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Phosphorylation-dependent regulation cytosolic localization and oncogenic function of Skp2 by Akt/PKB

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Abstract

Skp2 is an F-box protein that forms the SCF complex with Skp1 and Cullin-1 to constitute an E3 ligase for ubiquitylation. Ubiquitylation and degradation of the p27 is critical for Skp2-mediated cell cycle entry, and overexpression and cytosolic accumulation of Skp2 have been clearly associated with tumorigenesis although the functional significance of the latter has remained elusive. Here we show that the Akt/PKB interacts with and directly phosphorylates Skp2. We find that Skp2 phosphorylation by Akt triggers SCF complex formation and E3 ligase activity. Importantly, a phosphorylation-defective Skp2 mutant is drastically impaired in its ability to promote cell proliferation and tumorigenesis. Furthermore, we show that Akt-mediated phosphorylation triggers 14-3-3- β -dependent Skp2 relocalization to the cytosol, and we attribute a specific role to cytosolic Skp2 in the positive regulation of cell migration. Finally, we demonstrate that high levels of Akt activation correlate with Skp2 cytosolic accumulation in human cancer specimens. Our results therefore define a novel proto-oncogenic Akt/PKB-dependent signaling pathway.

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The ubiquitin-proteasome system regulates the cell cycle through control of protein ubiquitylation and degradation^{1,2}. One of the key ubiquitin ligases (E3 ligase) in this process is the Skp1/Cul-1/F-box (SCF) complex, which consists of Skp1, Cullin-1 (Cul-1), RBX1, as well as an F-box protein, all required for its E3 ubiquitin ligase activity. Disruption of this complex severely ablates its enzymatic activity^{1,2}.

Skp2 (S-phase kinase associated protein-2) is a SCF F-box protein and is responsible for substrate recognition^{1,2}. It binds to p27 and targets it for ubiquitylation and degradation³⁻⁵. Overexpression of Skp2 induces cell cycle entry, and the degradation of p27 is required for Skp2-mediated cell cycle progression^{6,7}. *Skp2* deficiency displays elevated p27 protein levels and a profound impairment in proliferation accompanied by nuclear enlargement, polypoidy, and centrosome overduplication^{8,9}. Overexpression of Skp2 is frequently observed in human cancers of diverse histology, while in most human cancers reduced level of p27 represents an adverse prognostic marker^{1,2}. Skp2 cooperates with H-Ras^{G12V} to transform primary rodent fibroblasts¹⁰. Overexpression of Skp2 in the T-cell compartment cooperates with N-Ras to induce T cell lymphomas¹¹, while prostate specific expression of Skp2 leads to prostatic intraepithelial neoplasia (PIN)¹². These observations suggest that Skp2 overexpression may contribute to tumorigenesis.

Although substantial advances have been made in understanding the mechanisms that control its levels of expression, by contrast, the molecular mechanisms by which Skp2 activity within the SCF complex and its subcellular localization are regulated are currently unknown. This is of further relevance as, in human cancer, Skp2 is frequently found aberrantly localized in the cytosol. Here we demonstrate that phosphorylation of Skp2 by Akt/PKB constitutes a molecular switch that critically controls Skp2 SCF complex formation, localization and function.

Results

Akt/PKB interacts with and phosphorylates Skp2

Skp2 is phosphorylated during G1/S transition^{1,2,13}. Mitogens, such as epidermal growth factor (EGF), can also lead to Skp2 phosphorylation¹⁴. However, the functional relevance of this phosphorylation event is unclear and the kinases that execute it are still unknown. Since EGF can activate both phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen activating protein kinase (MAPK) pathways, we speculated that Skp2 might be the phosphorylation target of one of these two pathways. We therefore tested whether Akt/PKB might be a Skp2 kinase. Skp2 was found to interact with Akt1 in reciprocal co-immunoprecipitation experiments (Fig. 1a-c). Interestingly, the interaction between endogenous Skp2 and Akt1 was detected in the presence of Insulin-like growth factor-1 (IGF-1) while the interaction was abolished by PI3K inhibitor LY294002 (LY), suggesting that Akt activity might favor the formation of the Akt/Skp2 complex (Fig. 1d). In support of this notion, we found that Akt1 kinase dead mutant (K179A) interacted with exogenous Skp2 much less effectively than the constitutive active Akt1 (data not shown). In glutathione S-transferase (GST)-pull down assays, Akt1 was able to interact with Skp2 directly (Fig. 1f).

We next determined whether Skp2 was an *in vitro* substrate for Akt1. Skp2 was readily phosphorylated by recombinant active Akt1 (Fig. 2a). Skp2 phosphorylation by Akt1 was comparable to the phosphorylation of the TSC2 by Akt1, a well-known Akt substrate (Supplementary information Fig. S1b)¹⁵⁻¹⁸. Using the Scansite program [<http://scansite.mit.edu>; ¹⁹] analysis, we found that Skp2 Ser (S) 72 lies within an Akt consensus site [(RXRXXS/T, where X is any amino acid)] identified at “medium stringency”, which is conserved from rat to human (Fig. 2b). To determine whether S72 is a site for Akt-mediated Skp2 phosphorylation, we mutated this residue from serine to alanine

(S72A) and used this Skp2 mutant in *in vitro* kinase assays. Indeed, Akt-mediated phosphorylation of Skp2 S72A was markedly reduced (Fig. 2c, even though Skp2 S72A still interacted with Akt as efficiently as wild-type (wt) Skp2 (Fig. 1e). Similarly, *in vivo* phospho-labeling experiments performed in transfected cells with a constitutively active form of Akt1 (Mri-Akt) revealed phosphorylation of wt Skp2, but not of Skp2 S72A (Fig. 2d). We further confirmed that Akt1 induced Skp2, but not Skp2 S72A phosphorylation *in vivo*, by using a phospho-Akt substrate antibody (Fig. 2e).

In order to verify whether S72 is phosphorylated by Akt, we next performed mass spectrometry analysis. *In vivo* phosphorylated Skp2 (Skp2-P) and unphosphorylated Skp2 (Skp2-C) was isolated from 293T cells, digested with trypsin, and analyzed by MALDI-reTOF mass spectrometry (Figs. 2f and Supplementary information, Fig. S1a). Peptide patterns were then compared for differences. One *m/z* peak, at 1671.88 atomic mass units (amu), was observed in the spectra of “Skp2-P” that was absent from “Skp2-C.” The *m/z* value mapped to the predicted, monophosphorylated fragment of the Skp2 sequence (LKS⁷²KGS⁷⁵DKDFVIVR) with monoisotopic (¹²C) mass discrepancies of less than 40 ppm. Next, the same peptide was selectively retrieved by affinity metal chromatography²⁰ and reanalyzed by MALDI-TOF/TOF MS/MS sequencing. The presence of unique fragment ions confirmed the identity and the monophosphorylation state (characteristic loss of 98 amu) for the peptide and allowed us to narrow down the site of phosphorylation to either S72 or S75. In contrast, in similar experimental conditions, the phosphorylated peptide at 1671.88 amu was not identified when analyzing a Skp2 S72A mutant (designed as “S72A-P”), while the non-phosphorylated mutant peptide was detected, strongly suggesting that S72 is the site for Akt-mediated Skp2 phosphorylation *in vivo*. Similar results were also obtained when using Skp2 phosphorylated *in vitro* by recombinant Akt1 (data not shown and Supplementary information, Fig. S1c, d).

Lastly, to further demonstrate Akt-dependent Skp2 phosphorylation at S72 *in vivo*, we generated a phospho (S72)-Skp2 specific antibody. Once again, we found that Mri-Akt could phosphorylate Skp2, but not Skp2 S72A using this antibody (Fig. 2g). Importantly, in PC-3 prostate cancer cells, where Akt is constitutively active, endogenous Skp2 was phosphorylated at S72 (Fig. 2h), while LY drastically reduced its phosphorylation (Fig. 2h).

Since the p70 S6 kinase (S6K) is activated by Akt, we next determined whether S6K could mediate Akt-induced Skp2 S72 phosphorylation. Rapamycin, a well-known potent inhibitor of mTOR, abrogated S6K activity towards S6, while Skp2 S72 phosphorylation was unaffected (Fig. 2i), suggesting that S6K is not involved in Akt-dependent Skp2 S72 phosphorylation.

As aforementioned, EGF is known to induce Skp2 phosphorylation¹⁴. We therefore determined whether EGF would induce Skp2 phosphorylation at S72. EGF treatment induced Akt activation and S72 Skp2 phosphorylation, which was inhibited by Wortmannin (WN), but not by MEK1 inhibitor U0126, suggesting that EGF mediated Skp2 S72 phosphorylation occurs through an Akt-dependent pathway independently of MAPK-RSK activation (Fig. 2j). These results provide strong support for direct Skp2 phosphorylation at S72 by Akt/PKB both *in vitro* and *in vivo*.

S72 phosphorylation is critical for Skp2 biochemical and proto-oncogenic functions

We next determined whether Skp2 S72 phosphorylation modulates its E3 ligase activity toward p27. Wt Skp2 and the phosphomimetic Skp2 S72D mutant readily promoted endogenous and exogenous p27 ubiquitylation, whereas Skp2 S72A was profoundly impaired in this function (Fig. 3a and Supplementary information, Fig. S2a). Although p27 phosphorylation at T187 is proposed as an important event for the interaction of p27 with

the Skp2 SCF complex¹, we found that, surprisingly, Skp2-mediated ubiquitylation of wt-p27 and p27-T187A was comparable (Supplementary information, Fig. S3a). Likewise, the interaction of Skp2 with wt-p27 and p27-T187A was also comparable (Supplementary information, Fig. S3b) consistent with the results from the Roberts group²¹ showing that the interaction of p27 with Skp2 regulated by p27 T187 phosphorylation is cell type-dependent.

Conversely, while wt Skp2 and Skp2 S72D promoted p27 degradation, Skp2 S72A markedly reduced this activity (Fig. 3b and Supplementary information, Fig. S2b). It should be noted that within this time frame there was no significant change in cell cycle distribution, suggesting that p27 degradation is not caused by varied cell cycle status (Supplementary information, Fig. S2c). The inability of Skp2 S72A in promoting p27 ubiquitylation and degradation was also not due to its inability to interact with p27 or a reduction of its expression level (Fig. 3b and Supplementary information, Fig. S2d). Furthermore, LY or WN suppressed Skp2-mediated p27 ubiquitylation (Fig. 3a). These results suggest Akt activity and Skp2 S72 phosphorylation promotes Skp2 E3 ligase activity.

Skp2 overexpression is known to promote cell proliferation by triggering p27 degradation^{6,7}. We therefore determined whether S72 phosphorylation regulates Skp2-mediated cell proliferation. Both Skp2 and the S72D mutant promoted cells in S-phase, while Skp2 S72A was markedly impaired in this activity in 293T cells (Fig. 3c). Similar results were also obtained in COS-1 cells (Supplementary information, Fig. S3c). LY or WN suppressed Skp2-mediated an increase in S-phase cells (Fig. 3c).

To study the role of Skp2 and Skp2 mutants in tumorigenesis, we generated LNCaP prostate cancer cells with stable overexpression of Skp2 or Skp2 mutants for tumorigenic potential. Overexpression of wt and Skp2 S72D but not Skp2 S72A markedly enhanced LNCaP proliferation and reduced p27 protein levels (Fig. 3d, e). While Skp2 and Skp2 S72D profoundly promoted tumorigenesis when compared to the mock control, Skp2 S72A completely lost this activity (Fig. 3f, g).

Skp2 phosphorylation regulates SCF complex formation and activity

Since the integrity of Skp2 SCF complex is critical for Skp2 E3 ligase activity, we next determined whether activation of the PI3K/Akt pathway could regulate the formation of the Skp2-SCF complex. Mri-Akt enhanced the interaction of exogenous Skp2 with endogenous Skp1 and Cul-1, whereas WN suppressed the formation of the Skp2/Skp1 complex (Fig. 4a). In this cell culture condition, we did not observe an appreciable change in S-phase by either Akt activation or inhibition (Supplementary information, Fig. S4), suggesting that the effect of Akt on Skp2 SCF complex formation is not due to changes in cell cycle distribution.

Similarly, suppression of the PI3K/Akt pathway profoundly impaired endogenous Skp2-SCF complex formation, while we observed a slight increase in the formation of this complex upon Akt activation (Fig. 4b). In agreement with these findings, wt Skp2 and Skp2 S72D readily complexed with endogenous Skp1 and Cul-1, while Skp2 S72A significantly reduced its interaction with Skp1 and Cul-1 (Fig. 4c).

The Skp2 SCF complex isolated from cells transfected with wt Skp2 or its mutants was used for *in vitro* p27 ubiquitylation assay. Skp2-SCF complex readily promoted p27 ubiquitylation (Supplementary Fig. 5a, middle panel). Importantly, the Skp2 SCF complex failed to induce p27 ubiquitylation unless p27 was phosphorylated by the Cyclin A/CDK2 complex prior to this event (Supplementary Fig. 5a, right panel). Skp2 S72A ability to complex with Skp1 and Cul-1 and promote p27 ubiquitylation was markedly impaired when compared to wt Skp2 and Skp2 S72D mutant (Supplementary Fig. 5a). Furthermore, Mri-Akt significantly increased Skp2-SCF complex formation and p27 ubiquitylation, while LY

reduced this effect (Supplementary Fig. 5b). We further demonstrated that the SCF complex contained phospho (S72) Skp2 (Fig. 4d, e), while alkaline phosphatase (CIP) induced Skp2 S72 dephosphorylation and disrupted the Skp2 and Skp1 interaction (Fig. 4f). In addition, the lysates with Skp1 depletion reduced Skp2 S72 phosphorylation and Cul-1 (Lane 2 versus lane 1) (Fig. 4e). GST pull down assays demonstrated that while Mri-Akt, which induced Skp2 S72 phosphorylation (Fig. 2d, g) profoundly increased Skp1 and Skp2 interaction *in vitro*, Skp2 S72A dramatically reduced its interaction with Skp1 (Fig. 4g). These results strongly suggest that Akt positively regulates Skp2-SCF complex formation, therefore enhancing its E3 ligase activity through Skp2 S72 phosphorylation.

Akt-mediated phosphorylation induces Skp2 cytosolic localization

Skp2 normally resides in the nucleus; however, cytosolic relocation of Skp2 has been observed in several human cancers²²⁻²⁵. However, the molecular mechanisms underlying this phenomenon and its biological consequence are currently unknown. Loss or mutations of the tumor suppressor PTEN is frequently observed in human cancers, resulting in marked Akt activation^{26,27}. Akt/PKB may represent a potential candidate for triggering Skp2 cytosolic relocation given that phosphorylation of Akt substrates by Akt often results in change in protein localization^{28,29}. Immunofluorescence (IF) revealed that although Skp2 was localized in the nucleus, Mri-Akt induced Skp2 cytosolic relocation in 293T cells (approximately 40% of cells). In contrast, this effect was markedly reduced with Skp2 S72A (Fig. 5a and Supplementary information, Fig. S6a). Importantly, Skp2 S72D relocated to the cytosol even without Mri-Akt, but Mri-Akt did not further enhance the accumulation of S72D in the cytosol (Fig. 5a, Supplementary information, Fig. S6a and data not shown), suggesting Skp2 S72 phosphorylation regulates Skp2 cytosolic relocation. We further demonstrated that Akt activation upon IGF-1 or Mri-Akt induced endogenous Skp2 cytosolic relocation, as determined by IF and fractionation/Western blot analyses (Fig. 5b, c, f, and Supplementary information, Fig. S6b). Importantly, we found Skp2 S72 phosphorylation was predominantly detected in the cytosol, but not in the nucleus (Fig. 5d).

We next determined the kinetic of Skp2 S72 phosphorylation and cytosolic Skp2 localization upon IGF-1 stimulation. IGF-1 induced Akt activation and Skp2 S72 phosphorylation at 30 min, which lasted for several hours (Fig. 5e). Strikingly, WN markedly suppressed IGF-1-induced Skp2 S72 phosphorylation (Fig. 5e). Skp2 cytosolic accumulation already occurred at 1 hour after IGF-1 treatment, and was significantly blocked by WN (Fig. 5f). It should be noted that within this time frame there was no significant change in cell cycle distribution upon IGF-1 treatment (Supplementary information, Fig. S7), demonstrating that Skp2 S72 phosphorylation at S72 and cytosolic localization preceded cell cycle entry. We also obtained similar results in IMR90 cells. One hour after IGF treatment, up to 75-80% of cells displayed cytosolic Skp2 accumulation although the cell cycle distribution was minimally affected (Supplementary information, Fig. S8a, b, and d). Once again IGF treatment led to Akt activation accompanied by Skp2 S72 phosphorylation, which was abrogated by WN (Supplementary information, Fig. S8c). Our results therefore suggest that Akt induces Skp2 S72 phosphorylation in turn resulting in Skp2 cytosolic relocation.

14-3-3 β interacts with Skp2 and is required for Akt-mediated Skp2 cytosolic relocation

Akt phosphorylation is known to trigger protein nuclear export. We therefore tested whether Skp2 S72 phosphorylation would prevent its nuclear localization. In fact, the Skp2 sequence PRKRLKS (where S is at residue 72) was identified as a putative nuclear localization sequence (NLS) (Supplementary information, Fig. S9a). We fused this putative NLS and its mutant sequences (S72A or S72D) to GFP and examined their ability to promote GFP nuclear import. We found that neither of them promoted GFP nuclear localization, while the

NLS from SV40 large T-antigen fused to the GFP readily did (Supplementary information, Fig. S9a). These results suggested that the PRKRLKS sequence from Skp2 may not function sufficiently as an NLS and that the Akt-dependent Skp2 cytosolic accumulation may be due to enhanced nuclear export. In full agreement with this hypothesis leptomycin-B (LMB), which blocks protein nuclear export, significantly prevented Akt-mediated Skp2 cytosolic localization (Supplementary information, Fig. S9b). Although the PRKRLKS sequence from Skp2 is not functionally sufficient as a NLS in our assays, it is required for Skp2 nuclear localization as Gao *et al.* demonstrate that a Skp2 mutant devoid of this sequence primarily localizes in the cytoplasm (see Gao *et al.* submitted and accompanying paper).

The 14-3-3 protein controls a variety of biological processes through regulation of partner protein localization³⁰. It binds to phospho-serine/threonine containing motifs in a sequence-specific manner³⁰. As several Akt substrates interact with 14-3-3 upon Akt-mediated phosphorylation, which in turn results in a change of protein localization³¹⁻³⁴, we therefore examined whether 14-3-3 mediates Skp2 cytosolic localization upon Akt activation. Co-immunoprecipitation experiments revealed that endogenous Skp2 interacted with endogenous 14-3-3 β , but not with 14-3-3 γ and 14-3-3 θ (Fig. 6a), although we observed a weak interaction between exogenous Skp2 and 14-3-3 γ (data not shown). Remarkably, suppression of the PI3K/Akt pathway by LY or PTEN significantly abolished this interaction (Fig. 6b). We further demonstrated that the interaction of Skp2 with 14-3-3 β was enhanced by IGF-1 through the PI3K/Akt-dependent pathway (Fig. 6c). GST pull down assays revealed that 14-3-3 β interacted with Skp2 directly and Akt profoundly enhanced this interaction (Fig. 6d).

While Skp2 and Skp2 S72D effectively interacted with 14-3-3 β , a Skp2 S72A mutation compromised this interaction (Fig. 6e), suggesting that Skp2 S72 phosphorylation is critical for the interaction of Skp2 with 14-3-3 β . We next determined whether 14-3-3 β is required for Akt-mediated Skp2 cytosolic relocalization. 14-3-3 β and 14-3-3 γ siRNAs profoundly suppressed 14-3-3 β and 14-3-3 γ protein expression, respectively (Supplementary information, Fig. S10a). Notably, silencing 14-3-3 β expression inhibited Akt-mediated Skp2 cytosolic relocalization (Fig. 6f and Supplementary information, Fig. S10b). Similarly, silencing 14-3-3 β expression also reduced Skp2 S72D cytosolic localization (Supplementary information, Fig. S10c). As expected, silencing 14-3-3 γ expression failed to affect Akt-mediated Skp2 cytosolic relocalization (Fig. 6f). These results suggest that 14-3-3 β is required for Skp2 cytosolic relocalization induced by Akt.

Cytosolic Skp2 mediates cell migration

Skp2 overexpression is frequently observed in advanced human cancers. Skp2 may play a role in tumor invasion and metastasis. We examined whether Skp2 plays a role in cell migration, which underlies these steps in tumor progression. Overexpression of Skp2 and Skp2 S72D in MEFs markedly enhanced cell migration, whereas Skp2 S72A failed to promote cell migration (Fig. 7a). As Skp2 overexpression promotes cell migration, we determined whether Skp2 is required for cell migration. Strikingly, *Skp2*^{-/-} MEFs displayed a profound defect in cell migration (Fig. 7b, c). Similarly, in wound scratch assays, loss of Skp2 significantly delayed wound closure (Supplementary information, Fig. S11a). As advanced cancers often display cytosolic Skp2²²⁻²⁵, we hypothesized that Skp2 relocalization to the cytosol would favor cell motility. To test this possibility, we created a Skp2-NES (nucleus export signal) construct where the NES was fused to the Skp2 C-terminal end. IF reveals that Skp2-NES was predominantly localized in the cytosol (Fig. 7d, upper panel). Remarkably, restoration of Skp2-NES rescued the migration defect in *Skp2*^{-/-} MEFs (Fig. 7c and Supplementary information, Fig. S11a). This function of Skp2-NES is likely independent from its ability to promote p27 degradation, as Skp2-NES did not form

Skp2 SCF complex efficiently and failed to induce p27 down-regulation (Fig. 7d and Supplementary information, Fig. S11b).

Cytosolic Skp2 correlates with pAkt levels, PTEN loss, and metastasis in human cancers

As Akt induced Skp2 cytosolic relocalization, we next determined whether Skp2 localization correlates with pAkt and PTEN levels in cancer specimens. We immunoassayed Skp2, pAkt, and PTEN in colon and prostate tumor microarrays (TMAs). Of 84 cases of colonic adenocarcinoma, 16 cases showed cytoplasmic Skp2 staining in infiltrating carcinoma cells compared to normal colonic mucosa from the same tissue whole section or control tissue cores on the TMA, which showed scattered nuclear staining in the crypts and glands (Fig. 8a). Sequential sections showed high pAkt (Fig. 8a, c, and Supplementary information, Fig. S12a; $p < 0.003$) and low PTEN levels (Fig. 8a, c, and Supplementary information, Fig. S12a; $p < 0.008$) in these tumors. The intensity of cytoplasmic Skp2 staining in prostatic adenocarcinomas was lower than in colonic adenocarcinomas (Fig. 8b versus Fig. 8a). However, the correlation of cytoplasmic Skp2 and high pAkt was highly significant (Fig. 8b, c, and Supplementary information, Fig. S12b; $p < 0.001$) and these cases also had low PTEN levels (Fig. 8b, c, and Supplementary information, Fig. S12b; $p < 0.008$). In agreement with the role of cytosolic Skp2 in cell migration, we found that cytosolic Skp2 strongly correlates with lymph node (LN) metastasis in colon cancers (Fig. 8c and Supplementary information, Fig. S12a, $p = 0.03$). These results demonstrate that cytosolic Skp2 strongly correlates with pAkt, PTEN loss, and metastasis in human cancers, further implying that Skp2 cytosolic relocalization may have a role in promoting tumor metastasis.

Evolutionary conservation of the Akt-Skp2 functional and biochemical cross-talk

S72 is conserved in many organisms from rat to primates (Fig. 2b). However, this residue is not conserved in the mouse Skp2 (mSkp2) raising the question on when and how this regulatory pathway has evolved. We therefore tested the consequences of Akt activation in murine cells. We found that both Akt-mediated mSkp2 phosphorylation and the functional consequences of this event still take place in murine cells. Although Akt did trigger mSkp2 phosphorylation *in vivo* and even in *in vitro* (Supplementary information, Fig. 13a-c), it phosphorylated mSkp2 *in vitro* much less efficiently than the human Skp2 protein (Supplementary information, Fig. 13a, right panel), demonstrating that mSkp2 is a poor direct Akt substrate. Importantly, however, mSkp2 SCF complex formation was also enhanced by Mri-Akt (Supplementary information, Fig. 13c). Akt-mediated mSkp2 cytosolic localization was also observed (Supplementary information, Fig. 13d). In agreement with these findings, in *Pten*^{-/-} MEFs, where Akt is constitutively active, mSkp2 was found to accumulate in the cytosol and to relocalize in the nucleus upon LY treatment (Supplementary information, Fig. 13e). These results therefore support the notion that the Akt-Skp2 pathway is evolutionarily conserved even though Skp2 may not be a direct target of Akt in murine cells.

Discussion

We show that Skp2 S72 phosphorylation by Akt is required for Skp2 E3 ligase activity and tumorigenesis. Skp2 S72 phosphorylation provides a molecular switch that orchestrates SCF complex formation. The proto-oncogenic PI3K/Akt pathway can therefore regulate Skp2 protein levels through the enhancement of Skp2 transcription³⁵ and stability (see the accompanying paper by Gao *et al.*), its subcellular localization, but, importantly, the function of the SCF complex through Skp2 phosphorylation. Collectively, these findings identify PI3K/Akt signaling as a major regulatory pathway of Skp2 function at multiple levels. Very recently, Skp2 was shown to be phosphorylated by Cdk2 at S64 whose phosphorylation also regulates Skp2 stability³⁶. However, we found that Skp2

phosphorylation at S64 by Cdk2 was not required for Skp2 SCF complex formation (Supplementary information, Fig. S14a).

It should be noted that varied Akt activity through the distinct culture conditions can affect the ability of Skp2 and Skp2 S72A to form a Skp2 SCF complex, as Skp2 S72A only exhibited a minor change in SCF complex formation compared to wt Skp2 in 0.1% FBS where Akt activity is low, while Skp2 S72A displayed an impairment in forming a SCF complex upon Mri-Akt activity compared to the wt Skp2 (Fig Supplementary information, Fig. S15a).

Additionally, it should be noticed that the scansite program also predicts Skp2 T21 as a possible additional Akt phosphorylation site at low stringency (data not shown). We indeed found that Akt-mediated phosphorylation was partially reduced towards a Skp2 T21A mutant *in vitro*. Moreover, the ability of Skp2 T21A mutant to form a SCF complex was also reduced (Supplementary information, Fig. S15b, c). These results suggest that T21 site on Skp2 may represent an additional site for the effect of Akt on Skp2 SCF complex formation, at least *in vitro*. However, since the stringency of this site as per scansite analysis is low, it remains to be determined if this site is at all relevant *in vivo*.

The crystal structure of Skp2 SCF complex has been resolved³⁷. However, the N-terminal Skp2 tail (aa 1 to aa 108 encompassing the S72 sites) was omitted in these structural studies, suggesting that the N-terminal Skp2 tail is not required for the formation of the SCF complex *per se*. By contrast, it may contain an inhibitory domain that, in the context of the full-length protein, hinders the Skp2 interaction interface for Skp1 binding. Akt-mediated phosphorylation of the N-terminal Skp2 tail at S72 may induce its conformational change in turn allowing the interaction of Skp2 with Skp1. In full support of this notion, a Skp2 mutant (aa 91-424) devoid of the N-terminal tail forms a SCF complex much more efficiently than the wt Skp2 in cytosol and nucleus (Fig. 4h, i).

Skp2 normally localizes in nucleus and aberrantly localizes into the cytosol during cancer progression through mechanisms unknown to date²²⁻²⁵. We provide convincing evidence showing that cytosolic Skp2 localization is regulated by Akt through Skp2 S72 phosphorylation. Although Skp2 is also phosphorylated by Cdk2 at S64³⁶, Skp2 S64A did not affect Akt-mediated Skp2 S64A cytosolic localization (Supplementary information, Fig. S14b). Similarly, a Skp2 S64D mutation did not induce Skp2 S64D cytosolic localization, while Skp2 S72D did (Supplementary information, Fig. S14b), suggesting that Skp2 S64 phosphorylation does not regulate Skp2 cytosolic localization. Our results showing that Skp2 cytosolic localization strongly correlates with pAkt, PTEN loss, and cancer metastasis suggest that cytosolic Skp2 may play an important role in tumor metastasis. In full agreement with this notion, we find that Skp2 plays an essential and dose-dependent role in cell migration, while a cytosolic Skp2 mutant restores the migration defects in *Skp2*^{-/-} MEFs. In conclusion, we demonstrate that Akt-mediated Skp2 phosphorylation is critical for SCF complex formation, Skp2 cytosolic localization, and Skp2 biological functions thus providing the molecular basis and the biological relevance underlying the aberrant cytosolic localization of Skp2 in tumorigenesis.

Methods

Mice, cell culture, and reagents

Mouse embryonic fibroblasts (MEFs) from wild-type and *Skp2*^{-/-} mice were prepared as previously described^{8,38}. 293T, PC-3, COS-1 (from ATCC), and IMR90 (from M. Pagano) were cultured in DMEM containing 10% Fetal Bovine Serum (FBS), while LNCaP prostate cancer cells were grown in RPMI containing 10% FBS. To clone Xp-Skp2, Skp2 was

amplified by the polymerase chain reaction (PCR) using pcDNA3-Skp2 as a template and inserted into a pcDNA4/HisMax-TOPO vector (Invitrogen). To clone GFP-Skp2, Skp2 was amplified by PCR using pcDNA3-Skp2 as a template and inserted into pcDNA3.1/NT-GFP-TOPO vector (Invitrogen). pcDNA3-Skp2 S72A, pcDNA3-Skp2 S72D, Xp-Skp2 S64A, Xp-Skp2 S64D, Xp-Skp2 S72A, Xp-Skp2 S72D, and GFP-Skp2 S72A constructs were generated by the site-directed mutagenesis kit (Stratagene) according to the manufacturer's standard procedure using pcDNA3-Skp2, Xp-Skp2, and GFP-Skp2 as templates, respectively. The Skp2-NES construct was generated by fusing the nuclear export signal (NES) (ELALKLAGLDINKTE) to the C-terminal end of Skp2. MSCV-PIG-Skp2-NES was generated by subcloning Skp2-NES into the XhoI site of the MSCV-PIG vector. pBabe-Skp2, pBabe-Skp2 S72A, and pBabe-Skp2 S72D were generated by subcloning Skp2 and Skp2 S72A into the EcoRI site of the pBabe vector. GFP-PRKRLKS (from Skp2), GFP-PRKRLKA (from Skp2 S72A), GFP-PRKRLKD (from Skp2 S72D), or GFP-PKKKKRKVD (from SV40 large T-antigen) constructs were generated by introducing the peptide encoding sequence in frame at the 3' end of the GFP coding sequence in a pcDNA3.1/NT-GFP-TOPO vector (Invitrogen). (His)₆-ubiquitin, HA-p27, and pGEX-4X1-Skp1 constructs are gifts from Drs. D. Bohmann, M. Pagano, and P. Jackson, respectively. To clone Xp-mSkp2, the mouse Skp2 cDNA was amplified by RT-PCR from MEFs and was inserted into pcDNA4/HisMax-TOPO vector. Flad-Skp2 and Flag-ΔN-Skp2 (aa, 91-424) were from Dr. W. Wei. The p27-T187A construct was a gift from Dr. M.H. Lee. IGF-1, EGF, MG132, U0126, rapamycin and LY were obtained from Calbiochem. Wortmannin was purchased from Sigma. GST-Akt1 and recombinant active Akt1 were obtained from Cell Signaling.

Immunoprecipitation, immunoblotting and immunofluorescence

Immunoprecipitation (IP), immunoblotting (IB), and immunofluorescence (IF) were performed essentially as described with some modifications^{38,39}. For protein-protein interaction, cells were lysed by E1A lysis buffer (250 mM NaCl, 50 mM HEPES, [pH 7.5], 0.1% NP40, 5 mM EDTA, protease inhibitor cocktail (Roche)). The following antibodies were used for IP, IB or IF: anti-Skp2 (IP: 1:250; IB: 1:1000; IF: 1:100, Zymed), anti-Skp2 (IB 1:1000, Santa Cruz), anti-phospho (S473)-Akt (IB: 1:1000, cell signaling), anti-Akt1 (IB: 1:1000, Cell Signaling), anti-phospho-Akt substrate antibody (IB: 1:1000, cell signaling), anti-Skp1 (IP: 1:200; IB: 1:1000, BD Transduction Lab), anti-14-3-3β (IP: 1:100; IB: 1:2000, Santa Cruz), anti-14-3-3γ (IB: 1:500, Santa Cruz), phospho (S72) Skp2 antibody (IB: 1:1000, Pocono Rabbit Farm and Laboratory Inc.), anti-Xpress (IP/IF: 1:500; IB: 1:5000, Invitrogen), anti-HA (IB/IF: 1:1000, Covance, Upstate), anti-α-tubulin (IB: 1:1000, Sigma), anti-β-actin (IB: 1:1000, Sigma), anti-GFP (IP: 1:500; IB: 1:1000, BD Clontech), anti-p27 (IP: 1:100; IB: 1:1000, BD Transduction Lab), and anti-E2F1 (IB: 1:500, Santa Cruz).

In vitro and *in vivo* phosphorylation assay

In vitro phosphorylation assay was performed as described previously⁴⁰. In brief, GFP-Skp2 or GFP-Skp2 S72A was immunoprecipitated with GFP antibody from 293T cells cultured in DMEM medium containing 0.5% FBS. Immune complexes were washed three times in RIPA lysis buffer 150 mM NaCl, 10 mM Tris (pH 7.5), 1% NP40, 0.5 % deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche), then washed twice in 1X kinase buffer (25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, 10 mM MgCl₂, 2 μM cold ATP), and incubated with 1 μl recombinant active Akt1 kinase and 2 μCi [γ-³²P]-ATP in 50 μl total reaction buffer for 30 min at 30°C. Reactions were stopped by washing twice in kinase buffer and boiling in 2×SDS loading buffer. Proteins were resolved by 8% SDS-PAGE and transferred to the nitrocellulose membrane, and ³²P incorporation was detected by autoradiography. For *in vivo* labeling experiments, 293T cells cultured in DMEM containing 10% FBS were transfected with indicated plasmids for 24 h,

and the medium was changed to phospho-free DMEM with 0.25 % dialyzed FBS containing 200 μ Ci/ml ortho- 32 P for 4 h. Cells were lysed by RIPA buffer for IP and the Skp2 immunocomplex was subjected to 8% SDS-PAGE, followed by autoradiography.

Identification of phosphorylation sites by mass spectrometry

Gel-resolved proteins from *in vitro* and *in vivo* phosphorylation reactions were digested with trypsin, batch purified on a reversed-phase (RP) micro-tip, and an aliquot was analyzed by matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-reTOF) mass spectrometry (MS; UltraFlex TOF/TOF; BRUKER Daltonics; Bremen, Germany) for peptide mass fingerprinting, as described^{40,41}. This served to confirm the identity of the proteins and to locate possible differences between the tryptic peptide maps of the phosphorylated and unphosphorylated forms. The remainder of the RP-eluted digest mixtures was then subjected to immobilized gallium (III) affinity chromatography for selective capture of phosphopeptides, followed by elution with phosphate buffer, desalting over an RP tip, and a second round of MALDI-reTOF MS²⁰. Peak m/z values were matched to the protein sequence, allowing for the likely presence of one or more phosphate groups. Mass spectrometric sequencing of the putative phosphopeptides was then carried by MALDI-TOF/TOF MS/MS analysis using the UltraFlex instrument in "LIFT" mode. Fragment ion spectra were inspected for the characteristic partial loss of 98 Da, indicating presence of phospho-serine or -threonine, and for the a'' , b'' , and y'' ions to compare with the computer-generated fragment ion series of the predicted tryptic peptides to locate the exact position of phosphoamino acids.

GST-pull down assays

For *in vitro* GST-Skp1 and Skp2 interaction, GST-Skp1 proteins purified from the bacterial lysates of BL21 competent cells transformed with pGEX-4X1-Skp1 using the Glutathione-Agarose beads according to the manufacturer's standard procedures. The GST-Skp1 proteins bound to glutathione Sepharose beads (Amersham Biosciences) were then incubated with the *in vitro* translated [35 S]-Skp2, which was preincubated with recombinant Akt kinase for 30 min at 30 °C, for 4 hours at 4°C in the interaction buffer (20 mM HEPES, pH 7.9, 150 mM KCl, 5mM EDTA, 0.5 mM DTT, 0.1% (v/v) Nonidet p-40, 0.1 % (w/v) BSA, 1mM PMSF, and 10% Glycerol), washed by the NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 6 mM MgCl₂, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT, 8% glycerol, 1 mM PMSF) 4 times, and subjected to 8% SDS-PAGE, followed by autoradiography. For *in vitro* Akt and Skp2 interaction, GST-Akt1 bound in agarose beads (Cell signaling) were incubated with *in vitro* translated [35 S] Skp2 at overnight in the interaction buffer, washed either by 1XPBS or NETN buffer 4 times, and subjected to 8% SDS-PAGE, followed by autoradiography.

Generation of phospho (S72)-Skp2 antibody

The Skp2 phospho (S72) peptide (PPRKRLKSPKGSDKDF) was synthesized and injected into the rabbit, followed by affinity purification according to the manufacturer's standard procedures (Pocono Rabbit Farm and Laboratory Inc.). For the Western blot analysis, the Skp2 phospho (S72)-antibody was pre-incubated with the Skp2 non-phospho peptide (PPRKRLKSKGSDKDF) [5 (peptide): 1 (antibody); w/w] overnight at 4°C, prior to adding it to the Western membrane. The secondary antibody used for this phospho-antibody was Rabbit TrueBlot HRP (eBioscience).

Ubiquitylation assay *in vitro* and *in vivo*

In vitro p27 ubiquitylation assays was performed essentially as described⁴. In brief, the Skp2 SCF complex was immunoprecipitated from 293T cells and mixed with *in vitro*-translated

[³⁵S]-p27 that was previously incubated with cyclin E/CDK2 for *in vitro* phosphorylation along with methylated ubiquitin and ubiquitin aldehyde for 60 min at 30 °C. The reaction was stopped with 2X SDS sample buffer and run on acrylamide gels. *In vivo* ubiquitylation assays were performed as described⁴². In brief, 293T cells were transfected with the indicated plasmids for 24 h, treated with vehicle, 20 μM LY, or 100 nM Wortmannin together with 20 μM MG132 for 6 h, and lysed by the denatured buffer (6M guanidine-HCl, 0.1M Na₂HPO₄/NaH₂PO₄, 10 mM imidazole). The cell extracts were then incubated with nickel beads for 3 h, washed, and subjected to the Western blot analysis.

RNA interference

293T cells were transfected with 14-3-3β or 14-3-3γ or nonspecific control siRNA using Oligofectamine reagent (Invitrogen) according to manufacturer's instructions. In brief, 100 nM final concentration of siRNA was used to transfect cells at 50 % confluency. Four hours after transfection, cells were recovered in full serum. Twenty-four hours later, cells were transfected with indicated plasmids together with siRNA using Lipofectamine2000 reagent in DMEM medium containing 0.5% before harvest. Cells were harvested 48 hr after siRNA transfection for IB and IF.

Cell growth and *in vivo* tumorigenesis assay

BrdU-incorporation assays were performed by using the *In Situ* Cell proliferation Kit, Fluos (Roche). LNCaP stable transfectant cells were generated as previously described⁴³. In brief, LNCaP cells were transfected with pcDNA3, pcDNA3-Skp2, or pcDNA3-Skp2 S72A and selected by G418 (300 μg/ml). The individual clone was picked and verified by Western blot analysis (data not shown). Two positive clones were pooled and used for Western blot analysis, cell growth, and the *in vivo* tumorigenesis assay (Fig.3). For cell growth assay, 2×10⁴ cells were seeded in 12 wells in triplicates, harvested, and stained with trypan blue at different days. Numbers of viable cells were directly counted under the microscope. For *in vivo* tumorigenesis assays, LNCaP stable cells (1×10⁶) mixed with matrigel (1:1) were subcutaneously injected into the left flank of 6-week-old athymic male nude mice (NCRNU-M, Taconic Farms Inc.). Tumor size was measured weekly using a caliper, and tumor volume was determined by using the standard formula: L×W²×0.52, where L is the longest diameter and W is the shortest diameter.

Cell migration and *in vitro* wound scratch assay

Cell migration assay and was done in 24 well transwell plate with 8-μm polyethylene terephthalate membrane filters [Falcon cell culture insert (Becton-Dickinson)] separating the lower and upper culture chambers. In brief, MEFs were plated in the upper chamber at 5 × 10⁴ cells per well in serum-free DMEM medium. The bottom chamber contained DMEM with 10% FBS. Cells were allowed to migrate for 18 hours in a humidified chamber at 37°C with 5% CO₂. After the incubation period, the filter was removed and non-migrant cells on the upper side of the filter were detached using a cotton swab. Filters were fixed with 4% formaldehyde for 15 min and cells located in the lower filter were stained with 0.1% crystal violet for 20 min and counted from three random fields. For the *in vitro* wound scratch assay, MEFs were grown to confluency and wounds were made using sterile pipette tips. Cells were washed in phosphate buffer saline (PBS) and replenished with DMEM containing 10% FBS. After overnight incubation, cells were fixed and photographed.

Immunohistochemistry

Paraffin immunohistochemistry was performed on tissue microarrays (TMAs) of prostate carcinoma (101 cases), colonic carcinoma (84 cases), and selected whole section slides of colonic carcinoma. Slides were deparaffinized and heated for antigen retrieval (steamer),

followed by incubating with primary antibodies for overnight at 4°C. Biotinylated secondary and tertiary antibodies (avidin-biotin complex) were incubated and developed with a chromogen diaminobenzidine. Primary antibodies for Skp2 (2C8D9, 1:400, Zymed), PTEN (1:75, Cascade), and anti-phospho (S473) Akt (1:250, Cell Signaling) were used for this study. The statistic is analyzed by chi-square χ^2 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Skp2 interacts with Akt

(a, b) 293T cells were transfected with the indicated plasmids and harvested for co-immunoprecipitation experiments. (c) Exogenous Skp2 interacts with endogenous Akt1. 293T cells were transfected with the indicated plasmids and harvested for co-immunoprecipitation experiments. (d) Endogenous Skp2 interacts with endogenous Akt1. 293T cells were serum starved for 1 day, treated with IGF (100 ng/ml) in the presence or absence of LY (20 μ M) for 6 h and harvested for co-immunoprecipitation experiments and Western blot analysis. (e) Analysis of the interaction between Akt and WT Skp2 or Skp2 mutants. 293T cells were transfected with Xpress (XP)-Skp2, XP-Skp2 S72A (XP-S72A), or XP-Skp2 S72D (XP-S72D) along with Mri-Akt were immunoprecipitated (IP) with Xpress

(XP) antibody, washed, and followed by Western blot analysis. (f) Akt1 interacts with Skp2 *in vitro*. GST-Akt1 proteins were incubated with in vitro translated [³⁵S]-Skp2 at 4 °C for overnight, washed by PBS (lanes 2 and 3) or NETN buffer (lanes 3 and 4), and subjected to 8% SDS-PAGE.

Figure 2. Akt/PKB phosphorylates Skp2 at S72 *in vitro* and *in vivo*

(a) Skp2 is phosphorylated by recombinant active Akt1 (Rec Akt1) *in vitro*. GFP-Skp2 was immunoprecipitated from 293T, incubated with recombinant active Akt for 30 min, and subjected to SDS-PAGE analysis. (b) Skp2 contains a conserved Akt consensus site in rat, cow, dog, monkey, and human, but not in mouse. (c) Skp2, but not Skp2 S72A, is phosphorylated by Rec Akt1 *in vitro*. GFP-Skp2 or GFP-Skp2 S72A was immunoprecipitated from 293T, incubated with recombinant active Akt for 30 min, and subjected to SDS-PAGE analysis. (d, e) Skp2, but not Skp2 S72A, is phosphorylated by Akt *in vivo*. Skp2 phosphorylation was determined by phospho-labeling and Akt phospho-substrate antibody, respectively. (f) Akt induces Skp2 phosphorylation at Ser72 *in vivo*.

GFP-Skp2 or GFP-Skp2 S72A was isolated from 293T cells transfected with GFP-Skp2 alone (Lane 1, Skp2-C), GFP-Skp2 and Mri-Akt (Lane 2, Skp2-P), or GFP-Skp2 S72A and Mri-Akt (Lane 3, S72A-P), and analyzed by mass spectrometry (see Methods). Mass spectrometry analysis reveals that the LKS⁷²KGS⁷⁵DFVIVR peptide from GFP-Skp2, but not the A⁷²KGS⁷⁵DFVIVR peptide from GFP-Skp2 S72A, is phosphorylated by Akt *in vivo*. **(g)** Akt induces Skp2 phosphorylation at Ser 72 *in vivo*. 293T cells were transfected with the indicated plasmids, and harvested for Western blot analysis using an anti-phospho (S72)-Skp2 antibody. **(h, i)** Endogenous Skp2 is phosphorylated by Akt at S72. PC-3 prostate cancer cells treated with 20 μ M LY or Rapamycin (Rapa) 10 nM for 5 h were harvested for immunoprecipitation (IP), followed by Western blot analysis using a anti-phospho (S72)-Skp2 antibody. **(j)** EGF induces Skp2 phosphorylation at S72 via Akt. 293T cells were serum-starved (0.1 % FBS) for 2 days, treated with EGF (100 ng/ml) in the presence or absence of Wortmannin (100 nM, WN) or U0126 (20 μ M) for 3 hours, and harvested for Western blot analysis using an anti-phospho (S72)-Skp2 antibody.

Figure 3. Phosphorylation of Skp2 is required for Skp2 E3 ligase activity and function
(a) Skp2 and the Skp2 S72D mutant, but not Skp2 S72A, promote endogenous p27 ubiquitylation. 293T cells were transfected with the indicated plasmids, treated with vehicle, 20 μM LY, 100 nM WN along with 10 μM MG132 for 6 h and harvested for *in vivo* ubiquitylation assay (see methods). **(b)** Down regulation of p27 protein expression by Skp2 and Skp2 S72D, but not Skp2 S72A (S72A). 293T cells were transfected with the indicated plasmids for 48 h and harvested for Western blot analysis. **(c)** Loss of Skp2 phosphorylation at Ser72 significantly compromises Skp2-mediated an increase in S-phase cells. 293T cells were transfected with the indicated plasmids in a serum-starved condition (0.1% FBS) for 24 h, refreshed with 10% FBS for 16 h, incubated with 20 μM BrdU for 1 h, and harvested for

quantification of Brdu-incorporation. 200-300 cells were scored and a representative result presented as means $\pm$ standard deviation (s.d.) is shown from three independent experiments., ** $p < 0.01$ using Student's t-test, $n=3$. **(d)** S72 phosphorylation is critical for Skp2-mediated cell proliferation. LNCaP prostate cancer cells stably transfected with Mock, Skp2, Skp2 S72A, or Skp2 S72D were plated in 12 wells for cell growth analysis. Results are presented as means $\pm$ s.d. from a representative experiment performed in triplicate., * $p < 0.05$, ** $p < 0.01$ using Student's t-test, $n=3$. **(e)** p27 protein levels are regulated by Skp2 and Skp2 S72D, but not Skp2 S72A. p27 protein expression in LNCaP cells stably transfected with Mock, Skp2, Skp2 S72A, or Skp2 S72D. **(f, g)** S72 phosphorylation is critical for Skp2-mediated tumorigenesis. LNCaP cells stably transfected with Mock, Skp2, Skp2 S72A, or Skp2 S72D were injected into nude mice ($n=5$ for each group) and followed-up for tumorigenesis (see Methods). Pictures in **(g)** were taken 4 weeks after the injection. Results are presented as mean values $\pm$ s.d. from two independent experiments., * $p < 0.05$, ** $p < 0.01$ using Student's t-test.

Figure 4. Akt-mediated Skp2 phosphorylation regulates SCF complex formation

(a) Akt promotes the interaction of exogenous Skp2 with endogenous Skp1 and Cul-1. 293T cells transfected with Mock or His-Skp2 were serum starved for 1 day, treated with or without 100 nM Wortmannin (WN) for 6h, and harvested for co-immunoprecipitation experiments. His-Skp2 was pulled down by Nickel column, followed by the Western blot analysis. (b) Endogenous SCF complex formation is positively regulated by Akt. 293T cells were transfected with the indicated plasmids, serum starved for 1 day, treated with 20 μ M LY for 6 h, and harvested for co-immunoprecipitation experiments. (c) S72 phosphorylation is critical for SCF complex formation. 293 T cells transfected with XP-Skp2, XP-S72A, or XP-S72D in the presence of Mri-Akt were serum starved for 1 day and harvested for were

for co-immunoprecipitation experiments. **(d)** The Skp2 SCF complex contains Skp2 phosphorylated at S72. 293T cells were transfected with mock or Mri-Akt, serum starved for 1 day, treated with 20 μ M LY for 6 h, and harvested for co-immunoprecipitation experiments. **(e)** Skp1 depletion reduces the amount of the phospho (S72) Skp2 proteins in the cell lysates. Total cell lysates from 293T transfected with Skp2 and Mri-Akt were immunoprecipitated with control IgG (anti-His antibody) or Skp1 antibody, washed, and subjected to Western blot analysis with the indicated Abs. The inputs for lane 1 and lane 2 were the lysates depleted with anti-His antibody and Skp1 antibody, respectively. **(f)** CIP treatment leads to S72 Skp2 dephosphorylation and disruption of the Skp1 and Skp2 interaction. Cell lysates from 293T cells transfected with XP-Skp2 were IP using a XP antibody, treated with CIP for 1 h, washed by 1X PBS 3 times, and subjected to Western blot analysis using an anti-phospho (S72)-Skp2 antibody. **(g)** Skp2 S72 phosphorylation regulates Skp2 and Skp1 interaction *in vitro*. GST-Skp1 bound to Glutathione Agarose beads were incubated with cell lysates from 293T cells transfected with XP-Skp2 or XP-S72A with or without Mri-Akt and cultured in the serum-free medium, washed, and subjected to Western Blot analysis. **(h)** The Skp2 mutant with N-terminal deletion forms a SCF complex much more efficient than the wt Skp2. 293 T cells transfected with Flag-Skp2 (aa, 1-424) or Flag- Δ N-Skp2 (aa, 91-424) were lysed by RIPA buffer containing 1% NP40, immunoprecipitated with Flag antibody, washed, and followed by Western blot analysis. **(i)** A N-terminal deletion Skp2 mutant participate much more efficient in SCF complex formation than its wt counterpart in both cytosol and nucleus. Cytosolic and nuclear fractions were prepared from 293 T cells transfected with Flag-Skp2 or Flag- Δ N-Skp2, immunoprecipitated with Flag antibody, washed, and followed by Western blot analysis.

Fig. 5. Skp2 phosphorylation by Akt regulates Skp2 cytosolic localization

(a) S72 phosphorylation mediates Akt-induced Skp2 cytosolic localization. 293T cells were transfected with the indicated plasmids, and cells were harvested for immunofluorescence (IF) analysis. 100-200 cells were scored and a representative result is shown from three independent experiments. (b, c) Activation of the PI3K/Akt pathway promotes endogenous Skp2 cytosolic localization. 293T cells were serum-starved (0.1% FBS) overnight, treated with vehicle or IGF-1 in the presence or absence of LY or WN for 6 h, and harvested for IF (b) or fractionation, followed by Western blot analysis (c). (d) Phospho-S72 Skp2 localizes in the cytosol. 293T cells were serum-starved overnight, treated with vehicle or IGF-1 in the presence or absence of LY or WN for 6 h, and harvested for fractionation, followed by

Western blot analysis by using a phospho (S72)-Skp2 specific antibody. **(e)** Kinetics of Skp2 S72 phosphorylation. 293T cells were serum-starved for 2 days, treated with IGF (100 ng/ml) in the presence or absence of WN for the indicated times and harvested for Western blot analysis using an anti-phospho (S72)-Skp2 antibody. **(f)** Kinetics of Skp2 cytosolic localization. 293T cells were serum-starved (0.1 % FBS) for 2 days, treated with IGF in the presence or absence of WN for the indicated times and harvested for fractionation and Western blot analysis. Scale bar, 10 μ m.

Fig. 6. 14-3-3β interacts with Skp2 and is essential for Akt-mediated Skp2 relocalization in the cytosol

(a) Skp2 preferentially interacts with the 14-3-3β isoform. 293T cell lysates were IP with IgG, 14-3-3β, 14-3-3γ, or 14-3-3θ antibody, washed, and harvested for Western blot analysis. (b) The PI3K-Akt pathway positively regulates the interaction of Skp2 with 14-3-3β. 293T cells were transfected with the indicated plasmids, and cells were harvested for co-immunoprecipitation experiments. (c) IGF-1 promotes the interaction between endogenous Skp2 and 14-3-3β. 293T cells were serum starved for 1 day, treated with IGF in the presence or absence of LY or WN for 6 h and harvested for co-immunoprecipitation experiments and Western blot analysis. (d) 14-3-3β interacts with Skp2 directly *in vitro*. GST-14-3-3β bound to Glutathione Agarose beads were incubated with *in vitro* translated [³⁵S] GFP-Skp2, which was preincubated with recombinant Akt kinase for 30 min at 30 °C, washed, and subjected to 8% SDS-PAGE, followed by autoradiography. (e) Phosphorylation-dependent regulation of Skp2 interaction with 14-3-3β. 293T cells were transfected with plasmids as indicated, and cells were harvested for CO-IP. (f) 14-3-3β, but not 14-3-3γ, mediates Akt-dependent Skp2 cytosolic localization. 293T cells were transfected with siRNA and plasmids as indicated, and harvested for IF analysis. 100-200 cells were scored and a representative result is shown out of three independent experiments. Scale bar, 10 μm.

Fig. 7. Cytosolic Skp2 positively regulates cell migration

(a) Skp2 and Skp2 S72D, but not Skp2 S72A, promotes cell migrations. Wt MEFs were infected with pBabe, pBabe-Skp2, pBabe-Skp2 S72A, or pBabe-Skp2 S72D selected by puromycin (2 μ g/ml) for 3 days, and plated for cell migration assays (Methods). Migrated cells were counted from three random fields, and mean values $\pm$ s.d. were calculated. Representative results from two independent experiments are shown. ** $p < 0.01$, *** $p < 0.001$ using Student's t-test, $n = 3$. (b) Loss of Skp2 impairs cell migration. Skp2 wt and Skp2^{-/-} primary MEFs were plated for cell migration assays. Migrated cells were counted from three random fields, and mean values $\pm$ s.d. was calculated. Representative results from three independent experiments are shown. ** $p < 0.01$ using Student's t-test, $n = 3$. (c) Cytosolic Skp2 restores cell migration defect in Skp2^{-/-} MEFs. Skp2 wt and Skp2^{-/-} primary MEFs were infected with the indicated viral constructs for 2 days, selected with puromycin for 3 days, and plated for cell migration assays. (d) Skp2-NES displays cytosolic localization and does not induce p27 degradation. 293T cells were transfected with XP-Skp2-NES for 2 days and harvested for IF and Western blot analysis. Scale bar, 10 μ m.

Fig. 8. Cytosolic Skp2 correlates with activation of the Akt kinase, PTEN loss, and metastasis in human cancer specimens

(a, b) Cytosolic Skp2 localization correlates with high pAkt levels and PTEN loss in colon (a) and prostate cancers (b). Colon cancer TMAs (84 cases) and prostate TMAs (101 cases) were stained for Skp2, pAkt and PTEN (Methods). Representative examples are shown. (c) Correlation of cytosolic Skp2 with high pAkt levels, PTEN loss, and metastasis in colon and prostate cancers.