Developmental Cell

Supplemental Information

Connexin Controls Cell-Cycle Exit and Cell Differentiation by Directly Promoting Cytosolic Localization and Degradation of E3 Ligase Skp2

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Supplemental Information

Fig. S1

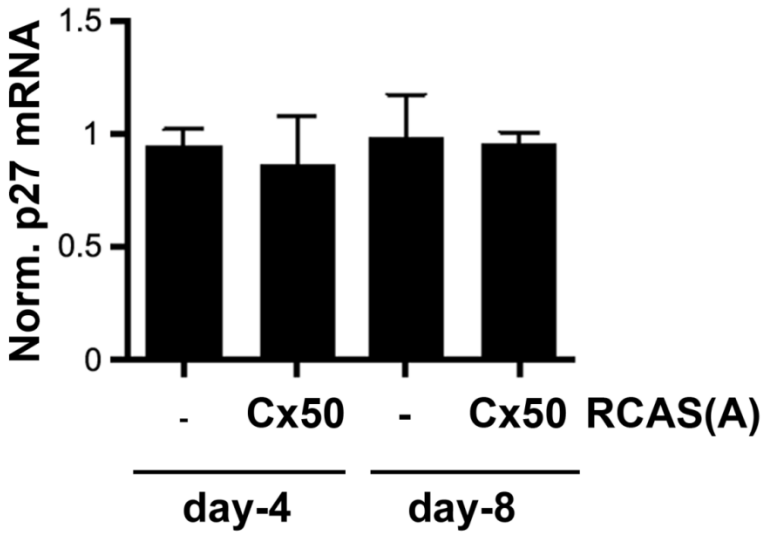

Figure S1. Cx50 expression does not change the RNA level of p27. Related to Figure 1. At culturing day 4 and day 8, total RNAs were isolated from lens primary cells and the p27 mRNA level was determined by quantitative real time RT-PCR. Data were normalized to β-actin mRNA levels by using efficiency ($2^{-\Delta\Delta Ct}$) method. The data were analyzed by student *t*-test, and were presented as the means ± SEM (n=3).

Fig.S2

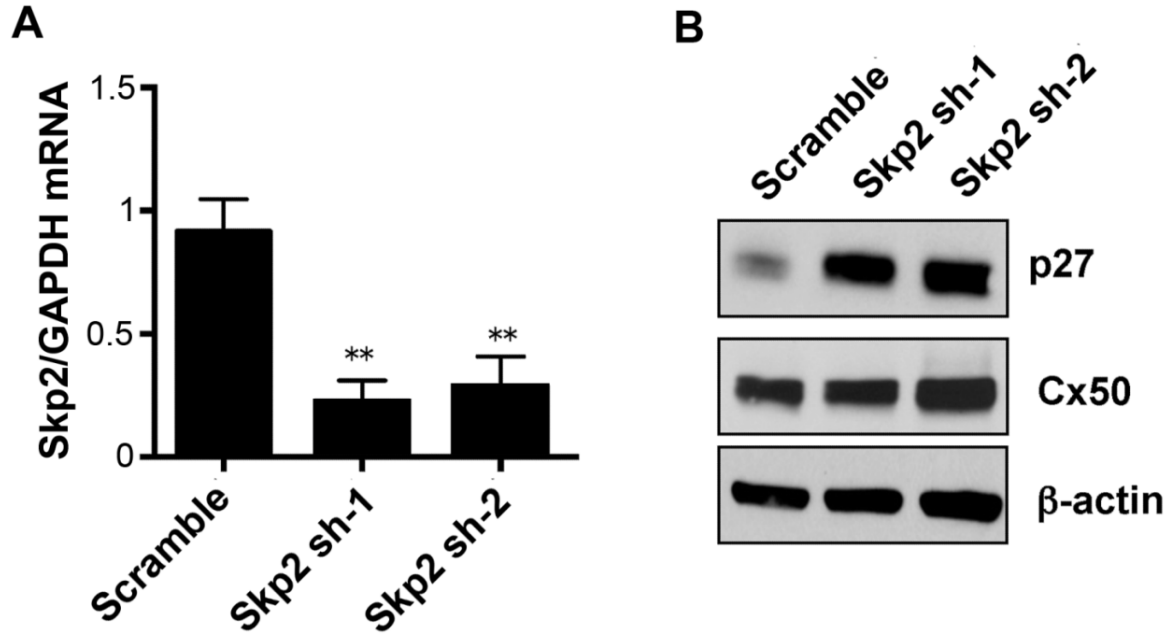

Figure S2. Skp2 shRNAs inhibit Skp2 expression, but enhance p27 levels in chick lens primary cells. Related to Figure 2. (A) mRNA level of Skp2/GAPDH was measured in chick primary culture with RCAS(A) or Skp2 shRNA retroviruses at culturing day 8. (B) Cell lysates of primary lens culture treated with Skp2 shRNA1 (Skp2-sh-1) or 2 (Skp2 sh-2) were immunoblotted with anti-p27, Cx50 or β-actin antibody. Both Skp2 shRNAs greatly enhanced p27 protein expression, but have no effect on endogenous Cx50 protein level.

Fig. S3

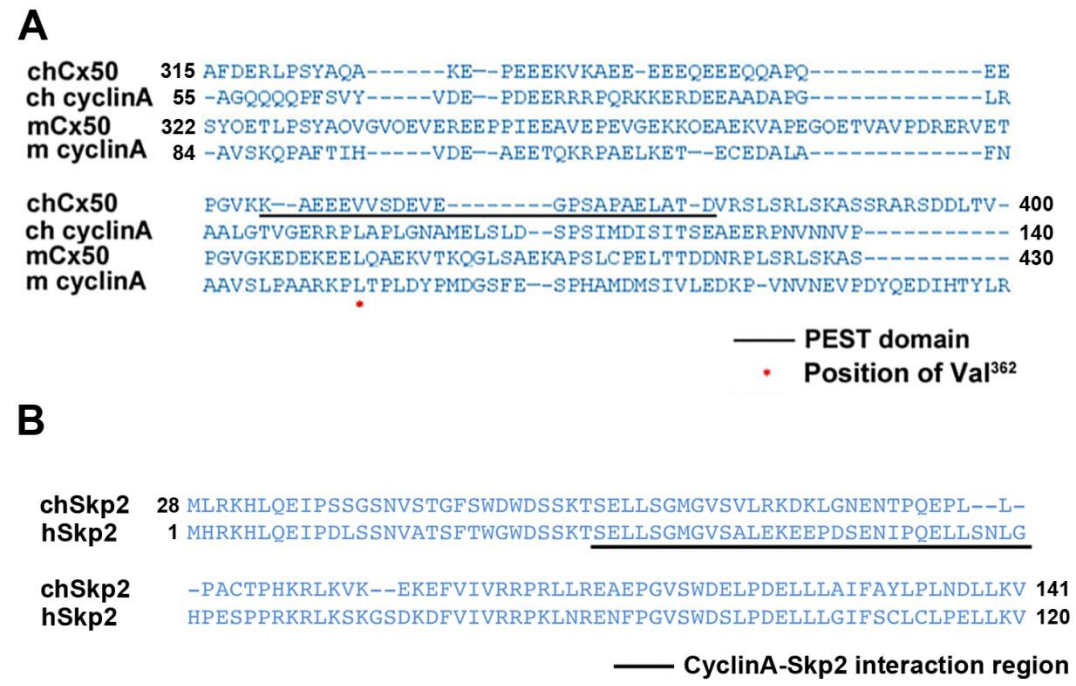

Figure S3. Sequence comparison between mouse and chick cyclin A and Cx50, and human and chick Skp2. Related to Figure 4. (A) The sequence alignment in the related regions of chick (ch) Cx50 and cyclin A and mouse (m) Cx50 and cyclin A was performed by FASTA2 program. The underline indicates PEST region and an asterisk in red indicates the position of Val362 in chick Cx50. (B) The sequence alignment in the related regions of chick (ch) and human (h) Skp2 was performed by FASTA2 program. The underline indicates a known human cyclin A and Skp2 interaction region.

Fig. S4

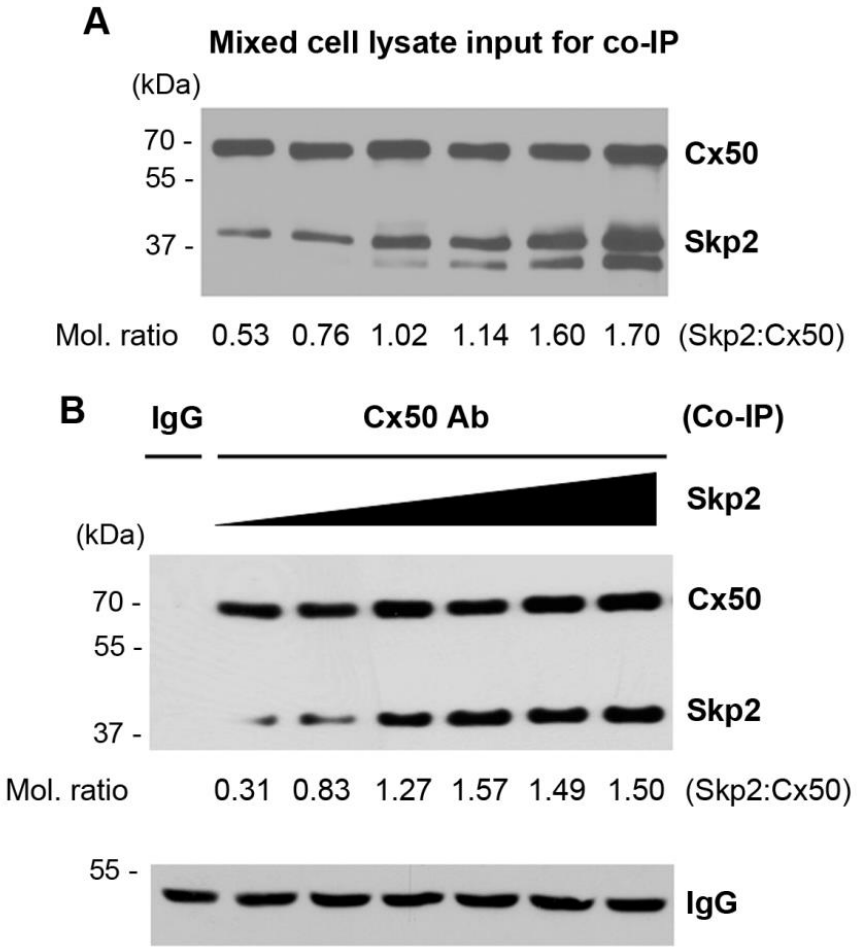

Figure S4. Stoichiometry of the interaction between Cx50 and Skp2. Related to Figure 5. The binding stoichiometry was determined by co-immunoprecipitation with saturation of the binding between Cx50 and Skp2. 500 μg of lysate of the CEF cell expressing FLAG-Cx50 were incubated with 500 μg, 750 μg, 1 mg, 1.5 mg, 1.75 mg, 2 mg of lysate of the cell expressing FLAG-Skp2, respectively. (A) The mixed cell lysates were immunoblotted with monoclonal anti-FLAG antibody. Both Cx50 and Skp2 were detected by FLAG antibody; thereby the levels of both proteins can be comparably assessed. The molar ratio of input proteins was indicated below the blot. (B) Co-immunoprecipitation assay was performed with the above mixed cell lysates using anti-Cx50 antibody against intracellular loop domain of Cx50. The co-immunoprecipitates were immunoblotted with anti-FLAG antibody. The binding stoichiometry was determined with relatively constant level of Cx50 and the increased Skp2 in the co-immunoprecipitates. Although input levels of Skp2 continued to increase **(A)**, the binding saturation was achieved in last three lanes with the molar ratio of Skp2 to Cx50 around 1.5. The molar ratio of Cx50 to Skp2 was calculated by relative intensity of the band normalized to molecular weight.

Fig. S5

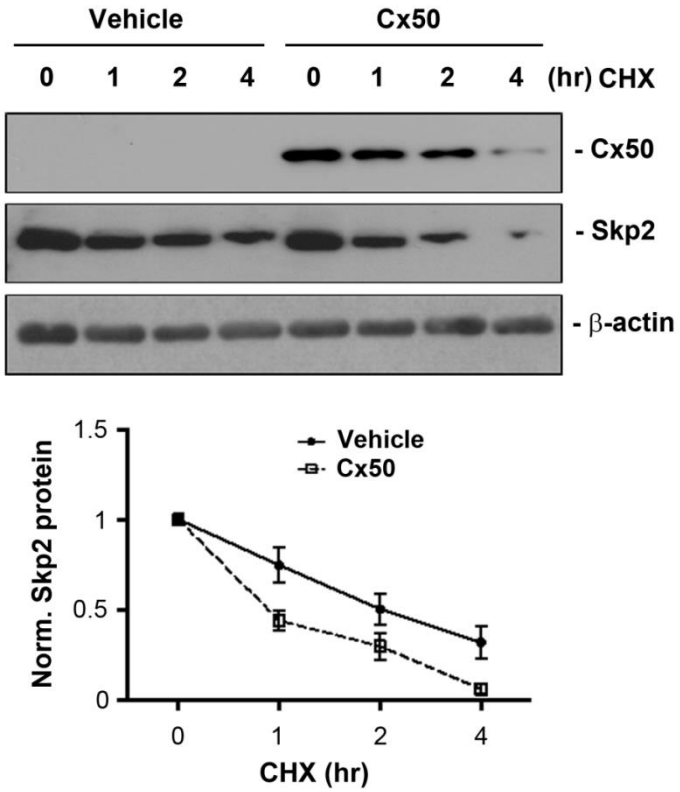

Figure S5. Cx50 de-stabilizes Skp2 in MEF cells. Related to Figure 6. Mouse Cx50 was exogenously expressed in MEF cells via lentivrial infection. The cells expressing GFP (vehicle) or Cx50-GFP were synchronized by thymidine-nocodazole block and then were treated with cycloheximide (CHX) (50 ng/ml) for up to 4 hours. Cell lysates were immunoblotted with anti-mouse Cx50, Skp2 or β-actin antibody. The protein levels were quantified (NIH Image J) and normalized based on the level at 0 hour.

Supplemental Experimental Procedures:

Materials

Fertilized, white leghorn chicken eggs were purchased from Ideal Poultry (Cameron, TX) and incubated for 10-11 days in a humidified 37°C egg incubator. Anti-Cx50 antibody against the intracellular loop domain was generated as described (Yin et al., 2001). Anti-CP49 antibody was a generous gift from Dr. Paul Fitzgerald at the University of California at Davis (Davis, CA); anti-AQP0 polyclonal antibody was generated from rabbit against chicken AQP0 CT domain (223–262 amino acids) (Shi et al., 2010). Rhodamine-conjugated goat anti-rabbit IgG and chemiluminescence kit (ECL) were purchased from GE Healthcare (Piscataway, NJ); paraformaldehyde (16% stock solution) from Electron Microscopy Science (Hatfield, PA); trypsin, tissue culture reagents; fetal bovine serum from Hyclone Laboratories (Logan, UT); QuikChange^{TM} site-directed mutagenesis kit from Stratagene (La Jolla, CA); Vectashield fluorescence mounting medium from Vector Laboratories (Burlingame, CA); protein A/G plus agarose beads, anti-p57 (Kip2) antibody, anti-Skp2 (for mouse) and anti-Skp1 antibody (C19) from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal anti-FLAG antibody from Rockland Immunochemicals (Gilbertsville, PA) and monoclonal anti-FLAG antibody from Sigma. Anti-p27 (Kip1) antibody was purchased from BD Transduction Laboratories (San Diego, CA). Anti-Cul1 and anti-Roc1 antibodies were obtained from Bethyl laboratories (Montgomery, TX). Laemmli sample buffer (non-reducing 2X, (Bio-Rad), containing 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue) was used for immunoprecipitation experiments. siRNAs for knocking down chick p27 were a pool of 3 target-specific nt 19-25 siRNAs, designed by and purchased from Santa Cruz Biotech (Cat# sc-270505). Mouse Cx50 cDNA construct was a generous gift from Dr. Bruce Nicholson at the University of Texas Health

Science Center (Chandrasekhar et al., 2013). All other chemicals were obtained either from Sigma or Fisher Scientific (Pittsburgh, PA).

Preparation of Recombinant Retroviral and Lentiviral Constructs

Recombinant avian retroviral constructs and high-titer retroviruses were prepared based on the previously published protocol (Jiang, 2001). Recombinant RCAS(A) DNA constructs containing Cx50, Cx50(V362E), Cx46 or Cx43 cDNA were generated as described (Shi et al., 2010; Jiang and Goodenough, 1998; Yin et al., 2000). For Skp2, we sub-cloned full-length Skp2 cDNA (GeneBank ID: AY947341.1) into RCAS(A) retroviral vector at EcoRI site with FLAG tag inserted at Skp2 C-terminus (sense primer: 5'-GAATCCATGCTCAGAAAACATCTCC-3'; anti-sense primer: 5'-GAATCCTTACTTGTCATCGTCGTCCTTGTAGTCGCCACTAAGGC TTCTC-3'). The Myc-ubiquitin was released from pCS2-myc-ubiquitin plasmid (a generous gift from Dr. P. Renee Yew at UTHSCSA) at BamH1 and XbaI sites, and then inserted into RCAS(A) vector. Then, truncated form of Skp2, Skp2 (ΔF), was generated by deleting aa 133-172, which is corresponding to aa 113-152 in human Skp2 sequence, with the primer pairs (sense primer: 5'-AGAAACACCTGGCTCTGCCT-3'; anti-sense primer: 5'-AGAAACACCTGGCTCTGCCT-3'). The recombinant RCAS(A) containing Skp2 RNAi is generated by using the method described previously (Harpavat and Cepko, 2006). Briefly, single-stranded oligonucleotides corresponding to the 5' and 3' ends of the hairpin were annealed together. The 5' fragment was constructed with an Apa I site at 5' end and a HindIII site at 3' end, whereas the 3' fragment was constructed with a HindIII site at 5' end and an EcoRI site at 3' end. Both fragments were cloned into a pBS/U6 vector cleaved with ApaI-EcoRI in a triple-ligation reaction. Correct construct would produce a ~465 bp insert detected using T3 and T7 primers by PCR. The resulting BS/U6-

hairpin fragment was generated by KpnI and EcoRI digestion, blunted, and cloned into the ClaI site (blunted) of G-RCAS(A) (Addgene plasmid #15182). Inserts in both the forward and reverse direction were identified by XbaI digestion. The correctness of inserted shRNA oligonucleotides in RCAS(G) vector was confirmed by DNA sequencing (UTHSCSA, DNA Core). Chick p27 was first cloned from cDNA, by using primer pairs (sense primer: 5'-AATGTCCATGGAGATGTCAAACGTCCGCA-3'; anti-sense primer: 5'-AATGTGAATTCTTACGTTTGATGTCGTCTCG-3'). HA tag was added into C-terminus of chick p27 sequence and the entire sequence of HA-p27 was cloned into RCAS(A) vector. p27 mutant (T187A) was generated with the QuikChange site-directed mutagenesis kit (La Jolla, CA) according to the manufacturer's instructions with the primer pairs (sense primer: 5'-CAGTTCAGTGGAGCAAGCCCCCAAGAAATCGAGC-3'; anti-sense primer: 5'-GCTCGATTTCTTGGGGGCTTGCTCCACTGAACTG-3').

For preparation of mouse Cx50 lentiviral construct, mouse Cx50 cDNA was cloned into a lentivirus transfer vector (pSDM-GFP), which was packed using pMD2.G (Addgene) and psPAX2 (Addgene) by co-transfection in HEK293T cells. Viral particles were then collected after 48 and 72 hours, and concentrated viruses were obtained after centrifugation at 20,000 x g for 2 hours. The viruses were used to infect MEF cells with polybrene (8 mg/ml) for 48 hours before biochemical analysis.

Preparation of Primary Lens Cell Culture

Primary chick lens cell cultures were prepared as described (BAnks et al., 2007). Lenses from 10-11 days old chick embryos were dissected, rinsed with TD buffer (140 mm NaCl, 5 mm KCl, 0.7 mm Na_2HPO_4, 5 mm glucose, and 25 mm Tris, pH 7.4), and digested with 0.1% trypsin at

37°C for 30 min. Lens cells were isolated by breaking the lenses apart and collecting them in M199 medium with 10% fetal bovine serum (FBS). Living cells were then counted and seeded at 3×10^5 cells per well of a 12-well culture plate. The following day after the primary culture was seeded, high-titter recombinant retroviruses containing wild-type and mutated forms of connexins and/or Skp2 or Skp2 shRNA were added to primary lens cultures. The cultures were incubated at 37°C, 5% CO_2 and fed every other day. At the start of culturing, only monolayer lens epithelial cells proliferated on the culture plates. After 3-4 days, lens epithelial cells reached confluency and differentiated to form fiber-like "lentoid" structures. The number of lentoids was counted every day during the culturing.

Immunofluorescence and Confocal Laser Microscopy

For immunolabeling of connexins and Skp2 expressed in CEF or primary chick lens cells, cells were cultured on a glass coverslip and fixed with 2% paraformaldehyde for 30 min and then incubated with blocking solution (2% goat serum, 2% fish skin gelatin, 0.25% Triton X-100 in Hanks' balanced salt solution) for another 30 min. Cells were labeled with anti-Cx50 polyclonal antibody from rabbit (1:500 dilutions) or anti-FLAG monoclonal antibody (1:2000) (M2 antibody, Sigma) followed by FITC or rhodamine-conjugated anti-rabbit or mouse IgG (1:400), respectively. The immunofluorescence-labeled cells were then mounted on slides using Vectashield. For immunolabeling assay in the mouse lens, postnatal day-1 mouse lenses were isolated and fixed with 2% paraformaldehyde for 30 min at room temperature, prior to being embedded in Tissue-Tek OTC compound (Miles Inc., Elkhart, IN). Frozen thin sections were prepared at 10-μm thickness and subjected to immunofluorescence labeling assay as described above. The lens sections were observed and analyzed under a confocal laser-scanning

microscope (Fluoview; Olympus Optical, Tokyo, Japan). Image acquisition parameters were maintained consistently for each sample.

Molecular Modeling and Computational Simulation of Cx50 and Skp2 Interaction

Three-dimensional models of Cx50-CT and Skp2-NT were generated based on the crystal structures of cyclin A (Bettayeb et al., 2007) (PDB code: 3BHT) and the Cul1-Rbx1-Skp1-F box (Skp2) SCF ubiquitin ligase complex (Zheng et al., 2002) (PDB code: 1LDK), respectively, using SWISS-MODEL (Schwede et al., 2003). The model of protein-protein interaction was constructed by the docking program MolDock (Thomsen and Christensen, 2006). First, the protein (Cx50) and the ligand (Skp2-NT) were automatically matched (charges and protonation states were assigned), and the automatic prediction of cavities was applied to the protein models to constrain predicted conformations during the matching process. The best predicted interaction interface (the lowest energy complexes) between Cx50-CT and Skp2-NT was searched by sampling positions, orientations, and torsion angles of the ligand as well as torsion angles of the protein side chains. Finally, the affinities, hydrogen bonds, and electrostatic energies were calculated and ranked to obtain the optimal solution.

Co-IP Assays and Protein Pull-down

The Co-IP and protein pull-down assays were based on our previous published protocols (Batra et al., 2012; Batra et al., 2014). For the co-IP assays, CEF cells infected with recombinant retroviruses were lysed with IP buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol) on ice for one hour and centrifuged for 45 min at 13000 *g*. The supernatant was incubated with first antibodies for 2 hours at 4°C and followed by incubating

with 20 μl protein A/G plus beads pre-washed twice with IP buffer for 16 hours at 4°C. The IP beads were then washed 3 times with IP buffer and eluted with 50 μl non-reducing Laemmli sample buffer before subjected to SDS-PAGE and western blotting.

For protein pull-down assay, CEF cells infected with Skp2-FLAG or Cx50-FLAG retroviruses were lysed with IP buffer and pre-cleared by centrifugation as described above. GST fusion proteins, Cx50-F1, Cx50-F2 and Cx50-Fs, were prepared as previously described (Yin et al., 2000; Yin et al., 2001). DNA construct of GST-fusion containing N-terminus of Skp2 (Skp2-NT) (aa: 1-120, or aa: 1-70) was prepared by PCR amplification using a chick Skp2 cDNA clone as a template (sense primer for both Skp2-NT-GST: 5'-GGGAATTCCATATGATGCTCAGAAAACATCTCCAGG-3'; anti-sense primer for Skp2 (1-120): 5'-ATCGGAATTCCCTCTTGCAAATCATGGAAAC-3'; anti-sense primer for Skp2 (1-70): 5'-ATCGGAATTCTTAGCCACTAAGGCTTCTCAGTG-3'). To prepare affinity matrix for protein pull down experiments, GST fusion proteins were incubated with GSH-sepharose beads (Sigma) and followed by washing three times with IP buffer. Lysates of cells expressing Skp2-FLAG or Cx50-FLAG were incubated with beads conjugated with GST-Cx50F1/F2/Fs or GST-Skp2, respectively, for 16 hours at 4°C and the beads were washed 3 times with IP buffer. The pull-down proteins were eluted with reduced Laemmli sample buffer before being analyzed by SDS-PAGE and western blotting.

RNA Extraction and Quantitative Reverse Transcription-PCR (qRT-PCR) Analyses.

Total RNA from cells was isolated by using Trizol (Invitrogen). Reverse transcription was performed with iScript cDNA Synthesis kit (Bio-Rad). Quantitative PCR was performed with SYBR Green PCR Master Mix (ABI system) on Applied Biosystems 7500 Fast Real-Time PCR

System. Sequences of primers used are listed as the following: β-actin (sense: 5'-AACACCCCAGCCATGTATGT-3', anti-sense: 5'-TGGGTAACACCATCACCAGA-3'), Skp2 (sense: 5'-AAGAGGTGGCATCGTCTGTC-3', anti-sense: 5'-CTGCAGGTAGCAACTGTCCA-3'), p27 (sense: 5'-ACTCCCTAAGGCGGAGGACT-3', anti-sense: 5'-CCATGGAGACCGACGATATG-3'). Data were normalized to β-actin mRNA content, by using efficiency ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001).

SDS-PAGE, Fluorography, and Western Blotting

Cultured cells were collected on ice with 0.5-1 ml of IP buffer and then lysed by sonication for 10 sec. Cell lysates were centrifuged for 45 min at 13,000 g to remove cellular debris. Total proteins in cell lysates were separated by 10-12% SDS-PAGE. Western blotting assay was performed by probing with antibodies, anti-AQP0 (1:1,000), anti-CP49 (1:1,000), anti-Cx50 (chick, 1:1,000), anti-Cx50 (mouse, 1:5000), anti-FLAG (monoclonal antibody, 1:2,000; polyclonal antibody, 1:7,500), anti-p27 (1:2,500), anti-ubiquitin (1:1000), anti-Skp2 (mouse, 1:1000), anti-Roc1 (1:2000), anti-Cul1 (1:1000), anti-GAPDH (1:5,000), and anti-β-actin (1:5,000). Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000) or anti-mouse IgG (1:10,000) using chemiluminescence reagent kit (ECL).

Supplementary References

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