfected with plasmids coding for full length EGFP-Ski WT or EGFP-SkiΔ491–728 (EGFP-SkiΔC). 24h after transfection, cells were subjected to immunofluorescence using anti γ-tubulin, a centrosomes marker. (B) Quantification of EGFP-Ski and EGFP-SkiΔ491–728 found in centrosomes was judged by co-localization with γ-tubulin. Mean±SD is shown. (*) $p<0.01$, Student's *t* test.

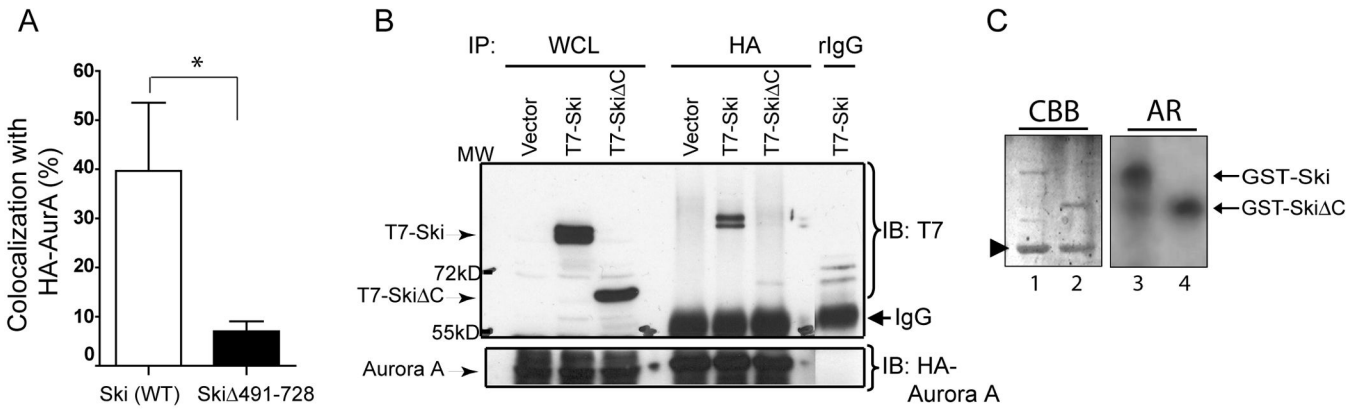

Figure 4. C-terminal portion of Ski is required for interaction with Aurora A

(A) U2OS cells were co-transfected with plasmids coding for EGFP-Ski, EGFP-SkiΔ491–728 and HA-Aurora A and analysed by immnunofluorescence and microscopy (as in Figure 2). Quantification of colocalization between HA-Aurora A and EGFP-Ski or EGFP-SkiΔ491–728 is shown. (B) HEK293T cells were co-transfected with plasmids coding for HA-Aurora A and T7-Ski or T7-SkiΔ491–728 (T7-SkiΔC). Cell lysates were subjected to immunoprecipitation (IP) with a rabbit anti-HA antibody or rabbit normal IgG (rIgG) and immunoblotted (IB) with anti-T7 and rat anti-HA antibodies. WCL: whole cell lysate. MW: molecular weight markers (55 and 72kD are shown). (C) *In vitro* Aurora A kinase assay. GST-Ski and GST-SkiΔ491–728 were used as substrates for purified active Aurora A. Coomassie bright blue staining of the purified proteins is shown (CBB, lanes 1 and 2). Autoradiography (AR, lanes 3 and 4) shows the incorporation of [γ^{32}P] in both GST-Ski and GST-SkiΔ491–728 proteins. Arrowhead shows a contaminant protein that is not phosphorylated by Aurora A. In (A), mean±SD is shown. (*) $p<0.01$, Student's *t* test.