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Journal of Cellular Biochemistry 117:334–343 (2016)

Journal of Cellular Biochemistry

The Ski Protein is Involved in the Transformation Pathway of Aurora Kinase A

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ABSTRACT

Oncogenic kinase Aurora A (AURKA) has been found to be overexpresed in several tumors including colorectal, breast, and hematological cancers. Overexpression of AURKA induces centrosome amplification and aneuploidy and it is related with cancer progression and poor prognosis. Here we show that AURKA phosphorylates in vitro the transcripcional co-repressor Ski on aminoacids Ser326 and Ser383. Phosphorylations on these aminoacids decreased Ski protein half-life. Reduced levels of Ski resulted in centrosomes amplification and multipolar spindles formation, same as AURKA overexpressing cells. Importantly, overexpression of Ski wild type, but not S326D and S383D mutants inhibited centrosome amplification and cellular transformation induced by AURKA. Altogether, these results suggest that the Ski protein is a target in the transformation pathway mediated by the AURKA oncogene. J. Cell. Biochem. 117: 334–343, 2016.

KEY WORDS: Ski; AURKA; AURORA KINASE; CENTROSOME AMPLIFICATION

The Oncogenic kinase Aurora A (AURKA) is a serine/threonine kinase that plays an important role in chromosome segregation and centrosome functions [Andrews, 2005; Nikonova et al., 2013]. AURKA overexpression has been found in a number of cancers, including breast, colorectal, glioblastoma, and melanoma [Sen et al., 1997; Bischoff et al., 1998; Mountzios et al., 2008; Lehman et al., 2012; Roylance et al., 2014; Caputo et al., 2015]. Increased Aurora A kinase activity is related with centrosome dysfunction, aneuploidy, chromosomal instability [Zhou et al., 1998; Roylance et al., 2014] and with aggressive clinical progression in several cancers, including colorectal, breast, gastrointestinal and others [Miyoshi et al., 2001; Goos et al., 2013; Roylance et al., 2014; Yeh et al., 2014].

One of the molecular targets of AURKA is the Ski protein [Mosquera et al., 2011], a transcriptional co-repressor that down-regulates TGFβ as well as other signaling pathways [Deheuninck and Luo, 2009]. Early studies demonstrated that the upregulation of Ski causes morphological transformation of several avian cellular lineages [Stavnezer et al., 1981; Li et al., 1986] and thus, it was considered an oncoprotein. Accordingly, a number of studies have shown increased expression of Ski in several types of cancers, including melanoma [Fumagalli et al., 1993; Reed et al., 2001; Reed et al., 2008], leukaemia [Ritter et al., 2006; Singbrant et al., 2014], colorectal [Buess et al., 2004; Bravou et al., 2009], gastric [Kiyono et al., 2009; Takahata et al., 2009; Nakao et al., 2011], pancreatic [Heider et al., 2007], esophageal [Fukuchi et al., 2004] and glioblastoma [Jiang et al., 2014]. However, decreased expression of Ski has also been shown in almost 50% of colorectal cancers [Buess et al., 2004] and some melanoma cell lines [Poser et al., 2005]; and, a tumor suppressor effect of Ski has been described in pancreatic [Wang et al., 2009; Wang et al., 2010], melanoma [Tulley

Grant sponsor: Fondo Nacional de Desarrollo Científico y Tecnológico; Grant number: 1120222; Grant sponsor: U.S. Public Health Service Grant; Grant number: CA42573.

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Manuscript Received: 24 June 2015; Manuscript Accepted: 29 June 2015
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 2 July 2015
DOI 10.1002/jcb.25275 • © 2015 Wiley Periodicals, Inc.

and Chen, 2014] and breast cancer [Azuma et al., 2005; Le Scolan et al., 2008; Theohari et al., 2012]. Studies in SKI knock-out mice have also identified Ski as a tumor suppressor protein in that SKI+/− mice were more susceptible to chemical carcinogenesis than wild type animals [Shinagawa et al., 2001]. Moreover, SKI−/− mouse embryonic fibroblasts (MEFs) exhibit high levels of chromosomal instability resulting from defects of chromosome alignment and segregation and ultimately aneuploidy [Marcelain et al., 2012]. This latest observation suggested a role for the Ski protein in mitosis, which is consistent with its localization in the mitotic spindle and centrosomes [Marcelain and Hayman, 2005; Mosquera et al., 2011; Chen et al., 2013] as well as higher levels of Ski during mitosis [Macdonald et al., 2004; Marcelain and Hayman, 2005; Chen et al., 2013].

It has been established that one mechanism determining the abundance of Ski is ubiquitin-proteasome degradation. Also, indirect evidences suggest that phosphorylation of Ski may affect its stability and abundance in mitosis [Macdonald et al., 2004; Marcelain and Hayman, 2005]. Ski is phosphorylated during mitosis by the cdk1(p34cdc2)/cyclin B kinase complex [Marcelain and Hayman, 2005]. Also, Mosquera et al., showed that AURKA interacts with Ski at the centrosome, and that AURKA can phosphorylate Ski in vitro [Mosquera et al., 2011]. However, the occurrence of Ski phosphorylation by AURKA in vivo, neither the effect of phosphorylation on the Ski protein abundance nor the specific phosphorylated residues have been described yet.

We hypothesized that AURKA phosphorylates Ski, affecting its intracellular levels and that the oncogenic effect of AURKA depends on the abundance of Ski. To test our hypothesis, we analyzed the specific sites in Ski phosphorylated by AURKA, the effect of phosphorylation on the stability of the Ski protein and defined whether Ski overexpression was able to interfere with the in vitro oncogenic transformation induced by AURKA.

MATERIALS AND METHODS

TISSUE CULTURE, PLASMIDS, AND TRANSFECTION

SKI−/− and SKI+/− MEFs have been described before [Marcelain et al., 2012]. HEK293T, MCF-7, MEFs, and U2OS cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 μg/ml). NIH3T3 cells were maintained in DMEM supplemented with 10% bovine calf serum. Expression plasmids were transfected with FuGENE 6 (Roche Applied Science, Pleasanton, CA) for HEK293cells, Lipofectamine 2000 and 3000 (Life Technologies) for MCF-7, U2OS and NIH3T3. pGEX2T-Ski, pCMV-T7-Ski, pFlag-Ski, pREBNA-EGFP, pREBNA-EGFP-Ski, and HA-Aurora A were described previously [Liu et al., 2004; Zhao et al., 2009; Mosquera et al., 2011]. pBABE-puro h-Aur, h-AurD274A and pWZL Flag AURKA were obtained from Addgene (8510) [Crane et al., 2004]. Human Ski short hairpin RNA (shRNA) expression plasmids were constructed by using pSHAG1 BseRI-BamHI (BSHAGGING-PCR) (B strategy as described before [Paddison et al., 2002]. The DNA sequence for the human Ski shRNA was: 5′-TTCCAGCCG-CACCCGGGGCTGCAGAAGAC-3′. The PCR product was cloned into RIGB retroviral vector [Ueki and Hayman, 2003].

SAMPLE PREPARATION AND MASS SPECTROMETRY

HEK293T cells were transfected with Flag-Ski or T7-Ski expressing plasmids. 48 hours after transfection, Ski protein was purified by affinity chromatography, using anti-Flag (Sigma-Aldrich, Chile) or anti-T7 antibodies (Merck Millipore). The purified proteins were separate by SDS-PAGE and the gel stained with Coomassie bright blue (CBB, Sigma). The bands corresponding to Ski were excised from the gel, trypsinized and analyzed by Liquid chromatography-coupled Tandem Mass Spectrometry (LC/MS/MS), using an Applied Biosystems Q-STAR system (Stony Brook Mass Spectrometer Facility).

SITE-DIRECTED MUTAGENESIS

For site-directed mutagenesis, hSki was subcloned in a pBlueScript II SK(+) plasmid. Standard protocols were used, but given high GC content of Ski sequence, PCR amplification was performed using PfuUltra HF (Agilent Technologies, CA) in a reaction buffer containing 5% DMSO and 10% Betaine. Point mutations and the absence of additional unwanted mutations, were assessed by complete sequencing of the hSki inserts. Mutated inserts were then subcloned into BamHI/HindIII sites of pCMV-T2A or pGEX2T plasmids.

IN VITRO AURORA A KINASE ASSAY

For in vitro phosphorylation assay, GST, GST-Ski, GST-SkiS326A, and GST-SkiS383A proteins were produced in BL21 cells and recovered on glutathione-Sepharose 4B beads (BD Biosciences, San Jose, CA). GST fusion proteins were incubated for 30 min at 30°C in reaction buffer (8 mM MOPS/NaOH pH 7.0, 0.2 mM EDTA) supplemented with 0.5 μCi [γ32P]ATP, 100 μM unlabeled ATP, and 200 ng active Aurora A (Specific Activity: 1,789 U/mg, Millipore.). Autographs were semi-quantified in ImageJ [Schneider et al., 2012]. Values were normalized to CBB staining of each substrate. For non-radioactive Aurora Kinase assay, 3 μg of GST-Ski and 600 ng of Aurora A were incubated in 100μM unlabeled ATP containing reaction buffer.

IMMUNOFLUORESCENCE

Immunofluorescence was performed as described before [Mosquera et al., 2011; Marcelain et al., 2012]. For centrosome and microtubule staining, cells were fixed in methanol at −20°C for 6 min. anti- γ-tubulin (T3559, Sigma. 1:2000) and α-tubulin (T5168, Sigma-Aldrich, Chile. 1:1000) were used. For Ski detection, cells were fixed in PFA 3,7% for 10 min. at room temperature. Anti-Ski (Santa Cruz Biotechnology, Santa Cruz, CA, 1:200) was used.

RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR ANALYSIS

Total RNA from shRNA expressing cells was extracted by using the RNeasy kit (Qiagen, Germany) and treated with DNase (DNA-free kit, Qiagen). Reverse transcription was performed by using Superscript III (Invitrogen). Quantitative real-time PCR was performed in an Applied Biosystems PRISM 7900HT Fast Real-Time PCR System with SYBR green PCR master mix (Applied Biosystems, Foster City, CA). Data was analyzed by averaging triplicates Ct (cycle threshold). Levels of RNA expression were determined according to the 2-ΔΔct method. The relative abundance of each Ski mRNA was determined

by using a standard curve generated from 10-fold serial dilutions of cDNA and normalized to βActin mRNA. The limits of a 95% confidence interval were determined to indicate variability of the mean ratios for each experiment. Significance was determined by using Student′s *t*-test.

PROTEIN STABILITY ASSAY

To determine the half-life of Ski, MCF-7 transiently transfected with Flag-Ski (WT or mutants) were treated with 50 μg/ml cycloheximide (CHX) (Sigma, St. Louis, MO) 24 h after transfection. For endogenous Ski′s determination, MCF-7 cells were pre-treated for 3 h with 1 mM Alisertib (MLN8237, Selleckchem) or vehicle (DMSO). Cells were harvested, lysed at 0, 4, 6, and 8 h in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 mg/ml phenylmethylsulfonyl fluoride, aprotinin [2 μg/ml], and 100 mM sodium orthovanadate) and evaluated via SDS–PAGE and Western blot. The pixels for each band were measured and normalized so that the number of pixels at t = 0 was 1. The relative pixels were plotted *versus* time and the t½ was calculated at 0.5.

COLONY FORMING ASSAY

NIH-3T3 cells were transfected with pREBNA-EGFP, pREBNA-EGFP-Ski, and/or pBABE-hAur (WT AURKA). After 24 h, cells were selected with 2 ug/ml puromycin for 2 days. Then, selected cells were reseeded in 60 mm dishes at 5% confluence and cultured for additional 18–21 days. For evaluation of mutants, cells were transiently transfected Flag-Ski WT, Flag-Ski S326D, and Flag-Ski S383D, seeded after 24 h at 5% confluence and cultured by 15 days. At the end, cells were fixed in methanol: acetic acid (3:1) and stained with 0.5% crystal violet.

STATISTICAL ANALYSIS

For centrosome number and mitosis quantification, at least 100 cells/nuclei were evaluated for each condition. For mitotic spindle analysis, about 50 cells were evaluated. All experiments were assessed independently three or more times. Significance of the differences was determined by using Student *t*-test or One/Two Way ANOVA with Bonferroni post-test, as depicted in the Legend of each figure.

RESULTS AND DISCUSSION

IDENTIFICATION OF SERINE 326 AND SERINE 383 AS IN VITRO AND IN VIVO TARGETS FOR AURKA

In order to identify the effect that phosphorylation by AURKA had on Ski, we identified the amino acids being phosphorylated by AURKA. We performed an in vitro cold AURKA kinase assay, using a purified recombinant GST-Ski as a substrate. A mobility shift of the protein incubated with AURKA indicated that it was indeed phosphorylated (Fig. 1A). Subsequent liquid chromatography followed by tandem mass spectrometry (LC/MS/MS) identified S326 and S383 as the phosphorylated residues in Ski protein.

The phosphorylation consensus motif for AURKA in budding yeast is [R/K]X[S/T][ILV] [Cheeseman et al., 2002]. This motif was further refined in higher eukaryotes, for example in the proteins TACC [Barros et al., 2005], CDC25B [Dutertre et al., 2004], PLK-1 [Macurek et al., 2008]. Later, the motif was defined as [R/K/N]RX[S/T]B, by in vitro phosphopeptide analysis, with B being any hydrophobic amino acid except for Pro [Ferrari et al., 2005] but this motif is not present in all AURKA substrates [Kettenbach et al., 2011]. The sequence surrounding S326 and S383 slightly fits to this motif. Although S383 is next to a P residue, this is also the case for S315 in p53, another target for this kinase [Katayama et al., 2004].

In order to confirm the specificity of MS findings, we used the mutant GST-SkiS326A and GST-SkiS383A proteins as in vitro substrates in an AURKA kinase assay. Figure 1B shows a representative autoradiograph. Phosphorylation of the mutants was evidently reduced when compared to the wild type protein (GST-WTSki). Quantification of the signal revealed that the mutation of S326 or S383 reduced Ski phosphorylation by AURKA to about 60% and less than 30% of control levels (GST-WTSki; Fig. 1C).

Next, to investigate whether phosphorylation on serine 326 and 383 were also found in vivo, we analyzed by LC MS/MS Flag-Ski and T7-Ski proteins immunopurified from exponentially growing HEK293T cells. In a total of 4 analyses using both tagged proteins, we identified eight serines phosphorylated in the Ski protein. Among these identified sites, we found S326 and S383 (Fig. 1D). As far as we know, there is no information concerning S326 phosphorylation, whereas phosphorylation of S383 has been previously described in proteomic discovery experiments in human cells. In addition, we confirmed S394, S432, and S480 phosphorylation, as previously reported in phosphoproteome screening studies in HEK293, Jurkat T-cell leukemia and ES cells [Gauci et al., 2009; Mayya et al., 2009; Phanstiel et al., 2011; Shiromizu et al., 2013] and, importantly, we identified three new phosphorylation sites in Ski: S343, S500 and S720. Our findings are also in accordance with early studies that reported serine phosphorylations in chicken c-Ski [Sutrave et al., 1990]. However, in that study, phosphorylation events in c-Ski were thought to be limited to the C-terminus domain of the protein [Sutrave et al., 1990], while we found that a truncated Ski (ΔC) was still phosphorylated at S383, S394, S480 (data not shown). Thus, these events seem to be not restricted to the C-terminal domain in human Ski. Nevertheless, Ski may be phosphorylated at other sites under different physiological conditions, and hence we cannot rule out the presence of additional phosphorylation events. Indeed, phosphorylation of Ski at T458 by Akt following activation of this pathway by specific growth factors [Band et al., 2009] and phosphorylation in S515, has been also described [Nagata et al., 2010].

PHOSPHORYLATION ON Ser326 AND Ser383 DECREASES THE STABILITY OF Ski

Ski protein levels are regulated through the cell cycle by ubiquitin-dependent proteasomal degradation, reaching highest levels at G2/M [Macdonald et al., 2004; Marcelain and Hayman, 2005]. In mitotic cells, proteosome inhibition increases the stability of the phosphorylated form of Ski in vivo [Marcelain and Hayman, 2005]. Thus, we evaluated whether phosphorylation of Ski by AURKA affected its stability. First, we transfected a Flag-Ski WT, Flag-SkiS326A, and Flag-SkiS383A expressing plasmids and assessed the stability of the WT and mutant proteins in MCF-7 cells, a breast adenocarcinoma cell line with amplified and overexpressed AURKA [Klein et al.,

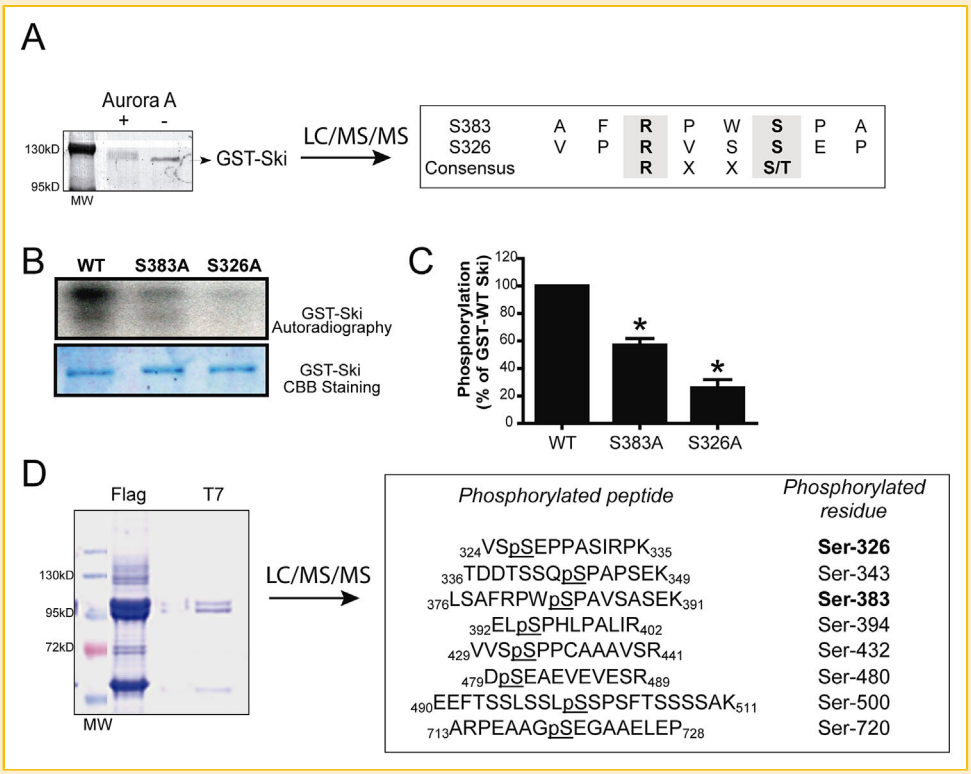

Fig. 1. Ski is phosphorylated at Serine 326 and 383 by AURKA. (A) Purified GST-Ski was incubated with or without Aurora A in an in vitro non-radioactive kinase reaction buffer. Phosphorylated Ski was analyzed by liquid chromatography-coupled Tandem Mass Spectrometry (LC/MS/MS). Identified phosphopeptides are shown to the left. R at position -3 is highlighted as it is shared by most Aurora A substrates. (B) In vitro AURKA kinase assay using GST-Ski WT and S326A and S383A mutants as substrates for purified active Aurora A. Autoradiography shows the incorporation of [γ^{32}P] in GST-Ski proteins. Coomassie bright blue staining of the purified proteins is shown (CBB). (C) GST-Ski phosphorylation was semi-quantified using CBB protein staining as loading control. Results represent median ± SD of three independent experiments and are expressed as percentage of GST-SKI WT protein phosphorylation. $^{*}P < 0.001$ versus WT protein. Student *t*-test. (D) Flag and T7-tagged Ski were immunopurified from HEK293 cells. A CBB gel of the purified proteins is shown. Bands corresponding to Ski were excised from the gel, trypsinized and analyzed by LC/MS/MS. A summary with the phosphopeptides and specific residues found to be phosphorylated in Ski in vivo is shown on right. S326 and S383 are highlighted. MW: molecular weight marker.

2005] (Fig. 2A). Point mutations S>A in S326 and S383 caused a significant increase of Ski half-life, indicating greater protein stability. Conversely, when serines were replaced by aspartics (S>D), half-life of Ski decreased to about 6 and 7 hours for S326D and S383D mutants, respectively (Fig. 2C and 2D).

Endogenous Ski protein was hard to detect by western blot in these cells. In fact, the protein was detected only after 4 h of CHX treatment (Fig. 2E). Conversely, when cells were pre-incubated with MLN8237 (Alisertib^{TM}), a small molecule inhibitor of AURKA, Ski became detectable in the absence of CHX (time "0 h"). Next, as Ski levels were hardly detected in MCF-7 cells, we evaluated the effect of AURKA overexpression on endogenous Ski in U2OS cells, a human cell line with higher levels of Ski. We found that ectopic expression of Flag-AURKA decreased Ski protein levels, when compared to vector transfected cells (Fig. 2F).

Overall, despite our data only indirectly implicates AURKA in phosphorylation of Ski in these experiments, these also indicate that phosphorylation on Ser326 and S383 indeed decreases stability of Ski.

The relationship between phosphorylation of Ski and stability has been explored before. Phosphorylation of Ski by Akt at threonine 458 has also been shown to cause destabilization of the protein in response to TGFβ [Band et al., 2009], while phosphorylation on serine 515 did not affect Ski's stability [Nagata et al., 2010].

Abovementioned results indicate that phosphorylation of S326 and S383 by AURKA reduces Ski half-life. Therefore, we further evaluated the effect of Ski protein abundance in the centrosome function and in the transformation activity of AURKA.

REDUCED LEVELS OF Ski RESULTS IN INCREASED CENTROSOMES NUMBER AND MULTIPOLAR MITOTIC SPINDLES

We have previously shown that absence of Ski results in chromosomal segregation defects driving cells into aneuploidy in mouse embryonic fibroblasts (MEFs) [Marcelain et al., 2012]. As AURKA over-expression has been shown to induce centrosomal amplification, we assessed whether decreasing Ski levels may result also in centrosomal amplification. Thus, we stained for γ-tubulin and performed indirect immunofluorescence in order to quantify the centrosomes in MEFs derived from SKI−/− and SKI+/− mice. We found that both SKI−/− and SKI+/− cells had centrosome amplification compared to wild type (WT) cells (Figs. 3A and 3B). When quantified number of centrosomes per cell, we found that

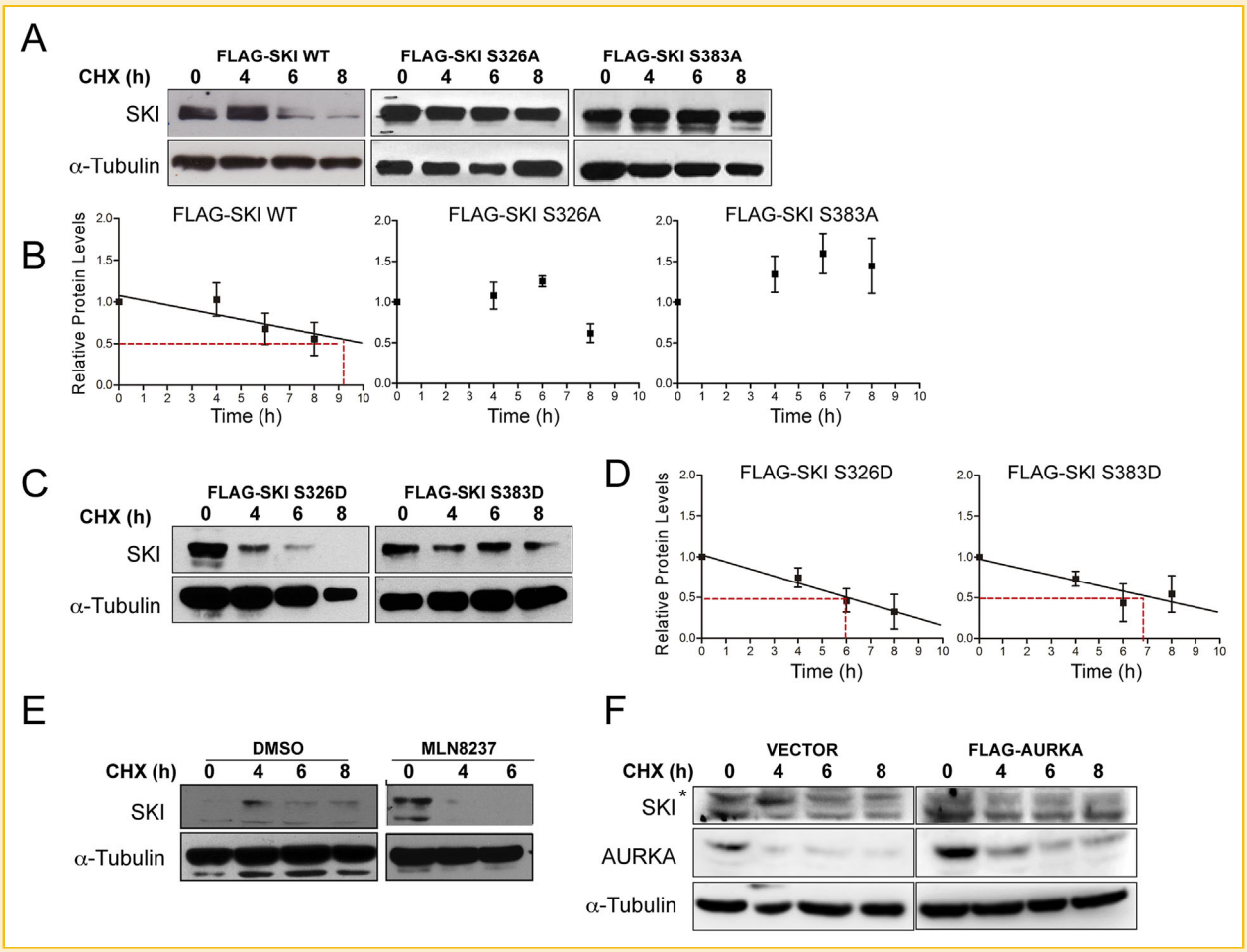

Fig. 2. Phosphorylation of Ski on S326 and S383 affected the stability of the protein. Stability of Flag-tagged SkiWT, SkiS326A, S383A (A); SkiS326D and SkiS383D (C) proteins were assessed in MCF-7 cells. Cells were incubated with cycloheximide (CHX) for up to 8 hours and Ski was detected by western blotting. In B and D, protein levels were semi-quantified and normalized by α-tubulin levels. Median ± SD of three independent experiments are shown and are expressed relative to time "0". Punctuated red line in D shows the time for 50% protein reduction according to a lineal regression model. (E) MCF-7 cells were pre-incubated with 1mM MLN8237 (Alisertib) or vehicle for 3 h before adding CHX. Ski was detected as in (A). MLN8237 treated cells were evaluated up to six hours after CHX treatment given mortality induced by the inhibitor at prolonged incubation times. (F) U2OS cells were transfected with an empty vector or a Flag-AurA plasmid. Ski and AURKA levels were evaluated as in (A).

more than 30% of the SKI+/− cells carried supernumerary centrosomes, and this percentage was significantly higher for the SKI−/− cells, reaching 50%. Importantly, the number of centrosomes per cell was also higher in the SKI−/− cells, with about 8% of cells having more than eight centrosomes (Fig. 3B). As SKI−/− cells were mostly binucleated and polyploid, the number of centrosomes was normalized to the number of nuclei. Similar results were obtained when we identified centrosomes by centrin staining (data not shown).

An increased number of centrosomes have direct consequences on the arrangement of multipolar spindles during mitosis, which leads to uneven cell division and consequently aneuploidy. Hence, centrosome amplification could contribute to the high percentage of aneuploidy reported previously for SKI−/− cells [Marcelain et al., 2012]. Thus, we analyzed the number of mitotic spindle poles in SKI+/− and SKI−/− cells that were in metaphase and anaphase stages of mitosis. We found that multipolar spindles arose in more than 35% of the SKI−/− cells. In contrast, although in the SKI+/− cells there was a significant increase in the number of centrosomes compared to WT cells, about 90% of these cells assembled bipolar spindles (Figs. 3A and 3C). Clustering of multiple centrosomes at a single spindle pole was frequently observed in both SKI+/− and −/− cells. Importantly, most of the time, the presence of multiple centrosomes did not interfere with normal chromosome segregation at anaphase in the SKI+/− MEF (see Fig. 3A).

To discriminate whether supernumerary centrosomes arise as a consequence of bona fide centriole overduplication or is secondary to polyploidy, we arrested early passage ($p \leq 3$) SKI WT and deficient MEFs in S-phase by hydroxyurea treatment and compared the induction of centrosome amplification to that in untreated exponentially growing cells (Fig. 3D). Prolonged S-phase did not have a significant effect of SKI compromised function on centrosome duplication, neither in SKI+/− nor in SKI−/− cells (Fig. 3D),

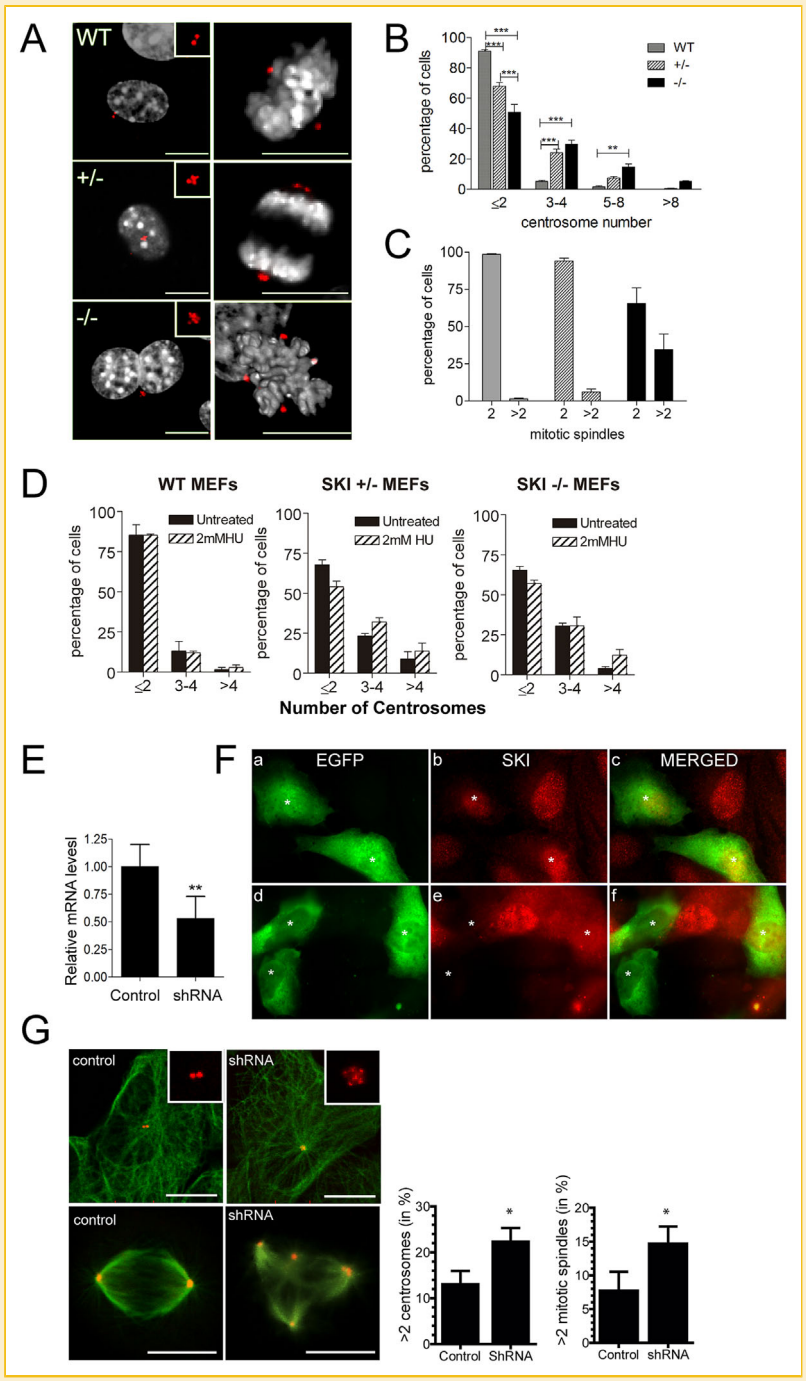

Fig. 3. Centrosomes amplification and multipolar mitoses in cells with reduced levels of Ski. (A) Centrosomes were detected by using an anti-γ tubulin antibody followed by immunofluorescence staining (red) in wild type (WT), *SKI*+/− and *SKI*−/− MEFs. DNA was stained with To-Pro3 (shown in gray scale). Magnified images of the centrosomes are shown in the insets. (B and C) Quantification of centrosomes and mitotic spindle poles. Each spot corresponding to γ-tubulin in interphase cells (left column in A) was considered as one centrosome or one spindle pole in mitotic cells (right panel in A). At least 500 interphase cells and 100 mitotic cells per experiment were counted and an average from two different cell populations from different animals of each genotype is shown. Significance was determined by using Student's *t*-test. $^{*}P < 0.05$ relative to WT; $^{**}P < 0.05$ relative to *SKI*+/− MEFs. *Bars*, 10 μm. (D) Primary, low passage (<4), Ski WT, SKI+/− and SKI−/− MEFs were treated with hydroxyurea (HU) for 24 h. Centrosomes number was evaluated as in A. Differences were not significant ($P > 0{,}05$; Studenťs *t*-test). (E) Ski mRNA levels were determined by qRT-PCR in U2OS cells stably expressing a control (scramble) or a shRNA targeting Ski. Cells from at least three independent experiments were harvested 10–20 days after antibiotic selection. mRNA levels were normalized to β-Actin mRNA levels and expressed as relative to control cells. (F) Expression of Ski protein was diminished in cells expressing the anti-Ski shRNA. Transduced cells (marked with an *) were identified by green fluorescence protein (EGFP) co-expression and Ski was detected by immunofluorescence (red). (G) Centrosomes and spindle poles were detected by using an anti-γ and α-tubulin antibody, followed by immunofluorescence in U2OS cells stably expressing a control and the shRNA targeting Ski. Quantification of centrosomes and spindles was performed as in A. The average of at least three independent experiments is shown. Significance was determined by using Student's *t*-test. $^{*}P < 0.05$. Bars, 10 μm.

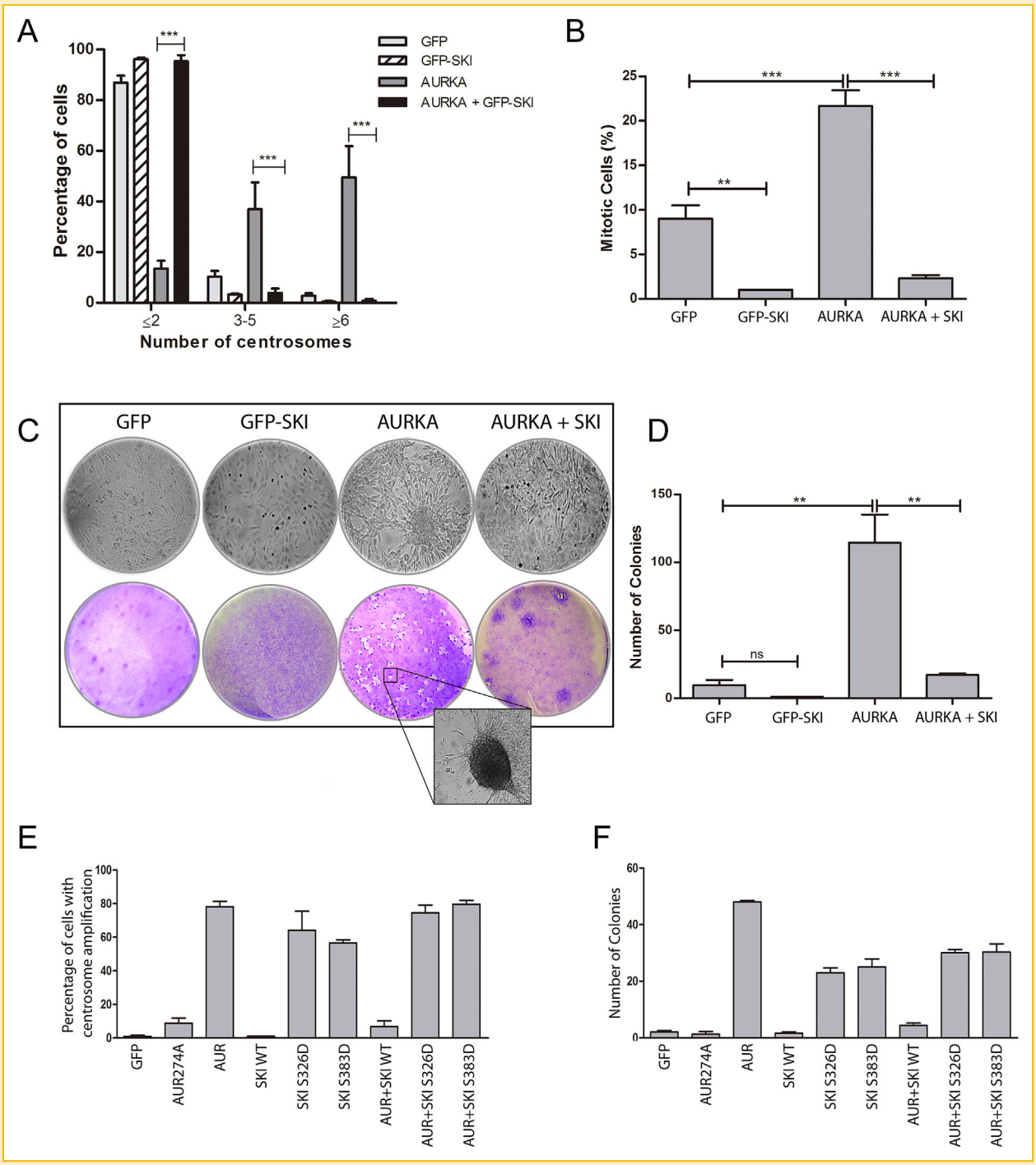

Fig. 4. Overexpression of Ski blocks AURKA induced cellular transformation. (A and B) U2OS cells were transfected with the indicated plasmids. Centrosomes number (A) was determined as in Figure 3. Mitotic index (B) was determined by counting mitotic cells. (C and D) NIH3T3 cells stably overexpressing the indicated proteins were seeded at 5% confluence and colony formation was evaluated 18 days after. In (C), a representative picture of the final cultures in phase contrast (up) and after staining with crystal violet (bottom). The inset shows a magnified picture of a colony that dettached from the plate, leaving empty spaces around the colonies. In D, quantification of colonies as depicted in C, is shown. In A, B, and D, mean of three independent experiments and SD are shown. $^{**}P < 0.001$; $^{***}P < 0.0001$; ns: not significant (One way ANOVA with Bonferroni post-test).

indicating that the increase in centrosome number in the SKI+/− and −/− MEFs is not caused by deregulation of centrosome duplication control during S phase. In full support of the previous observations, a high percentage of untreated SKI−/− and SKI+/− cells have centrosome amplification. As these experiments were carried out in MEF with a low number of passages, these observations indicate that centrosome amplification is an early event and may plausibly occur even before cell culturing.

In the absence of overduplication, centrosome clustering may indicate a mechanism of centrosome amplification in these cells and would explain the low aneuploidy observed in the SKI heterozygous cells [Marcelain et al., 2012], regardless of the increased number of centrosomes. This centrosomal clustering has been described before for p53 and Rb deficient cells [Borel et al., 2002] and it has been proposed as a mechanism to prevent the formation of multipolar spindles in cancer cells [Godinho et al., 2009; Yi et al., 2011]. A model explaining centrosome clustering induced centrosome amplification is shown in Supplementary Figure 1.

To establish whether reduced levels of Ski had the same effect on human cells, we reduced the levels of Ski in human cell lines by using a shRNA. shRNA targeting Ski was subcloned in a GFP-expressing retroviral vector and cells stably expressing the shRNAs were obtained by antibiotic selection. About a 50% reduction in the mRNA levels of Ski was achieved (Fig. 3E), which was also evident at the protein level (Fig. 3F). When analyzed the effect of the shRNAs on centrosome number and mitotic spindles, we found that a 50% reduction in Ski expression induced a significant increase in the percentage of cells carrying supernumerary centrosomes (i.e., from 12 to 25%) (Fig. 3G). Importantly, while virtually no control cell was found with more than 5 centrosomes (0.7%), a significant percentage of Ski-shRNA expressing cells had 6 or more centrosomes (4%). Reduction of Ski levels in human U2OS cells also induced centrosome clustering at single mitotic spindle poles and multipolar spindles in 14% of the cells (compared to 7% in cells expressing control shRNA) (Fig. 3G). Altogether, these results indicate that diminished levels of Ski cause supernumerary centrosomes and centrosome clustering.

OVEREXPRESSION OF Ski BLOCKS THE TRANSFORMATION EFFECTS OF AURKA

The oncogenic mechanism of AURKA has been proposed being initiated with the induction of supernumerary centrosomes. Hence, decreasing levels of Ski could be part of the oncogenic mechanism of AURKA. To further evaluate this possibility, we expressed AURKA in U2OS cells and quantified the number of centrosomes. We found that about 13% of control (EGFP expressing) cells had centrosome amplification and this percentage was significantly increased upon AURKA overexpression (86.5%). Interestingly, GFP-Ski expression decreased centrosomal amplification showed by control cells and, more importantly, inhibited the centrosomal amplification induced by AURKA (Fig. 4A).

In AURKA overexpressing cells, an increase in multipolar spindle formation was also evident (not shown). The effect of Ski expression could not be evaluated for this feature, because Ski overexpression reduced significantly the mitotic index. Moreover, GFP-Ski expression reduced the increased proliferation induced by AURKA overexpression (Fig. 4B).

To evaluate whether overexpression of Ski could also inhibits the oncogenic transformation mediated by AURKA, we stably overexpressed human AURKA in NIH3T3 cells, in the presence or absence of GFP-Ski and evaluated transformation by a colony formation assay. We found that AURKA overexpression induced cell transformation after two weeks of culturing, evidenced for a cell morphology change and an augmented colony formation capability, compared to control GFP expressing NIH3T3 cells (Figs. 4C and 4D). Overexpression of GFP-Ski did not have a significant effect on colony formation. However, expression of Ski decreased the effect of AURKA on colony formation, significantly reducing the number of colonies (114 ± 36 vs. 17 ± 1.7). Overall, these data suggest that decreasing levels of Ski is a mechanism implicated in centrosomal amplification and cellular transformation upon AURKA overexpression. To evaluate whether the effect of Ski was related to its phosphorylation, we expressed the Ski S326D and S383D mutants in U2OS and NIH3T3 cells, and evaluated their effect on centrosome number and colony formation, respectively. As expected, AURKA overexpression induced centrosome amplification and colonies formation while a kinase dead mutant (D274A) did not. Interestingly, neither Ski S326D nor Ski S383D was able to block the effect of AURKA overexpression. Moreover, unexpectedly, the expression of Ski mutants resulted in centrosome amplification and colonies formation even in the absence of AURKA (Figs. 4E and 4F; and Supplementary Figure 2).

Ski is phosphorylated in mitosis, localizes to the mitotic spindle and centrosomes in mitosis [Marcelain and Hayman, 2005] and interphase. These observations suggested a role for this protein in cell division. Accordingly, SKI−/− MEFs show several defects on chromosomal segregation during mitosis. Although these cells activate a spindle attachment or anaphase checkpoint (SAC) signaling when exposed to depolimerizing agents, they fail to stop anaphase when lagging chromosomes are present, indicating a weakened SAC [Weaver and Cleveland, 2005; Marcelain et al., 2012]. Here we showed that decreasing levels of Ski not only result in chromosome segregation defects but also in centrosomal amplification. Whether Skís role on chromosome segregation is functionally related to AURKA-mediated centrosome amplification and spindle formation, is currently under investigation. Although the data presented here indicate that decreasing Ski could be part of the mechanism of centrosomal amplification mediated by overexpressed AURKA, it is also plausible that Ski participates of an AURKA-independent mechanism. This would explain the strong effect that overexpression of this protein has on centrosome number and transformation (Fig. 4). Importantly, whatever the underlying mechanism, high levels of Ski could protect cells from genomic instability. Moreover, high levels of Ski could potentiate the effect of pharmacologic AURKA inhibitors, like MLN8237 (Alisertib), which have shown promising results on phase II trials for hematological and solid tumors [Farag, 2011; Kollareddy et al., 2012; Friedberg et al., 2014; Hilton and Shapiro, 2014]. More investigation in a clinical setting is needed to test these hypotheses.

ACKNOWLEDGMENTS

This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) Grant 1120222 and U.S. Public Health Service Grant CA42573 from the National Cancer Institute.

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