Supplementary Information

p27kip1 controls H-Ras/MAPK activation and cell cycle entry *via* modulation of MT stability

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Inventory of Supplementary Information

Supplementary Information includes: Supplementary Materials and Methods; Supplementary References; Supplementary Figures S1-S11 with Legends; and Supplementary Tables S1 and S2.

Supplementary Materials and Methods

As a general rule, all experiments described in this work have been performed by at least two independent researchers.

Cell cultures and PCR on genomic DNA

Primary wild type (WT), p27 knock-out (p27KO), stathmin knock-out (STMKO) and p27/stathmin double KO (DKO) mouse embryo fibroblasts (MEF) were prepared from embryos at day 13.5, according to standard procedures. Primary MEFs (at least 5 different preparations/genotype) were frozen at passage 1 and used in subsequent experiments between passage 3 and 5, without significant differences. The correct genotype of WT, p27KO, STMKO and p27/stathmin DKO cells was determined by PCR, as described *(1, 2)*. 3T3 fibroblasts were obtained from primary MEFs following up the 3T3 immortalization protocol, as described *(1, 2)*. 3T3 fibroblasts and MEFs were cultured in DMEM supplemented with 10% FBS (Sigma). 293T/17 (ATCC) and 293FT (Invitrogen, Life Technologies) were used for the production of retroviral and lentiviral particles, respectively, following manufacturer's instructions, and cultured in DMEM supplemented with 10% FBS (Sigma). MDA-MB-468 breast cancer cell line, used to study EGF uptake, was cultured in DMEM supplemented with 10% FBS (Sigma).

Generation of stable cell clones

Stable cell clones of 3T3 fibroblasts were obtained by retroviral transduction (murine stem cell virus retroviral vectors, MSCV; Clontech) of particles encoding for human $p27^{WT}$, human $stathmin^{WT}$, $p27^{KR}$ mutant (K165, R166 in Alanine) *(3)* or $p27^{CK-}$ mutant (R30, L32, F62, F64 in Alanine) *(3, 4)*, both produced by site directed mutagenesis (Stratagene), following manufacturer instruction.

Clones and pools were selected in complete medium supplemented with Puromycin

1.5 µg/ml or Hygromycin 0.4 mg/ml. The stable expression of the different constructs was tested by Western Blot analysis of the target protein. Each experiment has been performed using at least two independent clones or pools.

Stathmin and Cyclin D1 silencing

shRNAs for mouse Cyclin D1 have been delivered by lentiviral transduction using MISSiON system (pLKO lentiviral vector, Sigma) in 3T3 p27KO fibroblasts. Briefly, 293FT cells were transfected with pLP1, pLP2, pLP/VSV-G (Invitrogen recombinant lentivirus producing system) plus pLKOshRNA (sh1_ TRCN0000026881 and sh2_TRCN0000026883) by calcium phosphate protocol. After 48 and 72 hours, conditional medium containing lentiviral particles was harvested and used to transduce target cells. The adenoviral system used to silence stathmin expression has been described elsewhere *(5)*. Briefly, the Knockout RNAi System (Clontech) was used following the manufacturer's procedures and inserting the appropriate sequence: control: 5'-GATCCGTCCCATGAAGCTGAGGTCTTCAAGAGATTTTTTACGCG TG -3'; Mouse Stathmin AB064953: 5'-AGCCATCGAGGAGAACAAC -3'. Recombinant replication defective viruses were generated and amplified in HEK 293 cells. Viral stock was determined by Adeno-X Rapid Titer Kit (Clontech).

Proliferation and apoptosis assays

For growth curves, 0.5-1x10^5 cells/well (depending on the experiment) were seeded in 6-well plates in complete medium in triplicate. Fresh medium was added every other day. At the indicated times, cells were detached in trypsin-EDTA and counted by Trypan Blue exclusion test.

For MTS assay, 1,000 cells/well were seeded in 96-well plates in sextuplicate. Cell Titer 96-Aqueous Cell Proliferation Assay kit (Promega) was used for the detection of viable cells following the manufacturer's procedures.

Cell cycle distribution was analyzed by flow cytometry, under several culture conditions. Depending on the experiments, cells were analyzed in exponential growth, after serum deprivation (24 hours) and release in complete medium (10% FBS, different time points). Where indicated, cells have been treated with 10 μM U0126 (selective inhibitor of MEK1/2, Calbiochem), 2 μM PD0332991 (selective inhibitor of CDK4/6 complexes, Selleck) or 25 μM Roscovitine (selective CDK inhibitor, Calbiochem). Cells were collected and fixed in ice-cold 70% ethanol and stored at -20°C. Cells were then washed in PBS and resuspended in propidium iodide staining solution (50 μg/ml propidium iodide + 100 μg/ml RNase A, in PBS). Stained cells were subjected to flow cytometry analysis (FACS) with a FACScan or a FACSCalibur instrument (BD Biosciences). Distribution of cells in G1, S and G2/M phases of the cell cycle was calculated using the WinMDI2.8 software.

For BrdU incorporation assay, MEFs were seeded on 12-well plates (BD, Falcon) containing coverslips (Menzel-Glaser, 12 mm) and incubated under standard growth conditions for 1 hour with 10 μM BrdU (Roche). Then, cells were fixed in 4% paraformaldehyde (PFA) in PBS at room temperature (RT) and permeabilized in HCl 1.5 N for 30 minutes at 37°C. Coverslips were washed 2 times in Borate Buffer 0.1 M pH 8.5 and 2 times in PBS. Incubation with primary antibody anti-BrdU (Roche) was performed 1 hour at 37°C, then samples were washed in PBS and incubated with secondary antibody (anti-mouse AlexaFluor® 488-conjugated, Invitrogen) for 1 hour at RT or 30 minutes at 37°C. Finally, nuclear staining with propidium iodide (3 μg/ml + RNase 100 μg/ml, in PBS) was performed for 30 minutes at RT and coverslips were mounted on glass slides with Mowiol 4-88 (Calbiochem) containing 2.5% (w/v) DABCO (Sigma).

Detection of apoptosis was performed by TUNEL assay, using In Situ Cell Death

Detection Kit, AP (Roche) on cells adhered to coverslips or on cryostatic sections, according to the manufacturer's instructions. Apoptosis was calculated as the ratio of positive cells over the total number/field.

Adhesion assay

For adhesion experiments, cells have been starved overnight (ON) in DMEM-0.1% BSA and then detached and seeded onto Fibronectin (10 μg/ml, Sigma) or Collagen I (20 μg/ml, Purecol) -coated dishes. At different time points, cells have been collected and processed for protein expression analysis, as described.

Preparation of protein lysates, Immunoprecipitation and Immunoblotting

Cellular protein lysates were collected under several culture conditions, such as exponential growth, high confluence growth, serum starvation and release in complete medium (10% FBS), adhesion on Fibronectin or Collagen I and at different temperatures (37°C, 18°C, 4°C). Where indicated, cells have been also treated with the following molecules for different time points: EGF (3 ng/ml, Invitrogen), IGF-1 (3 ng/ml, Invitrogen), Roscovitine (25 μM, Calbiochem), U0126 (10-50 μM, Calbiochem), Taxol (100 nM), Dynasore (25 μg/ml, Sigma), Nocodazole (10 nM, Sigma) and EHNA (1mM, Sigma). To extract proteins from cells, at indicated time points, cells were scraped on ice using cold NP40 lysis buffer (0.5% NP40; 50 mM HEPES pH 7; 250 mM NaCl; 5 mM EDTA; 0.5 mM EGTA, pH 8) supplemented with a protease inhibitor cocktail (Complete™, Roche), 1 mM Na_3VO_4 (Sigma), 10 mM NaF (Sigma) and 1 mM DTT (Sigma). To extract proteins from mouse organs, the same procedure was used, except that tissue disruption was performed using the TissueLyser II (QIAGEN).

Immunoprecipitation (IP) experiments were performed using 0.5-1mg of total lysate in HNTG buffer (20 mM HEPES, 150 mM NaCl, 10% Glycerol, 0.1% Triton X-100,

protease inhibitor cocktail, 1 mM Na_3VO_4, 10 mM NaF and 1 mM DTT) with the specific agarose-conjugated primary antibodies, gently rocking ON at 4°C. When primary antibodies were not agarose-conjugated, Protein A or Protein G Sepharose™ 4 Fast Flow (GE Healthcare), was added during the last 2 hours of incubation. IPs were then washed six times in HNTG buffer and resuspended in 3X Laemmli sample buffer (5X Laemmli buffer composition: 50 mM TrisHCl pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue and 125 mM beta-mercaptoethanol). For immunoblot analysis, proteins were separated in 4-20% SDS-PAGE (Criterion Precast Gel, Biorad) and transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked with 5% not fat dried milk (NFDM) in TBS-0.1% Tween20 or in Odyssey Blocking Buffer (LI-COR, Biosciences) and incubated at 4°C ON with primary antibodies.

Primary antibodies were purchase from *BD:* p27kip1 (610242), CDK2 (610146), stathmin/metablastin (611146); *Santa Cruz*: cyclin B1 (sc-245), cyclin A (sc-751), cyclin E (sc481), cyclin D3 (sc-182), vinculin (sc-7649), ERK1 (sc-94), CDK4 (sc-260), AKT (sc-1618), p27kip1 (sc-527, sc-528), H-Ras (sc-520), *Cell Signaling*: pERK1/2 T202/204 (9101), pAKT S473 (9271), Rab5 (3547), Myc-Tag (2276), pS6 S235-236 (4858), pp70S6K1 T389 (9234), pp38 T180/182 (9211); *Sigma*: actin (A5060), OP18/stathmin (O0138), FLAG (A2220), HA (A2095), pS25 stathmin (SAB4300180); *Millipore*: CyclinD1 (04-1151); *Roche*: GFP (11814460001) and *Covance*: HA-Tag (MMS-101P). Membranes were washed in TBS-0.1% Tween20 and incubated 1 hour at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE, Healthcare) for chemi-luminescent detection (ECL, GE Healthcare) or with IR-conjugated (AlexaFluor® 680, Invitrogen or IRDye 800, Rockland) secondary antibodies for infrared detection (Odyssey Infrared Detection System, LI-

COR).

Band quantification was performed using the Quantity One application software or the Odyssey v1.2 software (LI-COR).

The Re-Blot Plus Strong Stripping Solution (Millipore) was used to strip the membranes, when reblotting was needed.

Kinase assay

Cell lysates were immunoprecipitated using anti-CDK2, -CDK4, -cyclin A, -cyclin B1, -cyclin E or control antibodies, as described above. After 5 washes in HNTG buffer, one tenth of the IP was resuspended in kinase buffer (20 mM TrisHCl pH 6.8, 10 mM $MgCl_2$), as previously described *(2, 6, 7)*. Where indicated, the IPs or the recombinant cyclin/CDK active complexes (SignalChem) were incubated 3 hours at 4°C with recombinant stathmin (purification of His-stathmin was performed after bacterial growth, using sonication and affinity in batch purification with resin NiNTA, Qiagen).

Then, a kinase reaction solution containing the sample plus 50 μM ATP, γ-P32 ATP and 2 μg of H1- Histone or recombinant retinoblastoma protein (RB) as substrate in buffered solution (20 mM TrisHCl pH 6.8, 10 mM $MgCl_2$) was prepared. The reaction was carried out at 30°C for 30 min and then 2X Laemmli sample buffer was added. After denaturation at 95°C for 10 minutes, proteins were loaded on a 4-20% SDS-PAGE (Criterion Precast Gel, Biorad). The gel was then dried and exposed on an autoradiographic film (GE, Amersham-Hyperfilm MP) at -80°C and developed after different time intervals. Band quantification was performed using the Quantity One application software.

Ras Pull-Down

Brains from 8-weeks-old mice C57BL/6 and MEFs of different genotypes were lysed

and at least 3 mg of protein were tested in pull-down assay, using glutathione S-transferase GST-Raf (Addgene plasmid 13338) *(8)*, bound to glutathione-Sepharose high performance (GE Healthcare) for 1 h at 4°C. After thorough washes, the samples were boiled for 10 minutes in Laemmli buffer to detach active GTP-bound Ras, then loaded on 15% SDS-PAGE gels (Criterion; Biorad) and immunoblotted using an anti-H-Ras antibody.

Gene expression profiling and data mining tools

RNA from MEF WT, p27KO and DKO grown at high confluence was extracted using RNeasy-Mini Kit (QIAGEN). Gene expression profiling (GEP) was performed by a dual-labeling strategy using Cy3-labeled amino-allyl RNA (aRNA) and Cy5-labeled aRNA as tester, hybridized to the Whole Mouse Genome (4 × 44 K) oligo microarray platform (Agilent Technologies); slides were analyzed by Agilent Microarray Scanner using the Agilent Feature Extraction Software 10.7.3 (Agilent Technologies). Pre-processing steps have involved quality check, exponential-normal convolution background subtraction, lowess and quantile normalization. After pre-processing, prefiltering was applied aimed at eliminating all genes whose interquartile range was below the 20th percentile of distribution (genes whose expression was overall not differentially modulated).

After pre-processing and pre-filtering steps, the final dataset was subjected to supervised analyses using a multi-SAM ("significance analysis of microarrays") method *(9)*. GEP results were visualized by hierarchical clustering applying Ward's method with Euclidean distance *(10)*.

The microarray data will be available in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=phgffgomyaoewvk&acc=GSE31533).

Quantitative Real-Time PCR

RNA was collected from cells in exponential growth, at high confluence, following starvation in serum free medium and release in complete medium (10% FBS). Where indicated, cells have been also treated with Roscovitine (25 μM), U0126 (10 μM), Taxol (100 nM) or Dynasore (25 μg/ml).

RNA was extracted using RNeasy-Mini Kit (QIAGEN), according to the manufacturer's instructions. RNA from tissue samples was extracted using TRIzol (Invitrogen, Life Technologies). Disruption of the tissue sample was achieved by grinding the frozen tissue thoroughly, with lead blocks. In some cases, the TissueLyser II (QIAGEN) was used. Complete homogenization was achieved by passing the lysate at least 5 times through a 23-gauge needle fitted to an RNase-free syringe. RNA was then quantified and retro-transcribed with AMV Reverse transcriptase (according to provider's instruction, Promega) to obtain cDNAs. Absolute expression of mouse cyclin D1, Cdkn1b (p27), stathmin, VEGFb, Mapre2, Hmga1, Sdpr, Rad23, MAPK7, Egr-1, Jun-B, c-Fos, was evaluated by qRT-PCR using SYBR Green dye-containing reaction buffer (Power SYBR® Green PCR Master Mix 2X, Applied Biosystems).

The following primers (Sigma) were used:

JUN-b FW: 5'-GCACTAAAATGGAACAGCCCTT-3'

JUN-b RW: 5'-GGCTCGGTTTCAGGAGTTTG-3'

EGR-1 FW: 5'-CCTTCCAGTGTCCAATCTGCA-3'

EGR-1 RW: 5'-CTGGCAAACTTCCTCCCACA-3'

c-FOS FW: 5'-TGGTGAAGACCGTGTCAGGA-3'

c-FOS RW: 5'-GCAGCCATCTTATTCCGTTCC-3'

cyclin D1 FW: 5'-TGGCCT CTAAGATGAAGGAGA-3'

cyclin D1 RW 5’-AGGAAGTGTTCGATGAAATCGT-3’

MAPK7 FW: 5’-CATAGGCAATGGGGCCTAC-3’

MAPK7 RW 5’-TCTTCTTGATGGCCACCTG-3’

Mapre2 FW: 5’-AGTCGCACCATGCAAACTC-3’

Mapre2 RW: 5’-GGATCCTGGCTTAGATGCAG-3’

Sdpr FW: 5’-CTTCAAAGTGCTCATCTTCCAG-3’

Sdpr RW: 5’-CCTCCAGGGACTTGTTCTCA-3’

Rad23 FW: 5’-GAGAGCCAGCTTCAACAACC-3’

Rad23 RW: 5’-GGTCAACCACAGCCTGACTT-3’

STM1 FW: 5’-GTTCGACATGGCATCTTCTGAT-3’

STM1 RW 5’-CTCAAAAGCCTGGCCTGAA-3’

CDKN1B/p27 FW: 5’-TCTCTTCGGCCCGGTCAAT-3’

CDKN1B/p27 RW: 5’-GGGGCTTATGATTCTGAAAGTCG-3’

GAPDH FW: 5’-TGAGGACCAGGTTGTCTCCT-3’

GAPDH RW 5’-CCCTGTTGCTGTAGCCGTAT-3’

18s FW: 5’-GGACCAGAGGCAAAGCATTTGC-3’

18s RW: 5’-TCAATCTCGGGTGGCTGAACGC-3’

βactin FW: 5’-AGGTGACAGCATTGCTTCTG-3’

βactin RW: 5’-GCTGCCTCAACACCTCAAC-3’

PGK1 FW: 5’-TACCTGCTGGCTGGATGG-3’

PGK1 RW: 5’-CACAGCCTCGGCATATTTCT-3’

HPRT FW: 5’-TCCTCCTCAGACCGCTTTT-3’

HPRT RW 5’-CCTGGTTCATCATCGCTAATC-3’

GUSB FW 5’- CAGTTGTTGTCACCTTCACCTC-3’

GUSB RW 5’- CTCTGGTGGCCTTACCTGAT-3’

VEGFb FW: 5'-GCCAGACAGGGTTGCCATAC-3'

VEGFb RW: 5'-GGAGTGGGATGGATGATGTCAG-3'

HMGA1 FW: 5'-GGTCGGGAGTCAGAAAGAGC-3'

HMGA1 RW: 5'-ATTCTTGCTTCCCTTTGGTCG-3'

Standard curves (10-fold dilution from 10^{1} to 10^{-4} attomoles) were prepared both for target genes and housekeeping genes. The incorporation of the SYBR Green dye into the PCR products was monitored in real time using the MyiQ2 Two Color Real-time PCR Detection System (Biorad) and the resulting threshold cycles (Ct) were computed. Ct values were converted into attomoles and the normalized target gene value was obtained by using at least two different housekeeping genes.

***In vivo* wound healing assay**

C57BL/6 p27 knock-out (p27KO) mice (The Jackson Laboratory) were grown in our animal facility at 22°C with 40-60% of humidity. Full thickness wounds were produced on both sides of the dorsal skin of 8 p27KO female mice, 9 to 17-weeks-old. Mice were anesthetized, shaved, disinfected with 70% ethanol and wounds were created using sterile biopsy punches (5mm diameter, Acuderm Inc.). Mice were treated with Taxol (5mg/kg) or vehicle (PBS) the day before, the day of the wound and the day after the wound and then sacrificed 24 hours later. Samples were collected by cutting the area around the wound, fixed in formalin and finally embedded in paraffin for immunofluorescence analyses, as described below.

Immunofluorescence analysis

For immunofluorescence (IF) on mouse skin, specimens were fixed ON in formalin and then embedded in paraffin. Specimens were sectioned with a microtome, rehydrated, immersed into 10 mM citrate buffer pH 6.0 and antigens retrieved by boiling in the microwave (550 W, 20 minutes). Samples were slowly cooled down to

RT and specimens permeabilized with 0.4% Triton X-100 (Sigma-Aldrich) in PBS for 10 minutes. Samples were then blocked with 10% normal goat serum in PBS with 0.1% Triton X-100 for 20 minutes, incubated ON at RT with anti-Ki67 polyclonal antibody (Abcam, Cambridge, UK). Incubation with anti-acetylated tubulin (mouse monoclonal, Sigma) 2hrs at RT was followed by incubation with anti-rabbit AlexaFluor® 488-conjugated (Invitrogen), anti-mouse AlexaFluor® 633-conjugated (Invitrogen) and propidium iodide 3 μg/ml + RNase A 100 μg/ml for 1 hour.

For immunofluorescence staining, cells seeded on coverslips were processed as described previously *(6, 11, 12)*. The following primary antibodies were used: pERK1/2 T202/204 (Cell Signaling), p27kip1 (BD), OP18/stathmin (Sigma), RAB5 (Cell Signaling) and anti-acetylated tubulin (Sigma). Secondary antibodies (AlexaFluor® 488-, 546- or 633-conjugated anti-mouse or anti-rabbit, Life Technologies) were incubated for 1 h at RT. Antibody incubation was followed by actin staining with phalloidin- AlexaFluor® 568 or 647-conjugated for 1hr at RT.

To analyze H-Ras localization, immortalized 3T3 fibroblasts of the three genotypes were seeded on coverslips, then transfected with GFP-H-Ras *(13)* using FuGENE HD (Promega) according to the manufacturer's protocol. 24 hours after transfection, cells were starved using DMEM 0.1% BSA for 18 hours and cycloheximide (30 μg/ml) was added 4 hours before stimulating cells with DMEM 10% FBS for the indicated time. Staining with the primary antibody (Rab5) was performed as described above. In some experiment, WGA (Wheat germ agglutinin AlexaFluor® 594 conjugated, Invitrogen) has been used, according to the manufacturer's instructions, as plasma membrane marker.

To analyze the endocytosis, immortalized 3T3 fibroblasts WT and p27KO, were seeded on coverslips, then transfected with pEGFP-Dynamin 1aa (Addgene plasmid

#22163) or pEGFPN1-human Dynamin 1aa K44A, (Addgene plasmid # 22197) *(14)*, using FuGENE HD (Promega). 48h after transfection, cells were serum starved using DMEM 0.1% BSA for 6h, then released in 10% serum for 15 minutes. Where indicated, immortalized 3T3 fibroblasts WT and p27KO, were seeded on coverslips and pre-treated with EHNA 1mM for 2h (selective dynein inhibitor, Sigma), then released in 10% serum for 5 and 15 minutes, as indicated. Immunofluorescence staining to evaluate pERK1/2 T202/204 was performed as described above. Protein lysates were also collected to perform Western Blot analysis.

Fluorescence intensity of selected regions of cell was measured with computer-assisted imaging software (LAS, Leica). Alternatively, fluorescence intensity and protein colocalization were studied using the Volocity® software (PerkinElmer).

EGF and Transferrin uptake assay

MDA-MB-468 breast cancer cells were seeded on coverslips, then transfected with EGFP-$p27^{WT}$ and EGFP-$p27^{1-170}$, using FuGENE HD (Promega). 48h after transfection, cells were serum starved using DMEM 0.1% BSA for 4h, then put on ice and labeled EGF (Tetramethylrhodamine-conjugate, Life Technologies) was added to the medium at a concentration of 0.5μg/ml. After 1h on ice, cells were washed to remove unbound EGF and incubated at 37°C for 10 and 30 minutes. Immunofluorescence analysis (phalloidin staining) was performed as described above. To evaluate Transferrin uptake, immortalized 3T3 fibroblasts, WT or p27KO, were seeded on coverslips and serum starved for 4h. Dishes were put on ice and labeled Transferrin (AlexaFluor® 488-conjugate, Life Technologies) was added to the medium at a concentration of 0.1mg/ml. After 1h on ice, cells were washed to remove unbound Transferrin and incubated at 37°C for 10 minutes. Immunofluorescence analysis (phalloidin staining) was performed as described above. Intracellular

fluorescence intensity of EGF and Transferrin was studied using the Volocity® software (PerkinElmer).

Ubiquitination Assay

In vivo ubiquitination assays were performed after transfection with HA-Ubiquitin *(7)*, FLAG-H-Ras, Myc-p27^{WT}, Myc-p27$^{1-170}$, EGFP-stathmin^{WT}, as indicated. 293T/17 cells were transfected by calcium phosphate protocol or using FuGENE HD (Promega), according to manufacturer's instructions. Alternatively, *in vivo* ubiquitination assays was performed on MEFs of the three different genotype, transfected with HA-Ubiquitin *(7)* using FuGENE HD (Promega) following the manufacturer's protocol. Cells were washed two times with ice-cold PBS then scraped in RIPA-lysis buffer (150 mM NaCl, 50mM Tris-HCl pH8, 1% NP-40, 0.5% SDC, 0.1% SDS supplemented with protease inhibitors). The lysates were cleared by centrifugation at 13200rpm for 15 min. Immunoprecipitation was performed as described, using HA-agarose (Sigma) or FLAG-agarose (Sigma) conjugated antibodies. Finally, the immunoprecipitated proteins were separated in 12.5% SDS-polyacrylamide gels.

Statistical analysis

Data were examined using the two-tailed Student t test or unpaired two-tailed Mann-Whitney U test. Differences were considered significant at $p < 0.05$. All graph and statistical analyses were performed using GraphPad PRISM version 4.00.

Supplementary References

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Supplementary Figures and Legends

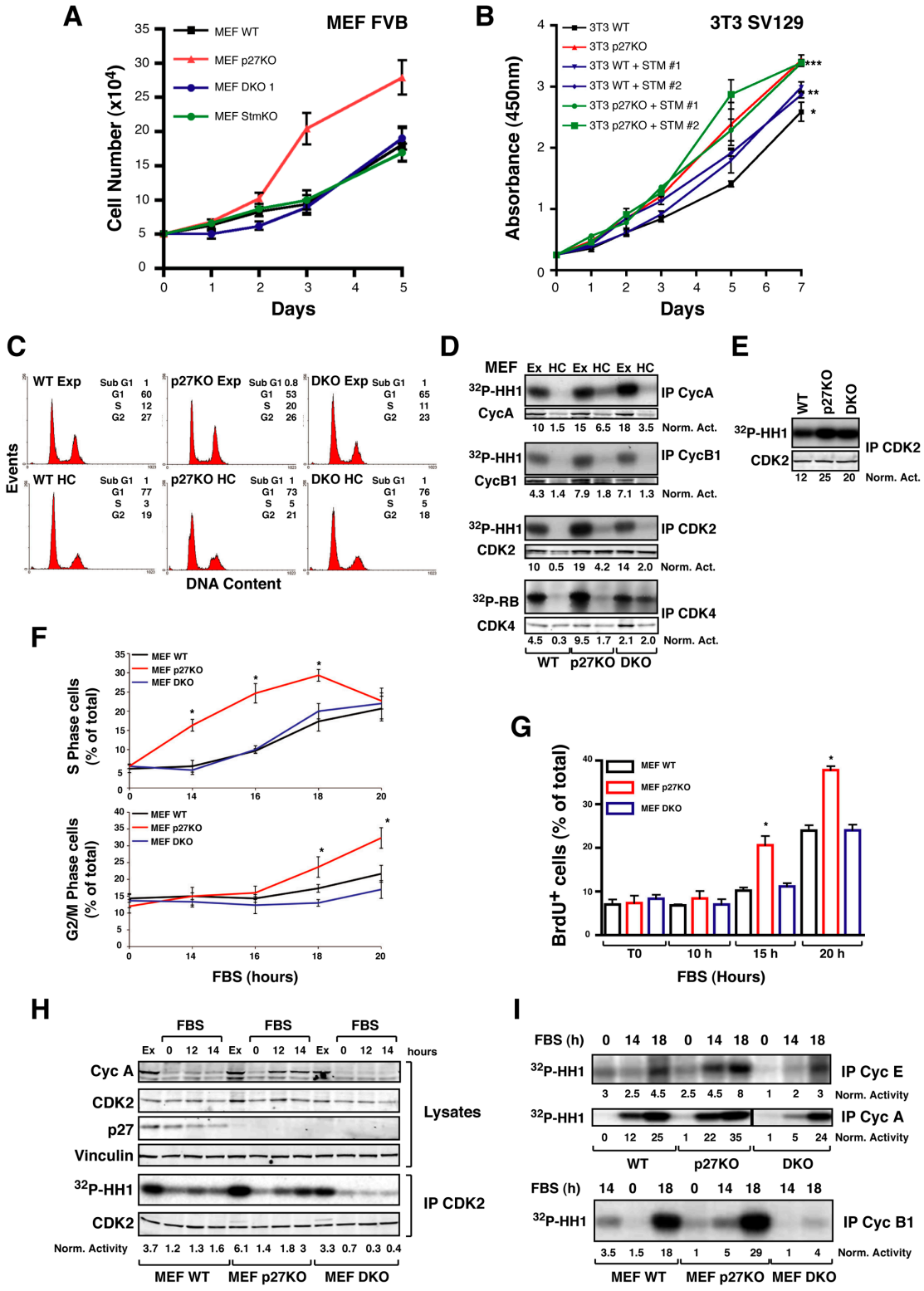

Fig. S1. The faster cell cycle entry of p27 null MEFs relies on stathmin expression

(A) Growth curves of FVB MEF WT, p27KO, DKO and StmKO. Cells, plated on day 0 and counted at indicated time points, using MTS assay.

(B) Growth curves of Sv129 3T3 WT and p27KO fibroblasts, expressing or not exogenous stathmin. Cells, plated on day 0, were analyzed at indicated time points, using MTS assay. Data represent the mean (±SD) of four experiments. * indicates p=0.002 in WT *vs* KO MEFs; ** indicates p≤ 0.01, in WT *vs* WT + STM; *** indicates p≤ 0.001 in KO *vs* WT + STM.

(C) FACS analysis of cell cycle distribution of primary MEF of the indicated genotypes, in exponential growth (Exp) or grown at high confluence (HC).

(D) Kinase activity on histone H1 (^{32}P-HH1) associated with the indicated immunoprecipitated (IP) proteins, in MEF populations exponentially growing (Ex) or grown at high confluence (HC). In the bottom panels, the same lysates were immunoblotted for the indicated proteins. Numbers at the bottom of panels indicate the normalized kinase activity for each sample (^{32}P-HH1 value/immunoprecipitated protein value).

(E) Kinase activity on histone H1 (^{32}P-HH1) associated with immunoprecipitated CDK2 (upper panel), in exponentially growing MEF populations of the indicated genotypes, different from those in (B). In the bottom panel, the same lysates were immunoprecipitated and immunoblotted for CDK2. Numbers at the bottom of panels indicate the normalized CDK2 activity for each sample (^{32}P-HH1 value/immunoprecipitated CDK2 value).

(F) Percentage of S (upper graph) and G2/M (lower graph) phases in MEFs of the indicated genotypes, measured by FACS analyses. Cells were serum starved and then stimulated with serum (FBS) for the indicated times.

(G) BrdU incorporation in MEF of the indicated genotypes, serum starved and then stimulated with FBS for the indicated times.

(H) Western Blot analysis of cyclin A, CDK2 and p27 and kinase assay of CDK2-associated kinase activity (^{32}P-HH1), in MEFs of the indicated genotypes, harvested in exponential growth (Ex) or serum starved (0) and then stimulated with FBS for the indicated times. Vinculin expression was used as loading control. Numbers at the bottom of panels indicate the normalized CDK2 activity for each sample (^{32}P-HH1 value/immunoprecipitated CDK2 value).

(I) Kinase activity using histone H1 as substrate (^{32}P-HH1) associated with the indicated immunoprecipitated (IP) cyclins, in MEF WT, p27KO, DKO from C57BL/6

mice, serum starved and then released with FBS for 14 or 18 hours (h), as indicated. Numbers at the bottom of panels indicate the normalized kinase activity for each sample (^{32}P-HH1 value/immunoprecipitated protein value).

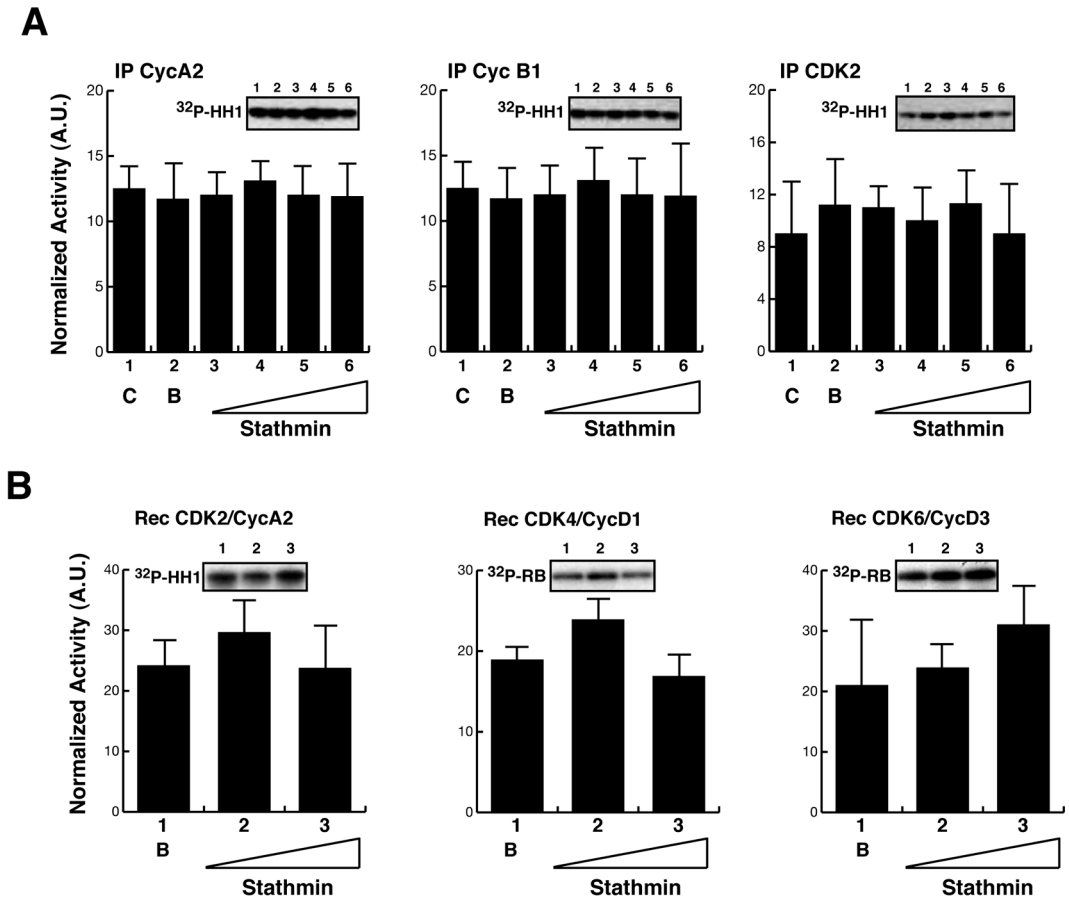

Fig. S2. p27 regulation of CDK2 activity is independent from stathmin expression.

(A) Kinase activity associated with the indicated immunoprecipitated (IP) proteins in lysates from exponentially growing SCC9 head and neck carcinoma cells, using histone H1 as substrate (^{32}P-HH1). Lane 1 is the control (C), lane 2 is the control with imidazole (B), lanes from 3 to 6 are lysates incubated with increasing doses of recombinant stathmin (0.25-0.5-1-2 μg), prior to being subjected to immunoprecipitation. Values in the graphs represent the mean of three independent experiments +/- SD. Differences in kinase activity in the presence of stathmin are not statistically significant.

(B) Kinase activity associated with the indicated recombinant Cyclins/CDKs complexes, using GST-RB as substrate (^{32}P-RB) in the presence of increasing doses of recombinant. Values in the graphs represent the mean of three independent experiments +/- SD. Differences in kinase activity in the presence of stathmin are not statistically significant.

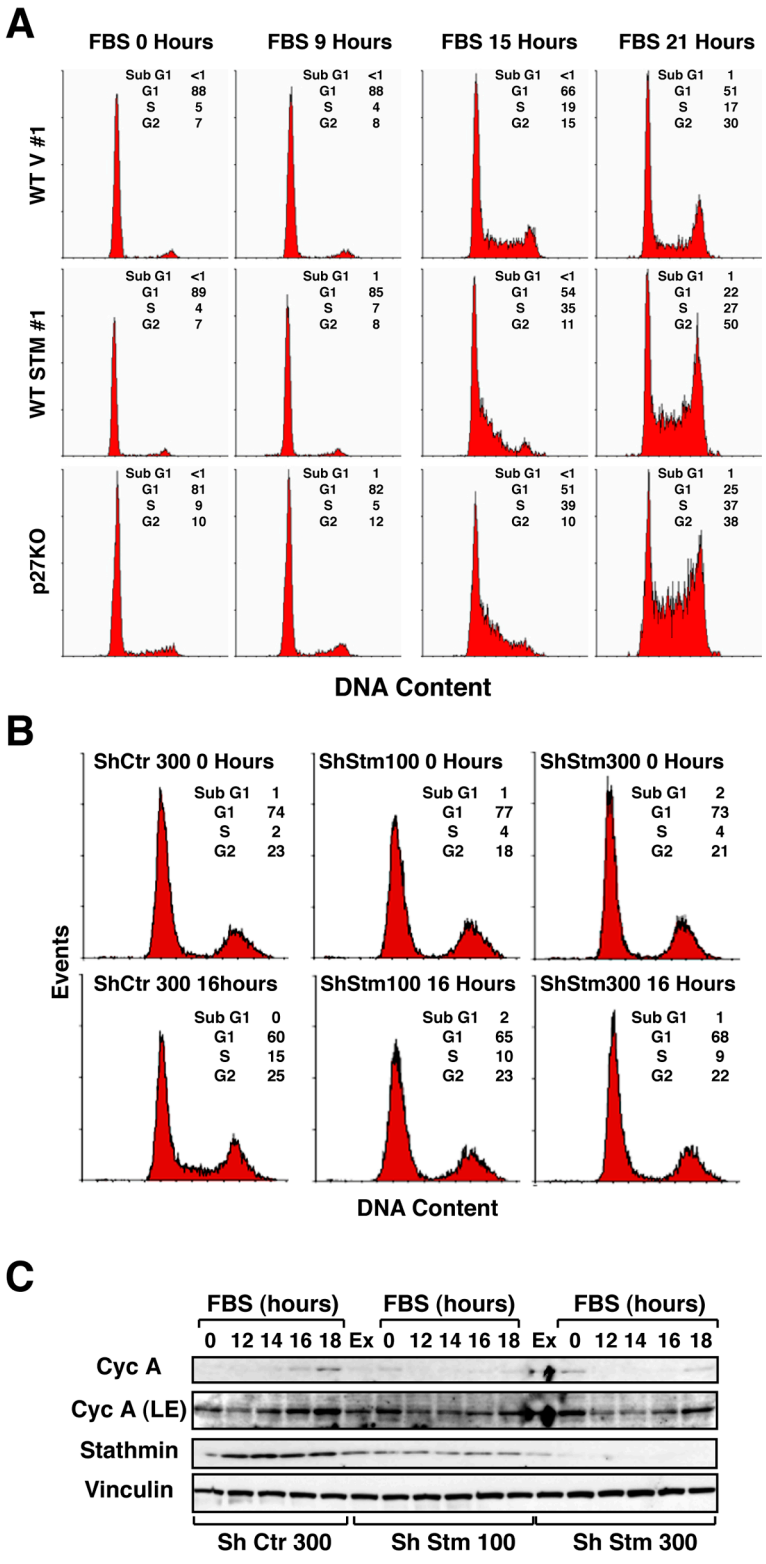

Fig. S3. p27/stathmin relative expression levels influence cell proliferation and cell cycle entry.

(A) FACS analyses of DNA content in 3T3 fibroblasts WT, p27KO and WT STM, serum starved (FBS 0 Hours) and then stimulated with serum (10% FBS) for the indicated times. The distribution in the different phases of the cell cycle is reported in each graph and represents the mean of 2 different experiments.
(B) FACS analyses of DNA content in p27KO fibroblasts transduced with control shRNA adenovirus (shCtr at multiplicity of infection, moi 300) or with anti stathmin shRNA, at two different moi (100 and 300). Cells were serum starved (FBS 0 Hours) and then stimulated with serum (10% FBS) for 16 hours. The distribution in the different phases of the cell cycle is reported in each graph.
(C) Western Blot analysis of cyclin A and stathmin in protein lysates from p27KO fibroblasts described in B, serum starved (0) and then stimulated with 10% FBS for the indicated times. Vinculin was used as loading control. LE indicates Long Exposure of the blot.

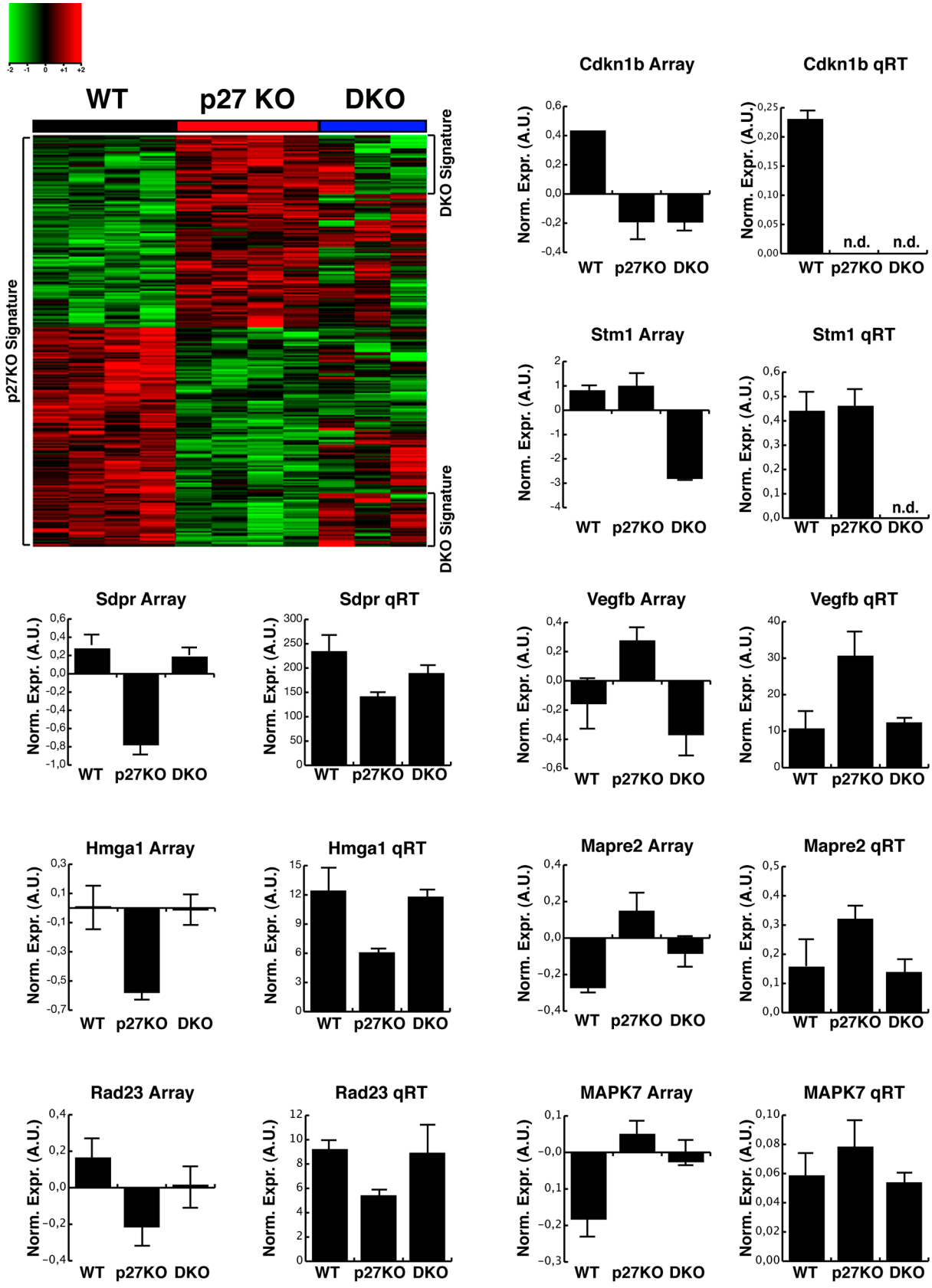
-2 -1 0 +1 +2
WT
p27 KO
DKO
p27KO Signature
DKO Signature
DKO Signature
Cdkn1b Array
Cdkn1b qRT
Norm. Expr. (A.U.)
WT p27KO DKO
n.d.
n.d.
Stm1 Array
Stm1 qRT
n.d.
Sdpr Array
Sdpr qRT
Vegfb Array
Vegfb qRT
Hmga1 Array
Hmga1 qRT
Mapre2 Array
Mapre2 qRT
Rad23 Array
Rad23 qRT
MAPK7 Array
MAPK7 qRT

Fig. S4. Gene expression profiles of WT, p27KO and DKO mouse embryo fibroblasts (MEF) identifies a DKO signature.

Gene microarray on RNA extracted from C57BL/6 MEF of the indicated genotypes. Four different WT and KO embryos/genotype were probed on the 44K microarray slides (Agilent), to identify genes differentially expressed in WT versus p27KO MEF (p27KO signature). The p27KO signature was then compared to the gene expression profile of DKO MEF (3 different preparations). This comparison identifies the DKO signature, representing the set of genes (n=55) differentially regulated between WT and p27KO cells and reverted in DKO cells.

Graphs report normalized expression of some of the genes identified in the array, evaluated by qRT-PCR, as indicated.

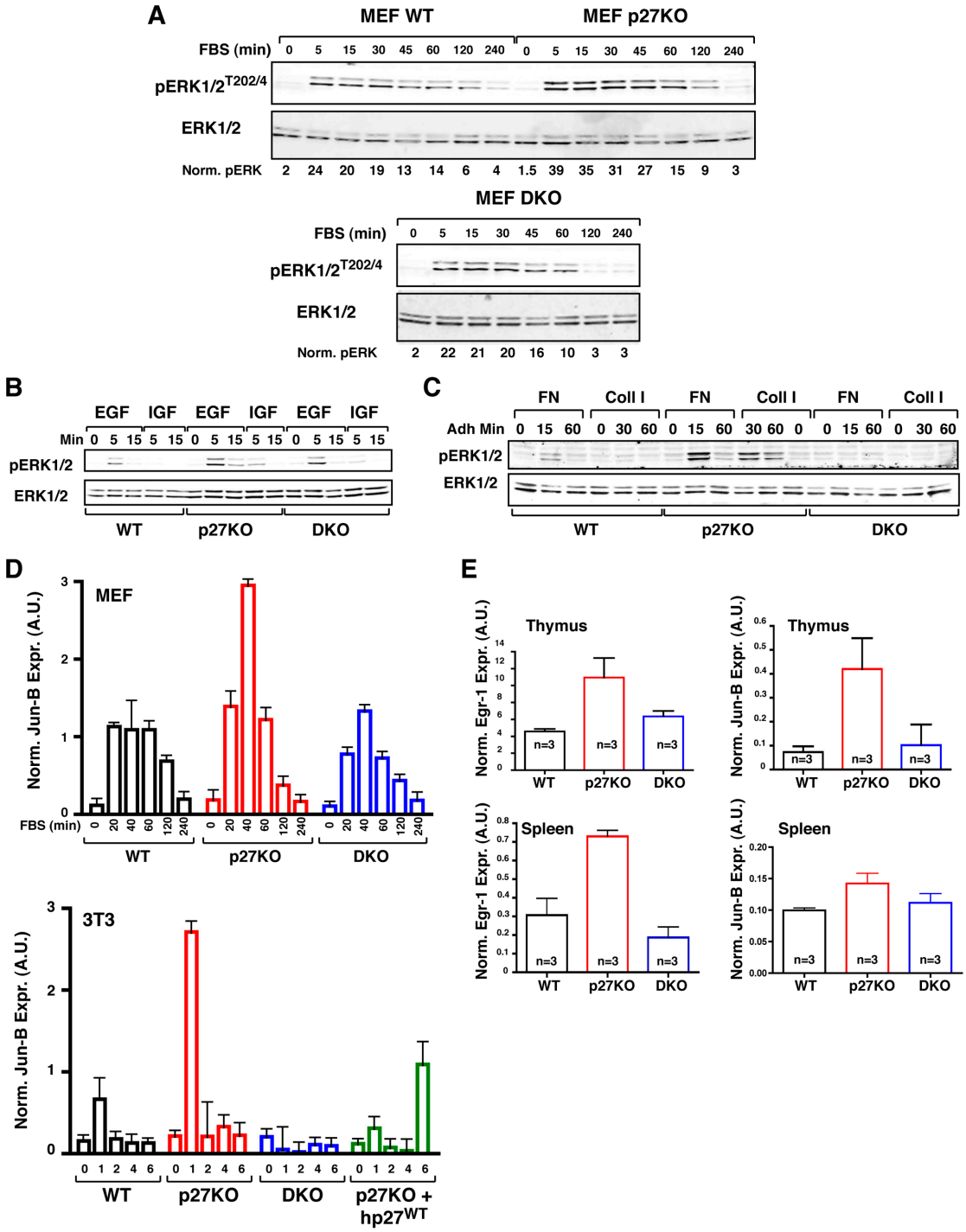

Fig. S5. p27 controls activation of the MAPK pathway.

(A) Time course analysis of ERK1/2 phosphorylation in MEFs of the indicated genotype, serum starved and then stimulated with 10% serum (FBS) for the indicated times. Numbers at the bottom of panels indicate the quantification of normalized ERK1/2 phosphorylation levels.

(B) Western Blot analysis of phospho-ERK1/2 in WT, p27KO and DKO MEFs, stimulated with EGF (3 ng/ml) or IGF (3 ng/ml) for the indicated times following serum deprivation.
(C) Western Blot analysis of phospho-ERK1/2 and ERK1/2in WT, p27KO and DKO MEFs, adhered to Fibronectin (FN) or Collagen I (Coll I) for the indicated times.
(D) qRT-PCR analysis of Jun-B on MEF (upper graph) and 3T3 (lower graph) fibroblasts of the indicated genotypes, serum starved and stimulated with 10% serum (FBS) for the indicated times. Values were normalized using Hprt and Pgk housekeeping genes and expressed as arbitrary units (A.U.).
(E) qRT-PCR analysis of Egr-1 and Jun-B on thymuses and spleens from WT, p27KO and DKO mice, as indicated. Values were normalized using Hprt and Pgk housekeeping genes and expressed as arbitrary units (A.U.).

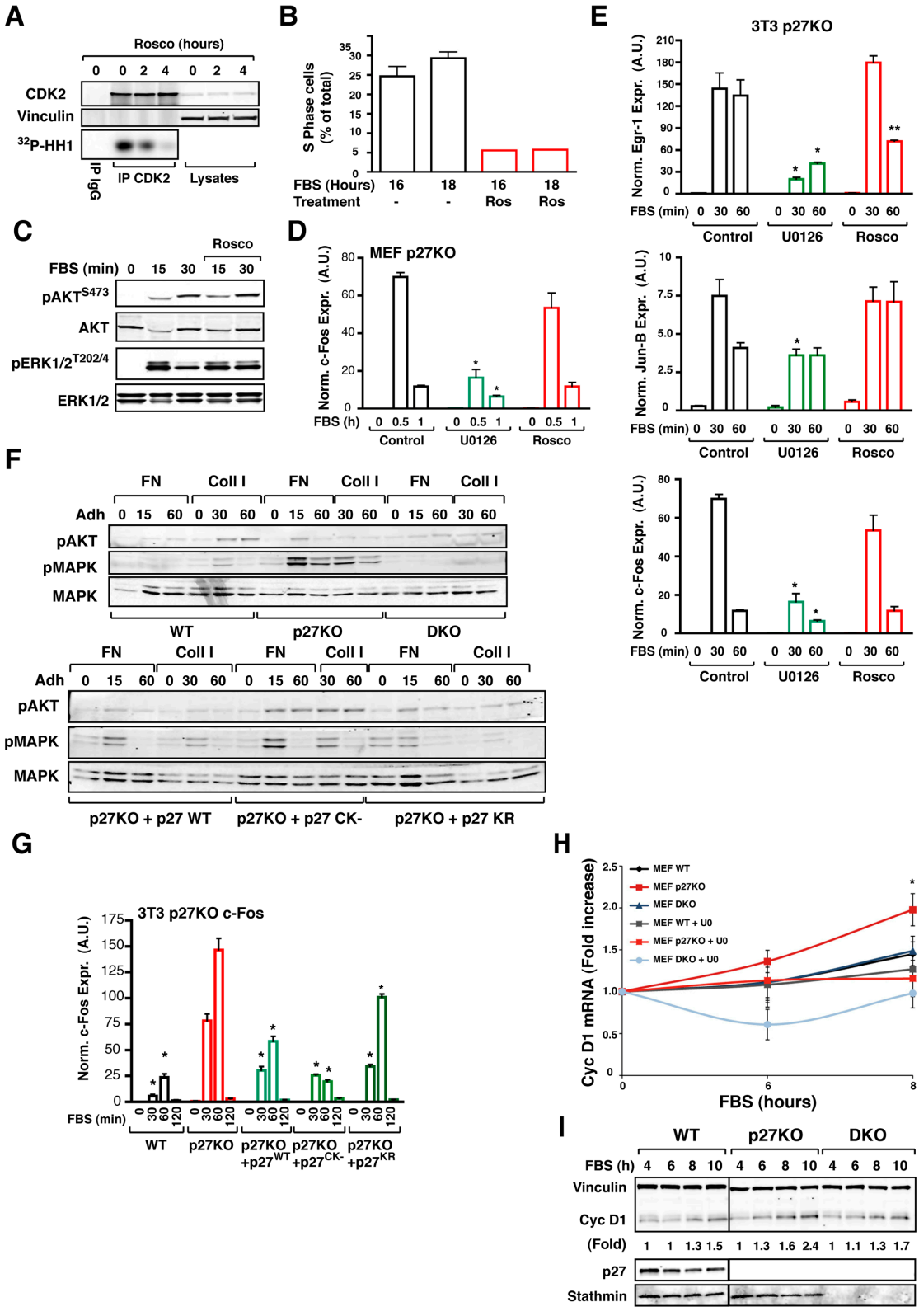

Fig. S6. p27 controls activation of MAPK pathway in a CDK-independent manner. (A) Western Blot analysis and kinase assay (^{32}P-HH1) of immunoprecipitated CDK2 in 3T3 p27KO fibroblasts treated or not (0) for the indicated times, in the presence of

Roscovitin. Vinculin expression was used as loading control. Non-specific IgG were used as control of immunoprecipitation experiments.

(B) FACS analysis of 3T3 p27KO fibroblasts serum starved and then stimulated with 10% serum (FBS) for the indicated times (16 and 18 hours), with or without Roscovitin, as indicated.

(C) Western Blot analysis of ERK1/2 and AKT phosphorylation status in p27KO MEF, serum starved and then stimulated with 10% serum (FBS) in presence or not of the CDK inhibitor Roscovitine, as indicated.

(D) qRT-PCR analysis of c-Fos in RNA extracted from MEF p27KO serum starved and then stimulated with 10% serum (FBS), for the indicated time, in presence or not of the MEK1 inhibitor U0126 or the pan-CDK inhibitor Roscovitine, as indicated. *$p \leq 0.007$

(E) qRT-PCR analysis of Egr-1 (upper panel), Jun-B (middle panel) and c-Fos (bottom panel) in RNA extracted from p27KO 3T3 fibroblasts serum starved and then stimulated with 10% FBS for the indicated times, in the presence of U0126 or Roscovitine, as indicated. Values were normalized using the expression of the Hprt and Pgk housekeeping genes and expressed as arbitrary units (A.U.). * $p \leq 0.02$; **$p=0.004$

(F) Western Blot analysis of phospho-AKT, phospho-MAPK and MAPK in cells serum starved and then adhered to Fibronectin (FN) or Collagen I (Coll I), for the indicated time. Experiment was carried out on 3T3 fibroblasts of the indicated genotypes (upper panels) and in 3T3 p27KO cells stably expressing $p27^{WT}$ or $p27^{CK-}$ (unable to bind cyclins and CDKs) or $p27^{KR}$ (mostly located into the cytoplasm) (lower panels), as indicated.

(G) qRT-PCR analysis of c-Fos in 3T3 fibroblasts of the indicated genotypes and in 3T3 p27KO cells stably expressing $p27^{WT}$, $p27^{CK-}$ or $p27^{KR}$. Cells were serum starved and then stimulated with 10% serum (FBS), for the indicated time. *$p \leq 0.04$

(H) qRT-PCR analysis of cyclin D1, in MEF of the indicated genotypes, serum starved and then stimulated with FBS for the indicated times. Where indicated, cells were also treated with U0126 (10 μM). Graph represents the fold induction over the value of cyclin D1 in serum starved cells. Values of gene expression were normalized using Hprt and Pgk housekeeping genes and expressed as fold-increase over the time zero value.

(I) Western Blot analysis of cyclin D1 expression in MEF of the indicated genotype, serum starved and then stimulated with FBS for 4-10 hours. Expression of p27, stathmin and vinculin (loading control) is also shown. Numbers at the bottom of the panel indicate the fold-increase of cyclin D1 expression, calculated on two different MEF preparations/genotype.

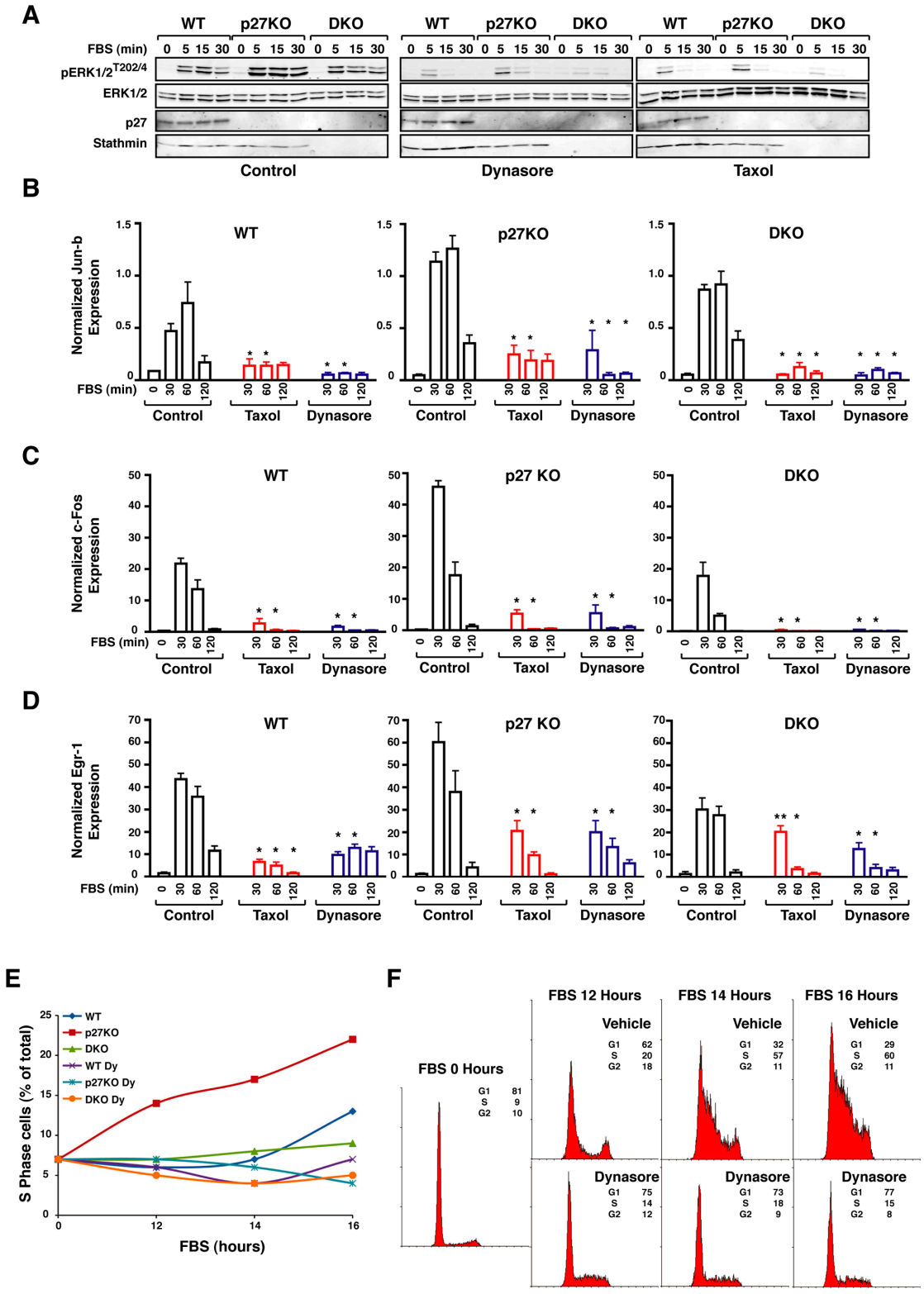

Fig. S7. Inhibition of microtubule-dynamics reverts the hyperactivity of MAPK pathway of p27KO cells.

(A) Western Blot analysis of phosho-ERK1/2 in the indicated MEFs, serum starved and stimulated with 10% serum (FBS) for 5-30 minutes. Cells were untreated

(vehicle, Control) or treated with Dynasore (25 μg/ml) or Taxol (100 nM), as indicated. Expression of total ERK, p27 and stathmin is also reported.

(B-D) qRT-PCR analysis of Jun-b (A) c-Fos (B) and Egr-1 (C) in RNA extracted from WT, p27KO or DKO MEFs serum starved (0) and then stimulated with 10% FBS for the indicated times, in the presence of vehicle (Control), Taxol or Dynasore, as indicated. Values were normalized using the expression of the Hprt and Pgk housekeeping genes and expressed as arbitrary units (A.U.). Data represent the mean of two independent experiments performed in duplicate. * $p \leq 0.02$;

(E) Graph reports percentage of MEFs in S phase, measured by FACS analyses, following serum starvation and stimulation with 10% serum (FBS) with or without Dynasore (25 μg/ml), for the indicated time.

(F) FACS analyses of p27KO cells, following serum starvation and stimulation with 10% serum (FBS), with or without Dynasore (25 μg/ml), for the indicated times.

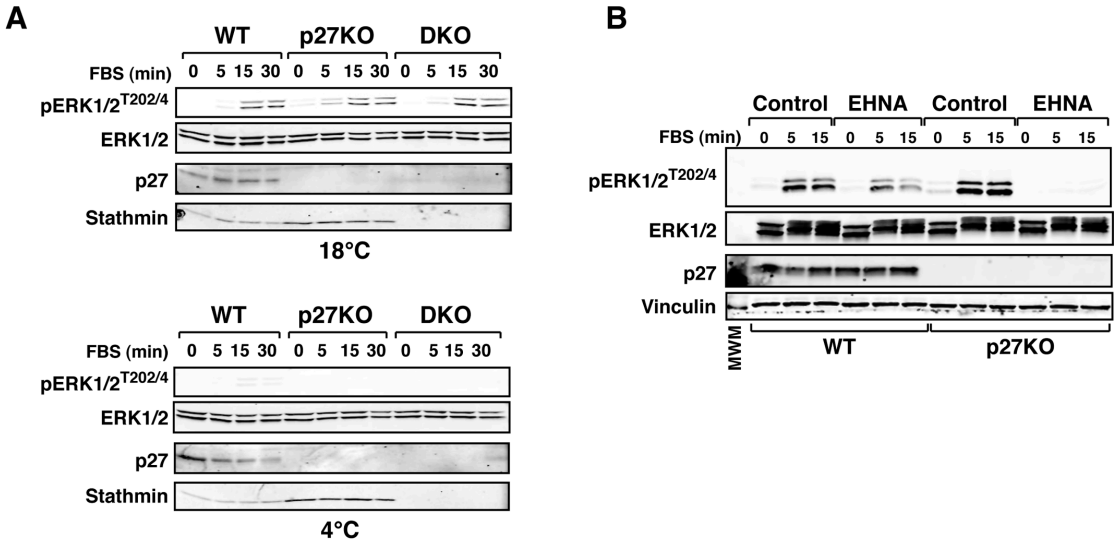

Fig. S8. Interfering with the endocytic transport abolishes MAPK activation.

(A) Western Blot analysis of ERK1/2 phosphorylation status in WT, p27KO and DKO MEF, serum starved and then stimulated with 10% serum (FBS) at 18°C (upper panels) and 4°C, as indicated (lower panels), for the indicated time points (minutes). Expression of total ERK1/2, p27 and stathmin is also reported.

(B) Western Blot analysis of ERK1/2 phosphorylation status in WT and p27KO fibroblasts, serum starved and then stimulated with 10% serum (FBS) for 5 and 15' in the presence or absence of the dynein inhibitor EHNA, as indicated, for the indicated time points (minutes). Expression of total ERK1/2, p27 and vinculin is also reported. MWM, molecular weight marker

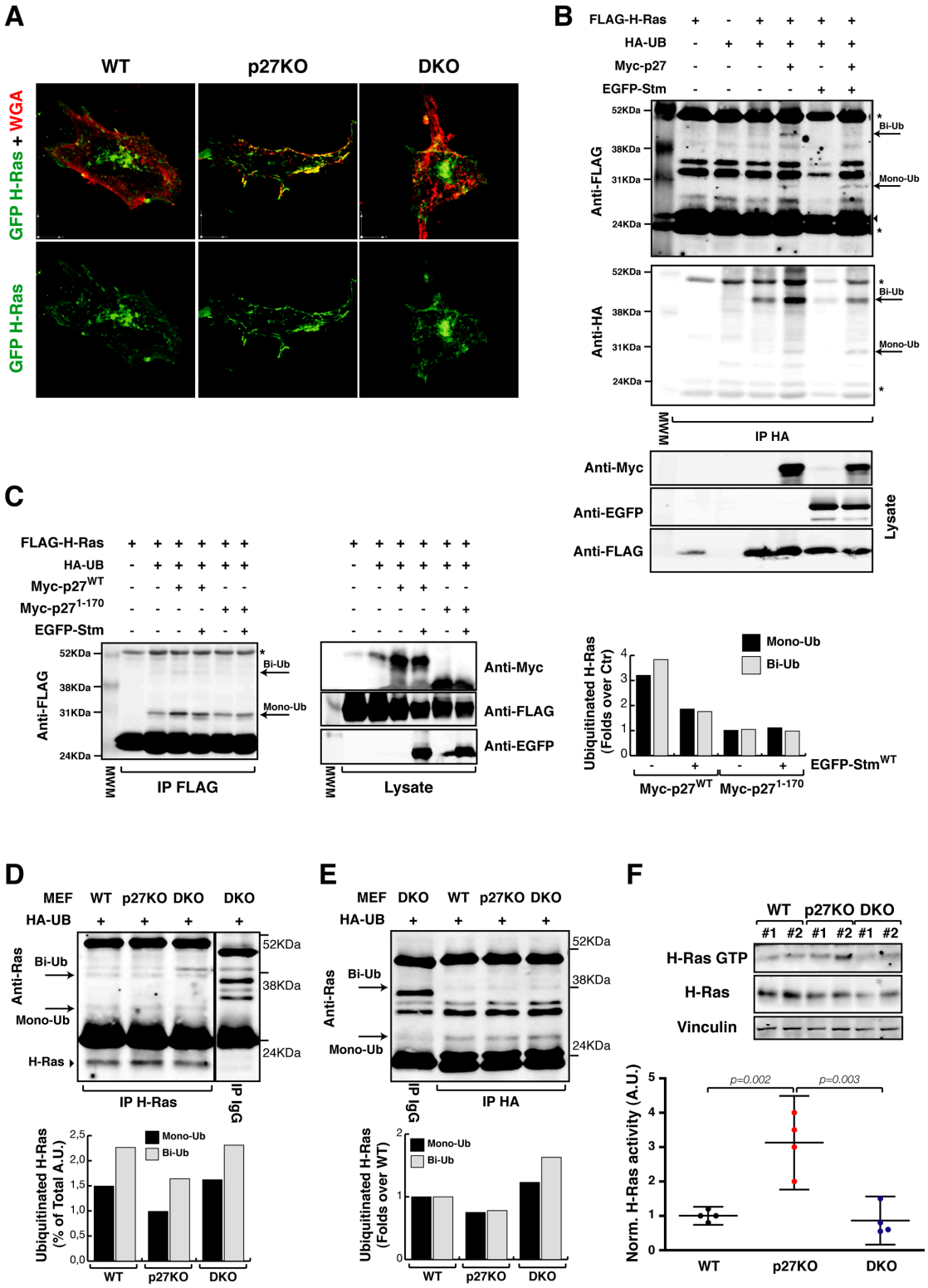

Fig. S9. p27/stathmin interaction controls H-Ras localization and ubiquitination.

(A) Immunofluorescence analysis of H-Ras (GFP, green) and WGA (Wheat Germ Agglutinin, used as membrane marker, red) in 3T3 WT, p27KO and DKO fibroblasts, serum starved and stimulated with 10% serum (FBS) for 15'.

(B) Immunoprecipitation analysis of H-Ras mono-bi ubiquitination in 293T/17 cells transfected with FLAG-tagged H-Ras in the presence or not of HA-tagged ubiquitin (HA-UB), Myc-tagged p27 and EGFP-tagged stathmin expressing vectors, as indicated. Total cell lysates were immunoprecipitated with an anti-HA antibody and probed with anti-FLAG (upper panel) or anti-HA (lower panel) antibodies, as indicated. Arrows indicate mono- and bi-ubiquitinated forms of H-Ras. Asterisks mark light and heavy IgG chains. Expression of transfected proteins is reported in the lower panels (Lysate).

(C) Immunoprecipitation analysis of H-Ras mono-bi ubiquitination in 293T/17 cells, transfected with FLAG-tagged H-Ras, in the presence or not of HA-tagged ubiquitin (HA-UB), Myc-tagged -$p27^{WT}$ or -$p27^{1-70}$ and EGFP-tagged stathmin expressing vectors, as indicated. Total cell lysates were immunoprecipitated and immunoblotted using an anti-FLAG antibody. Arrows indicate mono- and bi-ubiquitinated forms of H-Ras. Asterisk marks heavy IgG chains. Expression of transfected proteins is reported in the middle panels (Lysate). Graph on the right reports the amount of mono- and bi-ubiquitinated H-Ras, obtained by densitometric quantification of the ubiquitinated bands in the anti-FLAG blot and expressed as folds increase over the control (*i.e.* FLAG-H-Ras and HA-ubiquitin).

(D and E) Immunoprecipitation analysis of endogenous mono-bi ubiquitination of H-Ras in MEF populations of the indicated genotypes, transfected with HA-tagged ubiquitin (HA-UB). Total cell lysates were immunoprecipitated with anti-H-Ras (D) or anti-HA (E) antibodies and probed for anti-H-Ras. Arrows indicate endogenous mono- and bi-ubiquitinated forms of H-Ras. Graphs below report the amount of mono- and bi-ubiquitinated H-Ras, obtained by densitometric quantification of the ubiquitinated bands in the corresponding blot and expressed as percentage of total H-Ras (D) or as fold-increase over the WT (E).

(F) Pull down assays of active Ras (GTP-loaded Ras) in lysates from brains (E) collected from 8-weeks-old C57BL/6mice (n=4, only two are shown in the WB) of the indicated genotypes. H-Ras activity, normalized by H-Ras expression, is reported in the accompanying graph (n=4). Vinculin was used as loading control. Significance was calculated by Student's t-test and expressed by a p value ≤ 0.05.

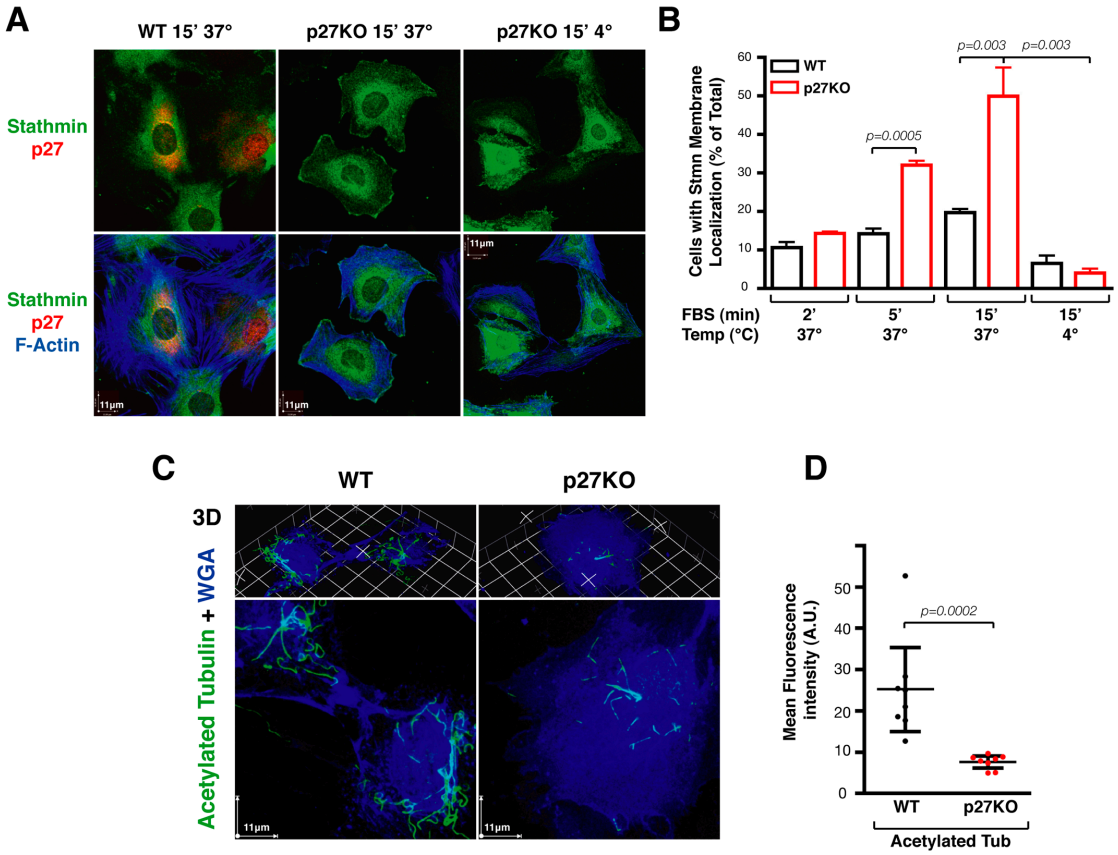

Fig. S10. p27 prevents stathmin relocalization at the plasma membrane following serum stimulation.

(A) Immunofluorescence analysis of stathmin (green) p27 (red) and F-actin (phalloidin, pseudocolored in blue) in 3T3 WT and p27KO fibroblasts, serum starved and stimulated with 10% serum (FBS) for 15', at 37° and at 4°C, as indicated.

(B) Graph reports the quantification of stathmin membrane localization from the experiment reported in (A), expressed as percentage of the total amount of stathmin expressed by the cell.

(C) Immunofluorescence analysis of acetylated tubulin (green) and WGA (Wheat Germ Agglutinin, used as membrane marker, pseudocolored in blue) in 3T3 WT and p27KO fibroblasts, serum starved and stimulated with 10% serum (FBS) for 15'.

(D) Graph reports the quantification of acetylated tubulin staining *per* cell, from the experiment described in (C), expressed as mean fluorescence intensity in arbitrary units (A.U.), calculated using the Volocity software. Each dot in the graph corresponds to one analyzed cell. Significant differences are reported in the graph and were calculated using the Mann-Whitney unpaired t-test.

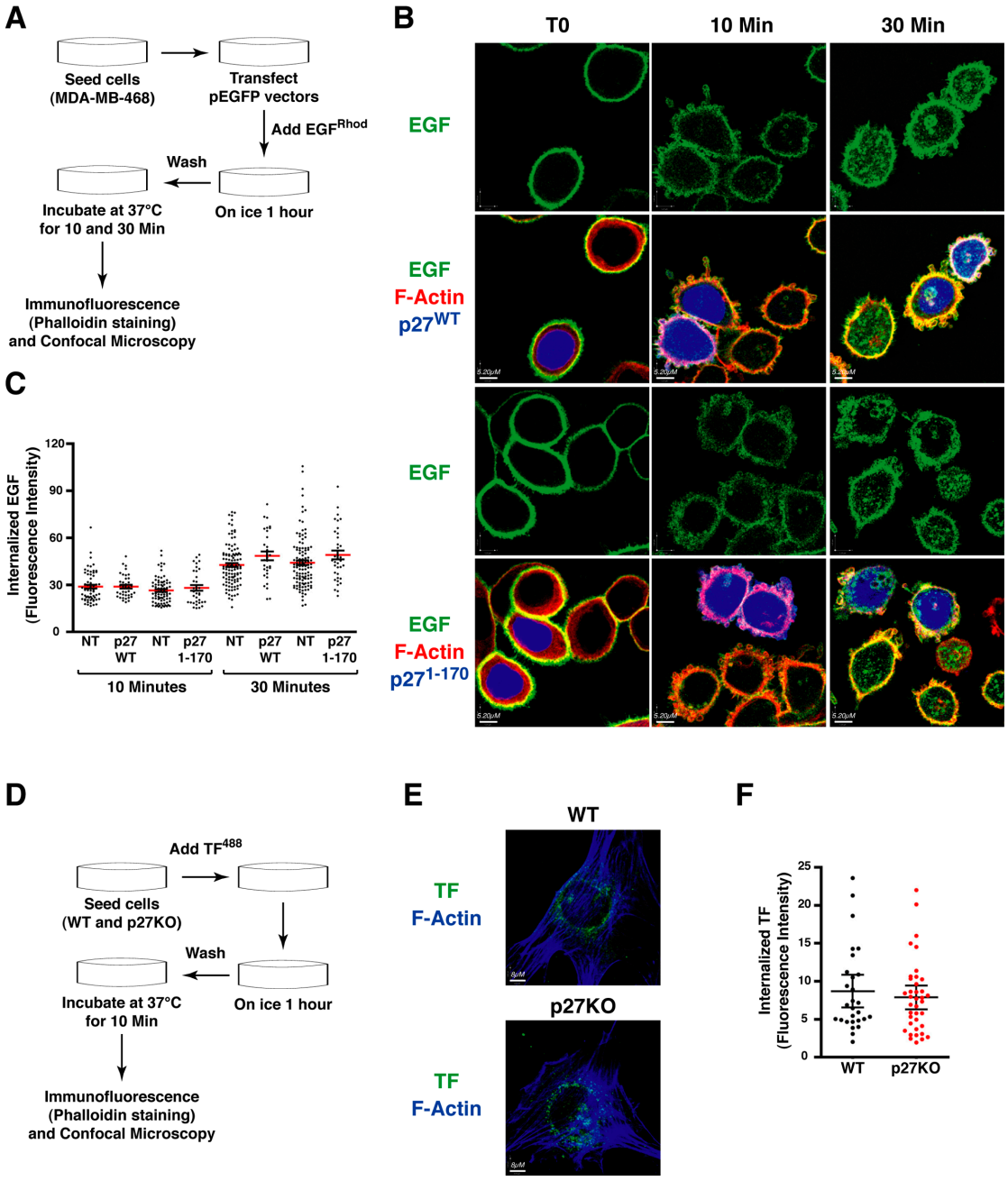

Fig. S11. Internalization of either EGF or transferrin is not altered by p27 absence.

(A) Schematic representation of the experiment reported in (B). MDA-MB-468 breast carcinoma cells were transfected with EGFP-empty, EGFP-p27^{WT} or EGFP-p27$^{1-170}$ vector. 48h after transfection, cells were serum starved, put on ice and stimulated with rhodamine-EGF (0.5µg/ml). After 1h on ice, cells were washed to remove unbound EGF and incubated at 37°C for 10 and 30 minutes.

(B) Immunofluorescence analysis of the experiment described in (A). Cells were stained for F-Actin (phalloidin, pseudocolored in red) and for p27 (EGFP, pseudocolored in blue). Internalized Rhodamine-EGF was pseudocolored in green.

(C) Graph reports the quantification of internalized EGF, measured by fluorescence intensity *per* cell, calculated using the Volocity software. Each dot in the graph corresponds to one cell. At least 30 transfected cells/sample were measured. NT indicates not-transfected cells, measured in the same fields. No significant difference was observed between the different samples at the same time point, as established using the Mann-Whitney unpaired t-test.

(D) Schematic representation of the experiment reported in (E). 3T3 p27WT and p27KO fibroblasts were serum starved, put on ice and stimulated with AF-488 conjugated transferrin (0.1mg/ml). After 1h on ice, cells were washed to remove unbound transferrin and incubated at 37°C for 10.

(E) Immunofluorescence analysis of the experiment described in (D). Cells were stained for F-Actin (phalloidin, pseudocolored in blue). Internalized AF-488 conjugated transferring was pseudocolored in green.

(F) Graph reports the quantification of internalized transferrin, measured by fluorescence intensity *per* cell, calculated using the Volocity software. Each dot in the graph corresponds to one cell. At least 30 cells/sample were measured. No significant difference was observed between the different samples, as established using the Mann-Whitney unpaired t-test.

Supplementary Table S1. Pathway altered in p27KO (p27 signature) and DKO (DKO signature) MEF, as evidenced using the Gather Gene Annotation online resource.

Rank	Pathway	G.O.	p value
p27 Signature			
1	*Regulation of JNK cascade*	GO:0046328	< 0.0001
2	*Detection of external stimulus*	GO:0009581	0.0001
3	*Response to external stimulus*	GO:0009605	0.0002
4	*Sensory perception*	GO:0007600	0.0002
5	*Response to stimulus*	GO:0050896	0.0004
6	*MAPKKK cascade*	GO:0000165	0.001
7	*Regulation of signal transduction*	GO:0009966	0.004
8	*JNK cascade*	GO:0007254	0.009
DKO Signature			
1	*Regulation of JNK cascade*	GO:0046328	0.0003
2	*Mesoderm development*	GO:0007498	0.004
3	*Mesendoderm development*	GO:0048382	0.007
4	*Mullerian duct regression*	GO:0001880	0.007
5	*Regulation of signal transduction*	GO:0009966	0.009

G.O. = Gene Ontology number

Only gene pathways displaying a p value <0.01 are reported.

Supplementary Table S2. Pathway altered in p27KO MEF (p27 signature), as evidenced using the online tool *Pathway Express* linked to the KEGG database.

Rank	Pathway	I. F.	Adj. p
p27 Signature			
1	*Apoptosis*	5.876	0.01
2	*mTOR signaling pathway*	5.262	0.03
3	*MAPK signaling pathway*	4.772	0.04

I.F. = Impact factor

Adj. p = Adjusted p value

Only significantly altered pathways are reported ($p<0.05$). For references on computational analyses please refer to *(15)*.