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ORIGINAL ARTICLE

Skp2 inversely correlates with p27 and tuberin in transformed cells

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Abstract The cyclin-dependent kinase inhibitor $p27^{Kip1}$ (p27) is a major gatekeeper of the mammalian cell cycle progression known to be regulated by both, its subcellular localization and its degradation. To allow entrance into S phase and thereby mammalian cell cycle progression p27 must be degraded by a skp2-containing E3 ubiquitin ligase whose task is to target p27 for degradation by the proteasome. The tumor suppressor gene product tuberin directly binds to p27 and protects it from degradation via skp2. Whereas, p27 and tuberin are known to be localized to both, the cytoplasm and the nucleus, the localization of skp2 remained elusive. Here we demonstrate that skp2 is a cytoplasmic and nuclear protein. In addition we found an inverse correlation of the endogenous protein levels of skp2 with p27 and tuberin in different transformed cells and under different growth conditions. These data allow new important insights into this molecular network of cell cycle control.

Keywords Skp2 · Tuberin · p27

Introduction

The cyclin-dependent kinase (cdk) inhibitor $p27^{Kip1}$ (p27) is a major regulator of the mammalian cell cycle progression. It accumulates in G0/G1 cells and is localized in the nucleus where it negatively regulates cdks. During the transition to S phase p27 is translocated to the cytoplasm and degraded by the ubiquitin-proteasome pathway. In the G1 phase of the cell cycle p27 is ubiquitylated by the ubiquitin ligase KPC followed by proteasome-mediated degradation. To allow entrance into S phase and thereby mammalian cell cycle progression p27 must be degraded by a Skp2-containing E3 ubiquitin ligase. Skp2 is the F-box protein, which together with Skp1, Cul-1, and Roc1/Rbx1, forms an SCF (Skp1/cullin/F-box protein)-type E3 ubiquitin ligase complex whose task is to target p27 for degradation by the proteasome (reviewed in Blain et al. 2003; Nho and Sheaff 2003; Sicinski et al. 2007).

Inactivation of the tumor suppressor gene TSC1 or TSC2 causes tuberous sclerosis (TSC), an autosomal dominant tumor syndrome affecting approximately 1 in 6,000 individuals. This human genetic disease is characterized by the development of hamartomas in the kidneys, heart, skin, and brain. The latter often cause seizures, mental retardation, and developmental disorders, including autism. TSC patients carry a mutant TSC1 or TSC2 gene in each of their somatic cells and loss of heterozygosity has been documented in a wide variety of TSC tumors. TSC1 encodes hamartin and TSC2 encodes tuberin (The European Chromosome 16 Tuberous Sclerosis Consortium 1993; The TSC1 Consortium 1997; Pan et al. 2004; Astrinidis and Henske 2005; Crino et al. 2006; Rosner et al. 2008).

Tuberin and hamartin form a heterodimer. Tuberin, which is assumed to be the functional component of this protein complex, is a multifunctional protein, which is involved in the regulation of cell size, cell cycle, translation, transcription, apoptosis, and cell differentiation. Tuberin is localized to both, the nucleus and the cytoplasm, and Akt phosphorylation of tuberin induces cytoplasmic tuberin localization (Rosner et al. 2007b). It has been implicated in the regulation of endocytosis (Xiao et al.

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1997), transcription (Henry et al. 1998), neuronal differentiation (Soucek et al. 1998a), and apoptosis (Inoki et al. 2003; Shah et al. 2004; Freilinger et al. 2006a, b, 2008). A wide variety of proteins, implicated in different regulations, have been demonstrated to interact with tuberin (Rosner et al. 2008). A major function of the TSC1/TSC2 complex is its role as a GTPase activating protein against Rheb (Ras homolog enriched in brain), which in turn regulates mTOR (mammalian target of rapamycin) signaling (Pan et al. 2004; Wullschleger et al. 2006; Corradetti and Guan 2006; Guertin and Sabatini 2007; Rosner et al. 2008).

A wide variety of different findings demonstrate that tuberin is also an important regulator of the mammalian cell cycle, mainly via controlling the transition from G0/G1 to the S phase by affecting the function of p27. Downregulation of tuberin expression induces quiescent fibroblasts to enter the cell cycle and TSC2−/− fibroblasts exhibit a shortened G1 phase. Overexpression of TSC1 or TSC2 triggers an increase in G1 cells and p27 protein levels (Soucek et al. 1997, 1998b; Miloloza et al. 2000). Tuberin also triggers an upregulation of the amount of p27 bound to CDK2 (Rosner and Hengstschläger 2004). Furthermore, in tuberin negative cells nuclear p27 is delocalized into the cytoplasm. Tuberin was found to induce nuclear p27 localization by inhibiting its 14-3-3-mediated cytoplasmic retention. Akt-mediated phosphorylation of p27, but not of tuberin, negatively regulates tuberin's potential to trigger p27 nuclear localization, demonstrating that p27 localization during the mammalian cell cycle is under the control of tuberin (Rosner et al. 2007a).

In addition, tuberin affects the function of p27 on another level. Tuberin was demonstrated to bind directly to p27. Still, tuberin is not in a complex with skp2. Furthermore, skp2 is not involved in the regulation of tuberin stability and tuberin does not affect skp2 protein levels. But binding of tuberin to p27 sequesters p27 from skp2 accompanied by an upregulation of the p27 interaction with cdk2. Skp2-induced p27 degradation and cell cycle progression is abolished by tuberin′s protective binding to p27. In summary, tuberin directly binds to p27 and protects it from degradation via skp2 (Rosner and Hengstschläger 2004).

The knowledge of this network of interaction between skp2, p27, and tuberin warrants the investigation of the correlation of these proteins. It could be speculated that cells or growth conditions representing high levels of the one player (skp2) express low protein levels of the other players (the target p27 and its "protector" tuberin) and vice versa. In this study we analysed the protein expression levels of skp2, p27, and tuberin in different cells and under different growth conditions to learn more about the relevance of this molecular network of cell cycle control.

Materials and methods

Cell culture

HEK293 (adenovirus transformed human embryonic kidney) cells, HeLa (human cervical carcinoma) cells and Rat-1 fibroblasts were obtained from the American Type Culture Collection and were grown in medium supplemented with antibiotics (30 mg/l penicillin, 50 mg/l streptomycin sulphate) at 37°C and 5% CO_2 and were routinely screened for mycoplasma. In detail the cells were grown as previously described (Rosner et al. 2007a; Burgstaller et al. 2008).

Total cellular protein extraction

Extracts of total cellular protein were prepared by physical disruption of cell membranes by repeated freeze–thaw cycles. Briefly, cells were washed with PBS and harvested by scraping. Pellets were lysed in buffer A containing 20 mM Hepes, pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4 supplemented with 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0,3 μg/ml benzamidinchlorid, 10 μg/ml trypsininhibitor by freezing and thawing. Supernatants were collected by centrifugation at 15,000 rpm for 20 min at 4°C and stored at −80°C (Rosner et al. 2003).

Cytoplasmic and nuclear fractionation

For cytoplasmic/nuclear fractionation, cells were harvested by scraping and collected by centrifugation. Remaining cell pellets were resuspended in 5 packed cell volume buffer F containing 20 mM Tris, pH 7.6, 50 mM 2-mercaptoethanol, 0.1 mM EDTA, 2 mM $MgCl_2$, 1 mM PMSF supplemented with protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.3 μg/ml benzamidinchlorid, 10 μg/ml trypsininhibitor) and incubated for 2 min at room temperature and for another 10 min on ice. Thereafter NP-40 was added at a final concentration of 1% (v/v) and lysates were homogenized by passing through a 20-gauge needle for three times. Nuclei were pelleted by centrifugation at 2,500 rpm for 5 min at 4°C and supernatant containing cytoplasmic proteins (C) was collected and stored at −80°C. Remaining nuclei were washed three times in buffer F containing 1% NP-40. During the last wash nuclei were stained with trypan blue and microscopically examined for number, purity, and integrity. The nucleic pellets were lysed in buffer A containing 20 mM Hepes, pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM DTT, supplemented with protease inhibitors by repeated freezing and thawing. Supernatants containing soluble nuclear proteins

(N) were collected by centrifugation at 15,000 rpm for 20 min and stored at −80°C (Rosner et al. 2007b; Rosner and Hengstschläger 2007). To obtain nuclear extracts containing both, soluble nuclear proteins and the nuclear envelope, pure nuclei, obtained through washing steps with NP-40 containing lysis buffer as described above, were resuspended in buffer F containing 0.3% SDS, vigorously vortexed and incubated at 4°C for 30 min under constant agitation. Remaining lysates (N+) were stored at −80°C. Equal amounts of cytoplasmic (C) and nuclear fractions (N, N+) were analysed to allow the comparison of a protein's cytoplasmic versus nuclear distribution within the cell.

Immunoprecipitation and immunoblotting

Immunoprecipitation of endogenous skp2 was performed as previously described (Rosner and Hengstschläger 2004). Denatured samples prepared from total lysates and immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were stained with Ponceau-S to visualize the amount of loaded protein. For immunodetection antibodies specific for the following proteins were used: skp2 (N-19, Santa Cruz or CL8D9, Zymed Laboratories), p27 (C-19, Santa Cruz or clone 57, transduction Laboratories), cyclin A (C-19, Santa Cruz), cdk2 (M-2, Santa Cruz), α-tubulin (DM1A, Calbiochem), topoisomerase IIβ (clone 40, Transduction Laboratories), lamin B (Ab-1, Calbiochem) and importin (karyopherin α, clone 2, Transduction Laboratories). Signals were detected with the enhanced chemiluminescence method (Amersham).

Western blot densitometry

The blots were densitometrically scanned. The results are given in relation to the intensity of the according protein (skp2, p27 or tuberin) in log HEK293 cells set as 1.

Results and discussion

It was the first aim of this study to compare the protein expression levels of skp2 and its target p27. As proven by cell doubling studies and flowcytometric DNA analyses (data not shown) all cells were growing logarithmically at the time point of protein extraction. After determination of the protein concentration using the Bio-Rad protein assay with bovine serum albumine as the standard, for each cell line exactly the same amount of total protein was run on an SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were stained with Ponceau-S to visualize the amount of loaded protein. Ponceau-S staining proved that the amount of protein loaded on each lane was equal in the Western blot presented in Fig. 1a. We co-analysed α-tubulin, which is neither a target nor an effector of the skp2/p27 regulatory network. This control analysis revealed that the observed differences in protein expression levels are not caused by loading differences. In this study,

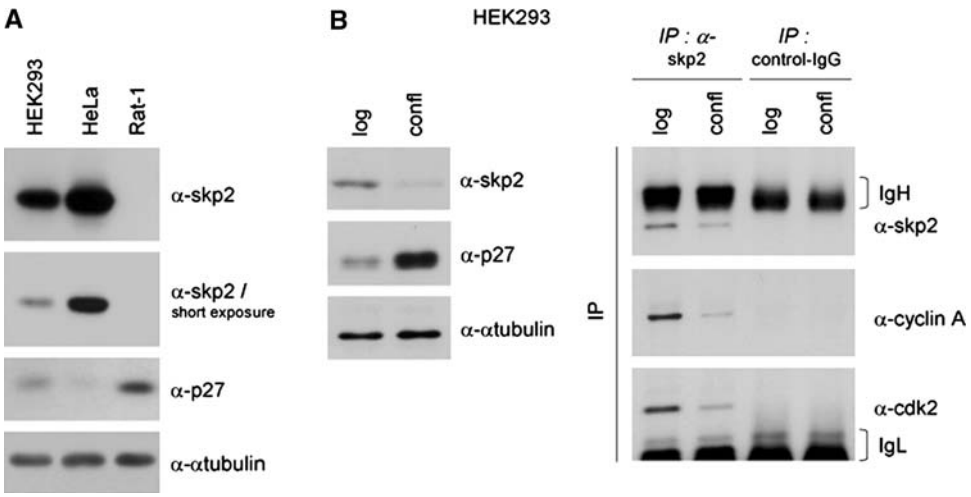

Fig. 1 Inverse correlation of skp2 and p27 protein levels in different cells and under different growth conditions. **a** Logarithmically growing HEK293 cells, HeLa cells and Rat-1 fibroblasts were analysed for the expression of endogenous skp2 and p27 protein levels via immunoblotting. Equal loading was confirmed by co-analysing α-tubulin. **b** HEK293 cells were grown under indicated growth conditions, logarithmically (*log*) and confluently (*confl*). Total lysates were prepared and protein levels of skp2 and p27 were analysed via immunoblotting. α-tubulin was co-analysed as a loading control *(left panel)*. Additonally, lysates were subjected to immunoprecipitation of endogenous skp2 and resulting precipitates were assessed for co-immunoprecipitation of cyclin A and cdk2. Immunoprecipitation with control-IgG was used as a negative control *(right panel)*

we found specific cells, such as Rat-1 fibroblasts, to express very low levels of skp2 and high levels of p27 (Fig. 1a). In contrast, the transformed human cell line HeLa expressed very high levels of Skp2 and very low levels of endogenous p27 (Fig. 1a). Another transformed cell line HEK293 represented a similar but not identical distribution. Although we found much higher levels of Skp2 and lower p27 expression compared to Rat-1 fibroblasts, the difference was not that pronounced (Fig. 1a). Taken together, these data provide evidence for a clear inverse correlation of endogenous skp2 and p27 protein levels in different cells. In addition, our findings suggest that transformed cells harbor high endogenous skp2 expression accompanied with low abundant p27, what is in agreement with the assumption that transformed cells should exhibit downregulated expression of a cell cycle inhibitor.

In the next step of this study we planned to investigate the expression of skp2 and p27 under different growth conditions. For these experiments we decided to use HEK293, because in these adenovirus transformed cells the endogenous protein levels of both, skp2 and p27, are clearly detectable under the experimental conditions chosen here (Fig. 1a). Restricted growth conditions were induced by growing logarithmically cycling HEK293 cells to confluency. In these experiments we observed striking inverse affects on skp2 and p27. Confluent growth triggered a significant downregulation of endogenous skp2 protein levels accompanied by a strong increase of endogenous p27 protein expression (Fig. 1b, left panel). Skp2—active in targeting p27 for proteasome degradation—was shown to be associated with cyclin A and cdk2 (Blain et al. 2003; Nho and Sheaff 2003; Sicinski et al. 2007). Immunoprecipitation approaches revealed that the observed downregulation of skp2 protein levels upon confluent growth indeed negatively affects the skp2/cyclin A/cdk2 complex formation (Fig. 1b, right panel). In summary, the data presented so far demonstrate that the strong inverse correlation of endogenous skp2 protein expression and p27 protein levels not only can be detected comparing different cell lines, but is also true within the same cell type as a consequence of different growth conditions.

The activity of the tumor suppressor protein tuberin is regulated by a variety of different phosphorylation events, but its protein level was assumed to be rather constant (Pan et al. 2004; Wullschleger et al. 2006; Corradetti and Guan 2006; Guertin and Sabatini 2007; Rosner et al. 2008). However, in a very recent study we observed that normal cell lines appear to exhibit higher endogenous tuberin protein levels than a variety of transformed cell types (Burgstaller et al. 2008). As already described above, one major target of tuberin is the cyclin-dependent kinase inhibitor p27 (Soucek et al. 1997, 1998b; Miloloza et al. 2000; Rosner and Hengstschläger 2004; Rosner et al. 2007a). Tuberin was shown to stabilize p27 via protecting it from Skp2-mediated degradation via its SCF-type E3 ubiquitin ligase complex (Rosner and Hengstschläger 2004). Accordingly, one could speculate that cells expressing high levels of tuberin should also exhibit high p27 protein levels probably accompanied with low skp2 levels. However, a lot of different aspects of the regulation of this network might interfere with such a simple correlation. (1) The endogenous levels of tuberin protein do not necessarily represent p27-regulating tuberin activity, because tuberin is activated and inactivated via a wide variety of different phosphorylation events (Pan et al., 2004; Wullschleger et al., 2006; Corradetti and Guan 2006; Guertin and Sabatini, 2007; Rosner et al. 2008). (2) Tuberin is clearly not the only regulator of p27 (Blain et al. 2003; Nho and Sheaff 2003; Sicinski et al. 2007). (3) Skp2 is not the only mediator of the proteasome-induced degradation of p27 (Blain et al. 2003; Nho and Sheaff 2003; Sicinski et al. 2007). (4) The relevance of the regulation of skp2 protein levels for its degradation-mediating function remains elusive. (5) Besides the control of p27 protein degradation, the function of p27 is also affected by the regulation of its nuclear and cytoplasmic localization. Importantly, a duality of function in tumorigenesis has recently been identified for p27. p27 acts as a tumor suppressor via inhibiting CDK activities in the nucleus. However in human malignancies, mutation/inactivation of p27 is only rarely detectable. Instead, p27 is degraded or relocalized to the cytoplasm. Several data provide evidence that cytoplasmic p27 might harbour oncogenic potential via regulating migration (Besson et al. 2004, 2007; Sicinski et al. 2007; Denicourt et al. 2007). (6) Last but not least, it has recently been shown that besides regulating p27 degradation, tuberin also affects the cytoplasmic/nuclear localization of p27. It was reported that tuberin, which is a nuclear and cytoplasmic protein, induces nuclear p27 localization by inhibiting its 14-3-3-mediated cytoplasmic retention (Rosner et al. 2007a; Rosner and Hengstschläger, 2007).

To investigate the correlation of skp2, p27, and tuberin levels we compared HEK293 cells and Hela cells. As already described above, HeLa cells express higher levels of skp2 and lower levels of p27 than HEK293 cells (Fig. 1a). Interestingly, we also observed lower levels of tuberin in HeLa cells compared to HEK293 cells (Fig. 2a, b). These data could suggest that the lower levels of tuberin are accompanied with less protection of p27 against skp2-mediated degradation leading to lower endogenous p27 expression in HeLa cells. However, that this correlation is not that simple can be seen by the analyses of the levels of these proteins under different growth conditions. Confluent growth triggered a downregulation of Skp2 and an increase of p27 levels in both, HEK293 and HeLa cells (Fig. 2a, b).

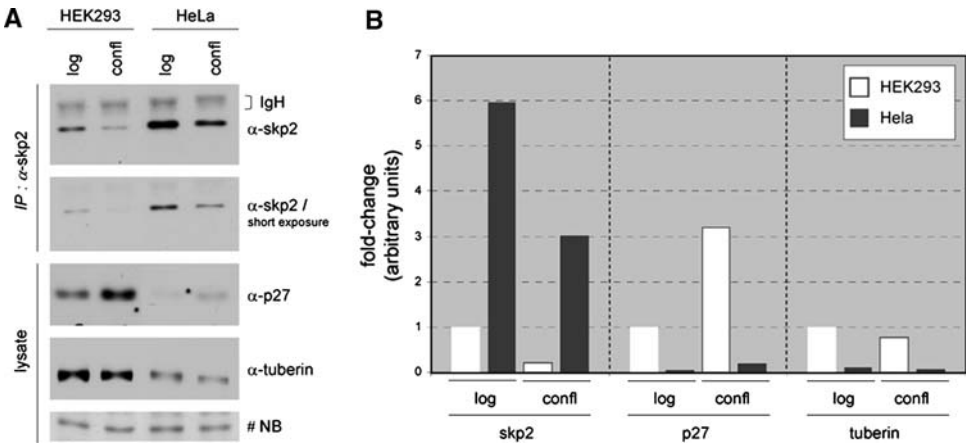

Fig. 2 Skp2 protein levels inversely correlate with p27 and tuberin protein levels in HEK293 and HeLa cells under different growth conditions. **a** HEK293 cells and HeLa cells were kept under indicated growth conditions, logarithmically (*log*) and confluently (*confl*). Total cell lysates were prepared and endogenous skp2 protein levels were analysed via immunoprecipiations approaches. In addition, p27 and tuberin protein levels were assessed via immunoblotting of lysates. *#NB* indicates a non-specific band, demonstrating equal loading of lysates. **b** Experiments performed in **a** were densitometrically analysed. The blots were densitometrically scanned. The results are given in relation to the intensity of the according protein (skp2, p27 or tuberin) in log HEK293 cells set as 1

However, in agreement with earlier reports (Soucek et al. 1997; Miloloza et al. 2000) we found the tuberin protein levels to be unaffected upon altered growth conditions (Fig. 2a, b). These findings provide evidence that while skp2 and p27 are inversely correlated in different cells and under different growth conditions, the direct correlation between p27 and tuberin levels is only true between different cells. Upon changed growth conditions the fluctuation of endogenous p27 levels cannot be explained by changed tuberin protein levels, but are rather the consequence of different additional levels of regulation of this network of interaction.

To allow a better understanding of this network, it was of importance to clarify the cellular localization of skp2. Both, tuberin, and p27 have been demonstrated to be cytoplasmic and nuclear proteins (compare Rosner et al. 2007a, band references therein). The nuclear transport of proteins occurs through the nuclear pore complex. The nuclear import starts with the recognition of the nuclear localization signal (NLS) of a cargo protein by its specific transport factor. The best characterized transport factor, importin α, recognizes the NLS and binds to importin β to form a "pore-targeting trimeric complex", which enters the nucleus through the nuclear pores. The small GTPase Ran regulates the dissociation of the complex after its entry into the nucleus by interacting directly with importin β, resulting in the release of importin α and the cargo protein (Xu and Massague 2004). p27 accumulates in G0/G1 cells and is localized in the nucleus where it regulates cdks. During the transition to S phase p27 is translocated to the cytoplasm and degraded by the ubiquitin-proteasome pathway. At least for the KPC-mediated degradation it is assumed that it occurs predominantly in the cytoplasm

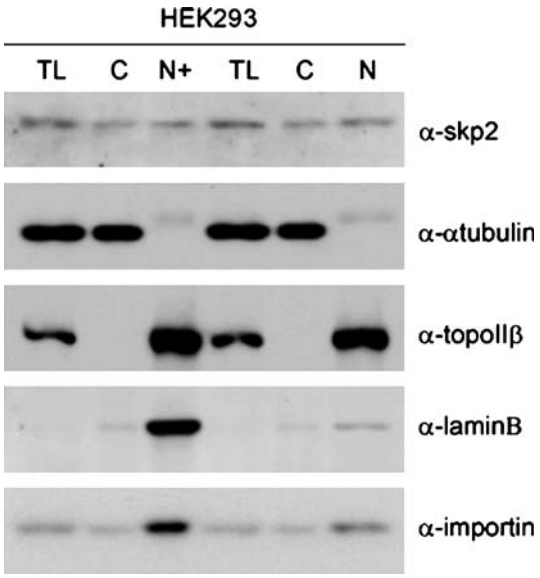

Fig. 3 Skp2 is equally distributed between the cytoplasm and the nucleus of HEK293 cells. Total lysates (*TL*) or cytoplasmic (*C*) and nuclear (*N*) fractions of logarithmically growing HEK293 cells were prepared and analysed for skp2 protein levels via Western blotting. *N* indicates nuclear extract containing soluble nuclear proteins; *N+* indicates nuclear extract containing both, soluble nuclear proteins and the nuclear envelope. To assess purity of so obtained fractions cytoplasmic (α-tubulin) and nuclear (topoisomerase IIβ and lamin B) marker proteins were analysed. In addition importin protein levels were co-analysed

(Kamura et al. 2004). However, whether skp2-mediated p27 degradation occurs in only one or both compartments remains elusive (Carrano et al. 1999; Sutterlüty et al. 1999).

We biochemically isolated extracts enriched for cytoplasmic proteins or for nuclear proteins from HEK293

cells. The purity of the obtained cytoplasmic and nuclear protein extracts was investigated by analysing the expression of α-tubulin (cytoplasmic), topoisomerase IIβ (nuclear) and lamin B (nuclear). In addition, we analysed the levels of importin, which is known to be higher expressed in the nucleus than in the cytoplasmic fraction. These experiments revealed for the first time that skp2 is almost equally expressed in the cytoplasm and in the nucleus (Fig. 3). These findings provide evidence that the regulation of skp2 localization is no additional level of the control of the skp2/p27/tuberin network.

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