HIPK2 is involved in cell proliferation and its suppression promotes growth arrest independently of DNA damage

S. Iacovelli^{*,‡}, L. Ciuffini^{*,§}, C. Lazzari^{*}, G. Bracaglia^{*}, C. Rinaldo^{*}, A. Prodosmo^{*}, A. Bartolazzi[†], A. Sacchi^{*} and S. Soddu^{*}

^{*}Department of Experimental Oncology, Regina Elena Cancer Institute, Rome, Italy, and [†]Department of Pathology, Sant'Andrea Hospital, Rome, Italy

Received 21 March 2008; revision accepted 4 June 2008

Abstract

Introduction/objectives: The serine/threonine kinase homeodomain-interacting protein kinase 2 (HIPK2) is a co-regulator of an increasing number of transcription factors and cofactors involved in DNA damage response and development. We and others have cloned HIPK2 as an interactor of the p53 oncosuppressor, and have studied the role of this interaction in cell response to stress. Nevertheless, our original cloning of HIPK2 as a p53-binding protein, was aimed at discovering partners of p53 involved in cell differentiation and development, still controversial p53 functions. To this aim, we used p53 as bait in yeast two-hybrid screening of a cDNA library from mouse embryo (day 11 postcoitus) when p53 is highly expressed.

Methods and results: In this study, we directly explored whether HIPK2 and p53 cooperate in cell differentiation. By measuring HIPK2 expression and activity in skeletal muscle and haemopoietic differentiation, we observed inverse behaviour of HIPK2 and p53 – excluding cooperation activity of these two factors in this event. However, by HIPK2 depletion experiments, we showed that drastic HIPK2 suppression promotes cell-cycle arrest by induction of the cyclin-dependent kinase inhibitor p21^{Waf-1/Cip-1}. HIPK2 activity is independent of DNA damage and takes place in cell-cycle-arresting

conditions, such as terminal differentiation, growth factor deprivation, and G₀ resting.

Conclusions: HIPK2 was found to be involved in cell-cycle regulation dependent on p21^{Waf-1/Cip-1} and independent of DNA damage.

Introduction

The homeodomain-interacting protein kinase 2 (HIPK2) was originally discovered, together with another two members of the HIPK family (HIPK1 and HIPK3), in yeast two-hybrid screening with the homeoprotein Nkx-1.2, and was shown to work *in vitro* as co-repressor of several mammary and *Drosophila* homeobox transcription factors (1,2). Subsequently, HIPK2 was found to interact with a large number of transcription factors and cofactors involved in development or in DNA damage response (3,4).

Most of the data on HIPK2 involvement in development come from studies of genetically modified mice and *in vitro* differentiating neurones. *Hipk1/Hipk2* double knockout embryos have defects in haemopoiesis and vasculogenesis (5,6), fail to close the anterior neuropore, they exhibit exencephaly (excess of neural tissue due to reduced cell death or increased cell proliferation, or both), and are progressively lost between 9.5 and 12.5 days postcoitus (7). In addition, survival and proliferation defects have been observed in specific neurones in single *Hipk2* knockout mice, as well as in developing sensory and sympathetic neurones on HIPK2 overexpression (7,8).

In response to DNA damage, HIPK2 modulates activity of several proteins directly or indirectly related to apoptosis, such as tumour suppressor p53 and its family members (9–11), p53 inhibitor MDM2 (12,13), CtBP transcriptional co-repressor (14), and β -catenin regulator Axin (15). More recently, we have identified HIPK2 as a critical target for the p53/MDM2 pathway in cell decision between cell cycle arrest and apoptosis in response to damage of different intensities (reparable, sublethal DNA damage

Correspondence: S. Soddu, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, Italy. Tel.: +39 065 2662492; Fax: +39 065 2662505; E-mail: soddu@ifo.it

[‡]Present address: San Raffaele Scientific Biomedical Park of Rome, Via Castel Romano 100, Rome, Italy

[§]Present address: Department of Biology, University of Rome 'Tor Vergata', Via della Ricerca Scientifica, Rome, Italy

S. Iacovelli and L. Ciuffini contributed equally to this work.

versus nonreparable, lethal damage) (16). Albeit we and others have shown that HIPK2 regulates p53 localization, phosphorylation, and acetylation in response to DNA damage (9,10,17), it is worthwhile mentioning that original identification of the HIPK2/p53 interaction was by using p53 as bait in yeast two-hybrid screening of a cDNA library generated from 11-day mouse embryos (10). The choice of this particular library was dictated by consideration that during mouse development, p53 mRNA is present at high levels in all embryonic cells from E8.5 to E10.5. At later stages, p53 expression becomes more pronounced during differentiation of specific tissues, and declines in mature tissues (18). Since the role of p53 in development and differentiation is still controversial, we sought to obtain clues on this issue by identifying cofactors involved in p53 activation/function during development. Although experiments performed thus far on the HIPK2/p53 interaction have focused on their role in the DNA damage response, suggested involvement of both proteins in development and differentiation prompted us to test directly whether HIPK2 and p53 cooperate in cell differentiation. We and others have shown a role for p53 in skeletal muscle and haemopoietic differentiation (19–23), while haemopoietic differentiation defects have been observed in *Hipk2* knockout mice. Thus, we used immortalized and primary mouse and human skeletal muscle and haemopoietic cells to evaluate whether HIPK2 has a direct role in their differentiation processes and whether there is cooperation between HIPK2 and p53 in this biological function. We found inverse correlation between HIPK2 and p53 expression during both types of differentiation, excluding cooperation activity of the two factors in these processes. Surprisingly, we observed strong and persistent repression of HIPK2 during cell cycle withdrawal associated to these types of differentiation. Similar HIPK2 behaviour was observed during cell cycle arrest induced by growth factor deprivation as well as in resting peripheral blood lymphocytes, while HIPK2 expression was reactivated in resting cells on growth factor stimulation. In addition, depletion of HIPK2 by RNA interference was associated with up-regulation of the cyclin-dependent kinase inhibitor p21^{Waf-1/Cip-1} and cell-cycle arrest, that could be rescued by inhibition of p21^{Waf-1/Cip-1} expression. Taken together, these results indicate cell-cycle-related activity of HIPK2, independent of DNA damage and requiring induction of p21^{Waf-1/Cip-1} expression.

Materials and methods

Cells and culture conditions

Primary human satellite cells were purchased from Cambrex Bio Science Walkersville Inc. (Walkersville, MD, USA) and

were cultured and differentiated following manufacturer's instructions to customers. Primary murine satellite cells and immortal C2C12 murine myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (hi-FBS). Muscle differentiation was induced by incubation in differentiation medium (DMEM with 10 µg/ml insulin, and 5 µg/ml transferrin) (19). Differentiation medium was replaced every 48 h. 32Dcl.3 murine haemopoietic precursor cells were cultured in RPMI-1640 supplemented with 10% hi-FBS and 10% conditioned medium from murine myelomonocytic cell line WEHI 3B, as source of crude interleukin 3 (IL-3), or 20 µg/ml granulocyte-colony stimulating factor (G-CSF) (R&D Systems, Minneapolis, MN, USA) to induce proliferation or granulocytic differentiation, respectively (19). HL60 human leukaemia cells were cultured in RPMI-1640 with 10% hi-FBS. Macrophage differentiation was induced by 50 µM phorbol 12-myristate 13-acetate (TPA) (Sigma, St. Louis, MO, USA) for 2 days while granulocytic differentiation by 1.25% dimethyl sulfoxide (Sigma) for 5 days. Primary murine bone marrow cells were obtained from long bones of sacrificed C3H mice, cultured in α -minimum essential medium supplemented with 20% hi-FBS, IL-3, stem cell factor and IL-6 (R&D Systems) as previously described (24). Immortalized human fibroblasts, HCT116 p53^{+/+} and p53^{-/-} were cultured in DMEM supplemented with 10% hi-FBS. Immortalized human mammary MCF-10 A cells (25) and their p21^{Waf-1/Cip-1} knockout derivative (26) were cultured in mammary epithelium basal medium supplemented with MEGM SingleQuots (Clonetics, Lonza Milano S.r.l., Treviglio, Italy). Peripheral blood lymphocytes were purified by Ficoll density gradient centrifugation using Lympholyte-H (CEDARLANE Laboratories Ltd, Burlington, ON, Canada) and were cultured in RPMI-1640 supplemented with 10% hi-FBS and 5 µg/ml phytohaemagglutinin (Sigma). For DNA damage, cells were treated with 20 µM bleomycin (Nippon Kayaku, Tokyo, Japan) or 2.5 µg/ml cisplatin (Teva Pharma-Italia, Milan, Italy). MG132 (Calbiochem, San Diego, CA, USA) was added to subconfluent cells at 10 µM.

Western immunoblotting analysis

Total cell extracts were prepared in RIPA buffer [50 mM Tris-HCl (pH 8), 300 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1% NP40, 1 mM EDTA], resolved on SDS-polyacrylamide gel electrophoresis, transferred on nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and analysed by Western blotting with the following antibodies: rabbit anti-HIPK2 (kindly provided by M. L. Schmitz); rabbit anti-p53 (FL-393), p21 (C-19), and anti- α -tubulin monoclonal

antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-myosin heavy chain (MyHC) monoclonal antibody (MF20, kindly provided by M. Crescenzi); anti-actin monoclonal antibody (Sigma); and horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit (Cappel West Chester, PA, USA). Immunoreactivity was determined using the ECL Western Blotting Detection (Amersham Corp., Arlington Heights, IL, USA) following the manufacturer's instructions.

Determination of cell differentiation

Granulocytic differentiation was assessed by morphological analysis on cytospin preparations fixed and stained using the May-Grünwald-Giemsa (Sigma) protocol. At least 200 cells were counted from each sample. Skeletal muscle differentiation was detected by indirect immunofluorescence for MyHC detection. Briefly, cells on Petri dishes were fixed with cold methanol:acetone (1 : 1) for 10 min, rehydrated and incubated for 1 h at 37 °C with MF20 monoclonal antibody. Immunoreactions were detected by incubation with an affinity-purified, fluorescein isothiocyanate-conjugated, goat anti-mouse immunoglobulin G (FAb)₂ fragment (Cappel). Nuclei were stained after immunofluorescence reaction by incubating the cells for 3 min with 1 µg/ml solution of Hoechst 33 258 dye in phosphate-buffered saline. Differentiation index was calculated as follows:

Differentiation index

$$= \frac{\text{Number of nuclei in differentiated cells}}{\text{Total number of nuclei}}$$

Cell cycle analysis and viability

Cells (5×10^5) were fixed in cold acetone:methanol (1 : 4) for 30 min at 4 °C and DNA was stained with 0.1 mg/ml propidium iodide (Sigma) in phosphate-buffered saline with 2 mg/ml RNase A (Roche Diagnostic Corporation, Monza, Italy) for 30 min at room temperature. DNA content was measured using an Epics XL analyser (Coulter Corporation, Miami, FL, USA). Cell viability was determined using the trypan blue exclusion test.

shRNA interference, recombinant viruses and viral infection

Lentiviral vector pLL3.7 (kindly provided by Dr L. Van Parijs), carrying sequences GCGTGTCAAGGAGAACT (HIPK2i-2496) or GAAAGTACATTTTCAACTG (HIPK2i-1376) for interfering murine *hipk2* RNA (27), and relative control virus, carrying the sequence for interfering β -galactosidase (LacZi sequence GTGACCAGCG-AATACCTGT), were produced into 293FT cells by

co-transfection of lentiviral and packaging vectors (ViraPower, lentiviral support kit; Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's instructions. Resulting supernatants were collected after 48 h, titrated on C2C12 cells, and were used to infect bone marrow cells (1.5×10^6 cell/sample) by co-culture of cells and supernatants in the presence of polybrene (Sigma) 8 µg/ml, IL-3, IL-6 and stem cell factor. After 48 h of co-culture, bone marrow cells were selected with 2 µg/ml puromycin (Sigma) and were plated in methylcellulose for colony-forming assay.

Colony-forming assay

Infected and selected bone marrow cells (2.5×10^3) were suspended in 2 ml of methylcellulose culture mixture with granulocyte-macrophage colony stimulating factor (GM-CSF) (Stem Cell Technologies, Vancouver, BC, Canada) and were plated in a 35-mm Petri dish. After 8 days incubation, bone marrow colonies were counted using an inverted microscope and were picked up for further analyses. Single cell morphology was evaluated after cytospin preparation and staining with May-Grünwald-Giemsa.

RNA extraction, RT-PCR and real-time RT-PCR

RNA was extracted using the SV Total RNA isolation system (Promega, Madison, WI, USA), following manufacturer's instruction. cDNA was synthesized in a total volume of 20 µl containing 250 ng of total RNA, using Omniscript Reverse Transcriptase (Qiagen, Hilden, Germany) and tested by semiquantitative polymerase chain reaction (PCR) as described (24) or by real-time PCR with the SYBR Green DNA Master Mix and the Applied Biosystems 7500 System SDS Software (Foster City, CA, USA). The following primers were used: upper human/mouse HIPK2 5'-AGGAAGAGTAAGCAGCACCAG-3'; lower human/mouse HIPK2 5'-TGCTGATGGTGATGACACTGA-3'; upper mouse aldolase 5'-TGGATGGGCTGTCTGAACGCTGT-3'; lower mouse aldolase 5'-AGTGACAGCAGGGGGGCACTGT-3'; upper human p53 5'-GTCTGGGCTTCTTGCAATTCT-3'; lower human p53 5'-AATCAACCCACAGCTGCA-3'; upper human GAPDH 5'-TCCCTGAGCTGAACGGGAAG-3'; lower human GAPDH 5'-GGAGGAGTGGGTGTCGCTGT-3'; upper mouse β -actin 5'-CGATGCCCTGAGGCTCTTT-3'; lower mouse β -actin 5'-TAGTTTCATGGATGCCACAGGAT-3'; upper mouse p21 5'-GGATTCCCTGGTCTTACCTTAGGC-3'; and lower mouse p21 5'-AGAAGTCACTGAGAACCCCAACTG-3'.

Each target amplification was performed in duplicate on two different RNA preparations.

Anti-HIPK2 monoclonal antibody and immunohistochemistry

Mouse monoclonal antibody specific for HIPK2 protein was generated by immunizing BALB/c mice with the adenoviral vector AdHIPK2 (intramuscular injection of 1×10^8 plaque forming units of virus per animal) (27). Somatic fusion, screening of positives clones by ELISA and purification from hybridoma supernatant by protein G chromatography were performed by Areta International S.r.l. (Gerenzano, Italy). For immunohistochemistry, normal skin specimens were fixed for 18–24 h in 4% buffered paraformaldehyde and then were processed through to paraffin wax. HIPK2 expression was evaluated by immunohistochemistry on 5- μ m-thick paraffin-embedded tissues. Sections harvested on SuperFrost Plus slides (Menzer-Glaser, Braunschweig, Germany) were deparaffinized and rehydrated in a 750 W microwave oven for 15 min in 10 mM citrate buffer (pH 6). Incubation with anti-HIPK2 monoclonal antibody (5 μ g/ml) was performed for 1 h at room temperature. Reactions were revealed using the SuperSensitive Link-Label Detection System (BioGenex Laboratories Inc., San Ramon, CA, USA), with 3-amino-9-ethylcarbazole (Dako, Glostrup, Denmark) as chromogen substrate. Sections were lightly counterstained with Mayer's haematoxylin and were mounted in aqueous mounting medium (Dako).

siRNA interference

HIPK2-specific (HIPK2i) and universal negative control siRNA were HIPK2i Stealth RNAi sequences (a mix of three different sequences) and Stealth RNAi Negative Medium GC Duplexes, respectively (Invitrogen). Cells were transduced using RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions.

Results

HIPK2 expression repressed during skeletal muscle and haemopoietic differentiation

To evaluate whether HIPK2 cooperates with p53 in cell differentiation, we tested HIPK2 expression in skeletal muscle and haemopoietic cells, in which we have previously shown that p53 is activated and required for differentiation whereas differentiation-associated apoptosis and cell cycle arrest are p53 independent (19,21,28). Western blot analyses were performed on total cell extracts from murine and human primary myoblasts (Fig. 1a,b), human HL60 leukaemia cells (Fig. 1c), and murine 32D myeloid progenitors (Fig. 1d). These cells were maintained in proliferative, undifferentiated conditions (Prol) or were

induced to terminally differentiate into different lineages. In particular, primary myoblasts were differentiated into myotubes, as confirmed by expression of MyHC, through incubation in differentiation medium for 3 days (Fig. 1a,b; upper panels). In this culture condition, both murine and human myoblasts reach differentiation index higher than 90%, as assessed by indirect immunofluorescence (data not shown). HL60 cells were differentiated into macrophages by TPA treatment for 2 days, or into granulocytes by dimethyl sulphoxide treatment for 5 days. 32D cells were differentiated into granulocytes by incubation in the presence of G-CSF and were analysed at different time points. Haemopoietic differentiation was assessed by May-Grünwald-Giemsa staining of cytopsin preparations and reported as percentage of terminally differentiated cells (Fig. 1d–e; lower panels). In each cell type tested, HIPK2 was detectable, although at different levels, in proliferating, undifferentiated cells; however, it was strongly suppressed during differentiation (Fig. 1a–d), showing opposite behaviour from p53, that is, induced during muscle differentiation (19,29) (Fig. 1a). This HIPK2 suppression was even more evident when its expression was assessed on removal of the portion of cells that undergo differentiation-associated apoptosis. As shown by time course analysis, HIPK2 expression persisted longer in total populations of G-CSF-treated 32D cells *versus* the same populations enriched for live cells by centrifugation on density gradient (Fig. 1e). Finally, HIPK2 protein suppression was not associated with reduction of *Hipk2* mRNA levels (Fig. 1a,g and data not shown) while treatment with the proteasomal inhibitor MG132 partially rescued HIPK2 protein levels in human primary myoblasts induced to differentiate (Fig. 1f), demonstrating presence of a protein degradation mechanism.

Taken together, these data indicate that, at least in the analysed conditions, HIPK2 has opposite behaviour to that of p53 (repression rather than activation) and excludes direct cooperation between HIPK2 and p53 in skeletal muscle and haemopoietic differentiation. This conclusion was further confirmed by absence of any evident modulation of p53 mRNA and protein expression upon induction of differentiation, in HIPK2 depleted human primary myoblasts (Fig. 1g,h).

HIPK2 expression induces apoptosis in terminally differentiated muscle and haemopoietic cells

To evaluate whether observed HIPK2 suppression is functional for the differentiation process, we tested the effect of HIPK2 overexpression in muscle differentiation. Mouse myoblasts of C2C12 cell line were used, as they can be grown and transduced more efficiently than primary cells. C2C12 cells were infected with adenovirus

Figure 1. HIPK2 expression in skeletal muscle and haemopoietic differentiation. Western blot analyses of indicated proteins were performed on total cell extracts from proliferating (Prol) and differentiated cells of different lines: (a) mouse primary satellite cells; (b) human primary satellite cells; (c) human HL60 leukaemia cells; (d) mouse 32D myeloid progenitor cells. '% TD cells' indicates percentage of terminally differentiated cells. (e) 32D cells were differentiated into granulocytes by incubation in the presence of G-CSF as in (d) and total cell extracts were obtained by total population or after density gradient purification of live cells. Relative percentage of viability is indicated below. (f) Human primary satellite cells were cultured in growth medium or differentiation medium in the presence or absence of MG132; p53 expression was tested as positive control of MG132 activity. (g, h) Human primary satellite cells were transduced with HIPK2-specific siRNA or with the relative control (UNC) and cultured in growth medium or differentiation medium as in (f). Real-time RT-PCR (g) and Western blot (h) analyses were performed on the indicated genes and proteins. Tubulin and actin were used as loading controls.

carrying the *Hipk2* gene (AdHIPK2) (27) and induced to differentiate 16 h post-infection by incubation in differentiation medium. Mock-infected cells and cells infected with the dL70.3 empty adenovirus were used as controls. Differentiation indices of the three different populations were calculated by indirect-immunofluorescence for MyHC and staining of nuclei. Mild and persistent reduction of the differentiation index was observed in HIPK2 overexpressing population (Fig. 2a). However, morphological evaluation of the AdHIPK2 infected population compared to controls showed presence of numerous cells with blebbing morphology and pyknotic nuclei, suggestive of apoptosis (Fig. 2b). Since HIPK2 overexpression can mimic DNA damage response and promote apoptosis (9,10), we tested whether apparent reduction of C2C12 differentiation was rather induced by an increase in cell death (reduction of the denominator in the differentiation index; see Materials and methods for details). Thus, C2C12 myoblasts, maintained in undifferentiated conditions or induced to differentiate for 3 days, were infected as above and level of cell death was evaluated by trypan blue incorporation (Fig. 2c) or TUNEL assay (data not shown). Strong increase in cell death was present in HIPK2 overexpressing cells, making it impossible to evaluate by this type of experiment, whether HIPK2 suppression observed during differentiation is functional in this biological process.

Lethal DNA damage induces HIPK2 expression also in terminally differentiated cells

Lethal DNA damage increases HIPK2 levels and promotes human p53Ser46 and mouse p53Ser58 phosphorylation in p53 expressing cancer cells and in normal cells (16,30). Skeletal muscle and haemopoietic terminally differentiated cells have undetectable levels of HIPK2 protein but presence of *Hipk2* mRNA, and we have shown that exogenous HIPK2 expression is still able to induce apoptosis in these cells; thus, we asked whether lethal damage of terminally differentiated cells is also associated to HIPK2 induction. To answer this question, HL60 and 32D cells were induced to differentiate by incubation in the presence of TPA or G-CSF, respectively, and then were treated with lethal doses of bleomycin or cisplatin, as tested by preliminary dose-response curves (data not shown). Undifferentiated, proliferating, HIPK2-expressing counterparts were used as positive controls. Although at lower magnitude than in undifferentiated cells, induction of HIPK2 level was observed in terminally differentiated cells with both drugs (Fig. 3a,b). In addition, p53Ser58 phosphorylation was detectable in wild-type p53-carrying 32D cells (Fig. 3c), suggesting that HIPK2 might play a role in response to DNA damage also in terminally differentiated cells that express undetectable levels of this kinase in basal conditions.

Figure 2. Effect of exogenous HIPK2 expression in C2C12 cells. (a) C2C12 myoblasts were induced to differentiate into myotubes by incubation in differentiation medium in the absence (mock) or presence of adenovirus infection with control virus (dl70.3) or HIPK2-carrying virus (AdHIPK2). Differentiation indices were evaluated as described in the Material and methods section at indicated days. One indicative experiment of three different infections is reported. (b) Morphological evaluation of the C2C12 cells treated as in (a). Day 5 cells are shown. (c) Undifferentiated (myoblasts) and differentiated (myotubes) C2C12 cells were infected as indicated and percentages of dead cells were evaluated using the trypan blue exclusion test. Mean $\pm$ standard deviation of three different experiments is reported.

HIPK2 depletion inhibits cell proliferation in primary bone marrow cells

Since we were clueless about the role of HIPK2 suppression in the differentiation process by HIPK2 overexpression,

Figure 3. HIPK2 expression in terminally differentiated (TD) haemopoietic cells after induction of DNA damage. (a) Proliferating (Prol) and TPA-differentiated HL60 cells were treated with 20 μ M bleomycin for the indicated times. Total cell extracts were analysed by Western blotting for the indicated proteins. (b) Proliferating (Prol) and G-CSF-differentiated 32D cells were treated with 2.5 μ g/ml of cisplatin for the indicated times. Total cell extracts were analysed by Western blotting for indicated proteins. (c) G-CSF-differentiated 32D cells were treated as in (b) and analysed for p53 expression and phosphorylation of the indicated serine residues. Actin was used as loading control.

we evaluated whether HIPK2 depletion, by RNA interference, might increase and/or accelerate cell differentiation. Two lentiviral vectors carrying HIPK2-specific shRNA were developed and employed to infect primary murine bone marrow cells (see Materials and methods for details). This cell system was selected because of the possibility of testing different types of differentiation in a single experiment and because, in the case of positive results, it allowed us to perform *in vivo* experiments by bone marrow transplantation, as we have previously done with p53-recombinant retrovirus-infected bone marrow cells (31). Bone marrow cells were explanted from 8-week-old mice, incubated for 24 h in the presence of cytokines, mock infected or infected with two different shHIPK2 sequences (HIPK2i-2496 or HIPK2i-1376) or sh β -galactosidase (LacZi) recombinant lentiviruses (see Materials and methods for details). Bone marrow cells were infected, selected with puromycin for 48 h, plated in methylcellulose in the presence of GM-CSF, and tested for *Hipk2* and *p21^{Waf-1/Cip-1}* mRNA expression (Fig. 4a, upper and middle panels). Colony formation efficiency of virus-infected cells showed significant reduction of colony number in the HIPK2i bone marrow cells compared to control (Fig. 4a, lower panel). In addition, size of each single colony was significantly smaller in HIPK2i populations relative to controls (Fig. 4b). To evaluate whether reduction in number and size of colonies associated with HIPK2 depletion was due, as suggested by our hypothesis, to increase in differentiation capacity of bone marrow cells, we assessed differentiation of each single colony by their staining with May-Grünwald-Giemsa stain, and morphological analysis by light microscopy. A mild reduction, rather than the expected increase, in percentage of terminally differentiated cells (macrophages and granulocytes) was reproducibly measured in HIPK2i colonies compared to

controls (Fig. 4b, right panel). Comparable results were obtained in human primary myoblasts in which HIPK2 depletion by siRNA slowed low MyHC expression (Fig. 1h). Overall, these data suggest that HIPK2 down-regulation associated with terminal differentiation is related to cell-cycle withdrawal rather than differentiation *per se*. This hypothesis might also be supported by the up-regulation of *p21^{Waf-1/Cip-1}* expression we observed in the HIPK2 depleted cells (see below).

HIPK2 is undetectable in growth-arrested cells and is reactivated in proliferating cells

To verify whether absence or presence of HIPK2 is linked to resting or to proliferating conditions, respectively, we evaluated HIPK2 expression by Western blotting on total cell extracts of *in vitro* cell cultures, or by immunohistochemistry on normal human skin, that contain both resting and proliferating cells. In the first set of experiments, we used human peripheral blood lymphocytes maintained in G₀ or reactivated by phytohaemagglutinin stimulation (Fig. 5a). HIPK2 was undetectable in resting cells while its expression increased on stimulation of cell proliferation (Fig. 5b). Comparable results were obtained by testing HIPK2 expression in 32D cells maintained in (i) growing condition by high amounts of IL-3 (Prol); (ii) G₁ arrest by low amounts of IL-3 (IL-3 Deprivation); and (iii) cell-cycle reactivation by re-addition of high amounts of IL-3 (Fig. 5c). As shown in Fig. 5d, HIPK2 protein was expressed in proliferating cells, strongly down-regulated in G₁ enriched cells and suddenly re-expressed upon IL-3 addition with timing that precedes cell entry into S-phase. Interestingly, *Hipk2* mRNA levels showed opposite behaviour, suggesting that HIPK2, as transcriptional co-repressor, might negatively regulate its own promoter.

Figure 4. HIPK2 depletion in mouse primary bone marrow cells. Bone marrow cells were explanted from long bones of sacrificed mice and were infected with recombinant lentiviruses carrying control (Ctr) or two different HIPK2-interfering sequences (2496 or 913). Infected cells were selected in puromycin for 48 h and then were plated in methylcellulose in the presence of GM-CSF. (a) Upper and middle panels: single colonies were picked-up from methylcellulose and employed to extract RNA. Real-time RT-PCR was performed on indicated genes. Lower panel: mean $\pm$ standard deviation of number of colonies present in each plate and counted at the inverted microscope. (b) Left panels: indicative morphology of each single colony in methylcellulose. Right panel: single colonies were picked-up from methylcellulose and their cells were cytocentrifugated and stained with May-Grünwald–Giemsa stain to assess granulocyte and macrophage differentiation. '% TD cells' indicates percentage of terminally differentiated cells.

To evaluate HIPK2 expression *in vivo*, we developed a monoclonal antibody that recognizes HIPK2 in immunohistochemistry (see Materials and methods for details). Analysis of human normal skin showed 'dotted' expression of HIPK2 in nuclei (Fig. 5e) (32) in cells of basal proliferating layers, while its expression disappeared in upper, nonproliferating ones (Fig. 5f).

Taken together, these results indicate that, in conditions that are independent of DNA damage, HIPK2 protein expression is strongly associated with cell proliferation while it becomes undetectable in cells arrested by non-damaging conditions.

Intense depletion of HIPK2 by siRNA induces cell-cycle arrest by p21^{Waf-1/Cip-1} expression

To test whether the HIPK2 suppression associated with cell-cycle exit is functional to this event, we acutely depleted HIPK2 from immortalized human fibroblasts and mammary cells (MCF10A) by HIPK2-specific siRNA transfection. Three different duplexes were used singly, or as a mix, with comparable results; data reported below were obtained with the mix. Consistent with observations we made with mouse bone marrow cells by shRNA, HIPK2 depletion by siRNA strongly reduced proliferation rate of both human fibroblasts and MCF10A

cells compared to their relative controls (Fig. 6a). No apoptosis was associated with these culture conditions until the last time point, 4 days after siRNA transduction (Fig. 6b), further supporting a role for HIPK2 in cell proliferation regulation.

To obtain initial clues about mechanisms that might be responsible for HIPK2 function in cell proliferation, we sought to test, by Western blot analysis, a series of cell-cycle-related proteins. Induction of cyclin-dependent kinase inhibitor p21^{Waf-1/Cip-1} was observed in HIPK2-depleted bone marrow cells (Fig. 4a) and this is known to contribute to cell-cycle withdrawal associated with different types of differentiation, including myogenesis (29,33), during which we observed HIPK2 suppression. Thus, we analysed p21^{Waf-1/Cip-1} expression in HIPK2-depleted cells. Up-regulation of p21^{Waf-1/Cip-1} was present in all HIPK2i populations we tested, including wild-type p53 expressing MCF10A (Fig. 6c, left panel), human fibroblasts (Fig. 6c, right panel), and HCT116 p53^{+/+} and p53^{-/-} cells (Fig. 6d), suggesting that p53 is not required for this event. However, in the last case, total amounts of p21^{Waf-1/Cip-1} did not even reach those present in p53-proficient HCT116 cells in basal conditions.

Next, we asked whether increased expression of p21^{Waf-1/Cip-1} induced by HIPK2 depletion contributes to the cell-cycle arrest. The p21^{Waf-1/Cip-1} knockout derivative

Figure 5. HIPK2 expression in proliferating and arrested conditions. (a) Cell-cycle profiles of normal peripheral blood lymphocytes purified by density gradient and maintained in the absence (resting) or presence (phytohaemagglutinin-stimulated) of phytohaemagglutinin. (b) Pair samples of lymphocytes were lysed and total cell extracts analysed by Western blotting for the indicated proteins. (c) Cell-cycle profiles of 32D cells maintained in proliferating condition (Prol); growth arrested by incubation in low levels of IL-3 for 48 h and stimulated to re-enter into the cell cycle by addition of high amounts of IL-3 for the indicated times. (d) Pair samples of cells analysed in (c) were analysed by real-time RT-PCR for *Hipk2* mRNA (upper panel) or lysed and total cell extracts analysed by Western blotting for indicated proteins (lower panels). (e, f) Immunohistochemistry of HIPK2 on normal human skin. (e) High magnification to evaluate nuclear 'dotted' morphology of HIPK2 is shown. (f) This panel shows low magnification to appreciate the HIPK2 positivity in basal, proliferating skin layers (Bl: basal layers) and HIPK2 negativity in the upper, nonproliferating skin layer (Sp, spinocellular layer; Co, corneal layer). HIPK2 is also expressed in the proliferating cells of a hair follicle (Hf).

of MCF10A cells were depleted of HIPK2 and were analysed for their proliferation rates (26). As shown in Fig. 6e, HIPK2 depletion was no longer able to inhibit proliferation in the absence of p21^{Waf-1/Cip-1}. Comparable results were obtained in human fibroblasts by concomitant depletion of HIPK2 and p21^{Waf-1/Cip-1}, indicating that p21^{Waf-1/Cip-1} up-regulation is required for the cell-cycle arrest. Interestingly, the p21^{Waf-1/Cip-1} induction that HIPK2 depletion was still able to induce in p53-null HCT116 cells promoted only mild reduction in cell proliferation (Fig. 6h,i), consistent with the model by which total amount of p21^{Waf-1/Cip-1} present in the cells, rather than its relative increment, are relevant for induction of growth arrest.

Overall, these results indicate that HIPK2 down-regulation might contribute to cell-cycle arrest by induction of p21^{Waf-1/Cip-1}.

Discussion

This study explored the possible role of interacting proteins HIPK2 and p53 in differentiation of skeletal muscle and haemopoietic cells. This particular investigation stems from our original identification of HIPK2 and p53 interaction. Indeed, we fished out HIPK2 using yeast

two-hybrid screening of an 11-day mouse embryo cDNA library using p53 as bait searching for p53 cofactors that might be involved in role(s) played by p53 during mouse embryo development, when p53 is highly expressed (18,20,34). Although the data reported thus far from several groups, including ours, highlighted relevance of HIPK2/p53 interaction in cell response to DNA damage (reviewed in 3,4), to our knowledge, there was no attempt to directly verify whether p53 and HIPK2 cooperate in cell differentiation. We tested this possibility by analysing HIPK2 expression during myogenesis and haemopoiesis in which p53 is known to trigger differentiation independently of regulation of differentiation-associated cell-cycle arrest and apoptosis (reviewed in 34). Surprisingly, we found strong suppression of HIPK2 expression at the onset of each differentiation condition we tested, clearly showing opposite behaviour between p53 and HIPK2 in this biological event. Thus, at least for skeletal muscle, granulocyte, and macrophage differentiation, HIPK2 and p53 do not have any cooperative function. At this stage, we cannot exclude the possibility that HIPK2/p53 interaction might be relevant in other types of differentiation, such as neuronal differentiation, or whether the interaction is only relevant in cell response to DNA damage, as previously shown. Indeed, we found that HIPK2 can be re-expressed

Figure 6. Effects of HIPK2 depletion in proliferating cells. Indicated cells were transduced with Stealth siRNA Duplex specific for HIPK2 (HIPK2i) or with the relative universal negative control (UNC). (a) Proliferation rates of MCF10A and human fibroblasts were calculated by direct counting. (b) Cell-cycle profiles of MCF10A treated as in (a). (c, d) Western blot analyses of indicated proteins were performed on total cell extracts from the indicated cells, 48 h post-transduction. Asterisk (*) indicates a nonspecific band. (e) MCF10A p21^{Waf-1/Cip-1} knockout derivative were transduced as in (a) and proliferation rate was calculated by direct counting. Relative cell viability of each time point is indicated in parentheses. (f, g) Human fibroblasts were depleted for the indicated proteins and analysed by Western blotting (f) or for cell proliferation (g). (h, i) Pair samples of cells analysed in (d) were tested for cell-cycle analysis (h) and proliferation rate (i).

in terminally differentiated cells upon stimulation with apoptosis-inducing doses of drugs, suggesting that damage-associated apoptotic function of HIPK2 is maintained in cells that permanently withdraw from the

cell cycle, and that it is distinct from the possible role HIPK2 suppression might have in cell-cycle arrest.

Although we obtained negative results relative to HIPK2/p53 interaction in differentiation, consistent

disappearance of HIPK2 we observed prompted us to evaluate whether it might be biologically relevant. We found that, besides differentiation-associated cell-cycle arrest, HIPK2 is suppressed during different types of cell-cycle exit and is re-expressed on cell-cycle re-entry, such as IL-3 stimulation of G₁-arrested 32D cells or phytohaemagglutinin stimulation of resting peripheral blood lymphocytes. In addition, HIPK2 depletion by RNA interference strongly inhibits cell proliferation in a p21^{Waf-1/Cip-1}-dependent manner. Increment of p21^{Waf-1/Cip-1} is regulated at transcriptional and/or post-transcriptional level(s) and is independent of p53, since it takes place also in p53-null HCT116 cells. However, at least in the HCT116 model, absence of p53 does not allow reaching a total amount of p21^{Waf-1/Cip-1} protein, comparable to that present in basal conditions in parental, p53-proficient cells, making subsequent inhibition of cell proliferation very mild. Interestingly, p53-independent expression of p21^{Waf-1/Cip-1} is induced in muscle and other terminally differentiating cells (29,33). Unfortunately, we could not directly verify whether inhibition of HIPK2 suppression during muscle differentiation can block p21^{Waf-1/Cip-1} up-regulation since forced expression of HIPK2 in myoblasts mimics DNA damage response and induces apoptosis. However, p21^{Waf-1/Cip-1} up-regulation induced by HIPK2 depletion might support the hypothesis that HIPK2 suppression at the onset of muscle and haemopoietic differentiation allows transcription of p21^{Waf-1/Cip-1}. This would also be compatible with transcriptional repression activity of HIPK2 (1,2,8,27,35) and with defects in cell proliferation observed in *Hipk2* and *Hipk1/Hipk2* knockout mice (5,8). This particular phenotype might not apply to all cell types; indeed, a mild proliferative advantage has been observed in epidermal cells of *Hipk2* knockout mice (36). However, our staining of normal human skin showed HIPK2 expression in proliferating, basal layer cells and HIPK2 disappearance in nonproliferating upper layers. Whether this apparent discrepancy depends on species specificity or is the result of a completely different approach (gene knockout *versus* analysis of protein expression) needs to be clarified. Characterization of molecular mechanism(s) through which HIPK2 contributes to cell proliferation, independently of its role in cell response to DNA damage, would provide tools to approach the issue.

Depletion of HIPK2 by anti-sense oligonucleotides (9,10) or by RNA interference (13,27,37) was shown to induce strong resistance to DNA damage-induced apoptosis. Here, we show that HIPK2 depletion inhibits cell proliferation while cell-cycle re-entry of arrested cells is associated with HIPK2 expression. These differences can be explained by the different levels of HIPK2 depletion. Indeed, mild reduction of HIPK2 expression is sufficient to induce resistance to damage-induced apoptosis (see

HIPK2 protein expression upon depletion in 9,10,13,30; data not shown). This type of behaviour is also consistent with the observation that HIPK2 expression is strongly suppressed by MDM2-mediated degradation upon sublethal DNA damage, when cell-cycle arrest is required to allow DNA repair (4,38). In contrast, inhibition of cell proliferation we have reported here, is obtained with deep depletion of HIPK2, such as those obtained by high efficient transduction of stealth siRNA that reduces *Hipk2* mRNA levels between 30- and 50-fold on average, compared to 10-fold reduction in the same cells by shRNA transfection (A.P. and C.R. unpublished results). In summary, our results support a role for HIPK2 in regulation of cell proliferation distinct from its role in DNA damage response.

Acknowledgements

The financial support of Associazione Italiana per la Ricerca sul Cancro and Ministero della Sanita are gratefully acknowledged. We also wish to thank all the people cited in the text for providing us with cells, reagents, and/or expression vectors.

References

- 1 Kim YH, Choi CY, Lee SJ, Conti MA, Kim Y (1998) Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. *J. Biol. Chem.* **273**, 25875–25879.
- 2 Choi CY, Kim YH, Kwon HJ, Kim Y (1999) The homeodomain protein NK-3 recruits Groucho and a histone deacetylase complex to repress transcription. *J. Biol. Chem.* **274**, 33194–33197.
- 3 Calzado MA, Renner F, Roscic A, Schmitz ML (2007) HIPK2: a versatile switchboard regulating the transcription machinery and cell death. *Cell Cycle* **15**, 6 (2), 139–143.
- 4 Rinaldo C, Prodosmo A, Siepi F, Soddu S (2007) HIPK2: a multi-talented partner for transcription factors in DNA damage response and development. *Biochem. Cell Biol.* **85** (4), 411–418.
- 5 Isono K, Nemoto K, Li Y, Takada Y, Suzuki R, Katsuki M, Nakagawara A, Koseki H (2006) Overlapping roles for homeodomain-interacting protein kinases *hipk1* and *hipk2* in the mediation of cell growth in response to morphogenetic and genotoxic signals. *Mol. Cell. Biol.* **26**, 2758–2771.
- 6 Aikawa Y, Nguyen LA, Isono K, Takakura N, Tagata Y, Schmitz ML, Koseki H, Kitabayashi I (2006) Roles of HIPK1 and HIPK2 in AML1- and p300-dependent transcription, hematopoiesis and blood vessel formation. *EMBO J.* **25**, 3955–3965.
- 7 Zhang J, Pho V, Bonasera SJ, Holtzman J, Tang AT, Hellmuth J, Tang S, Janak PH, Tecott LH, Huang EJ (2007) Essential function of HIPK2 in TGF β -dependent survival of midbrain dopamine neurons. *Nat. Neurosci.* **10**, 77–86.
- 8 Wiggins AK, Wei G, Doxakis E, Wong C, Tang AA, Zang K, Luo EJ, Neve RL, Reichardt LF, Huang EJ (2004) Interaction of Brn3a and HIPK2 mediates transcriptional repression of sensory neuron survival. *J. Cell Biol.* **167**, 257–267.
- 9 Hofmann TG, Moller A, Sirma H, Zentgraf H, Taya Y, Dröge W, Will H, Schmitz ML (2002) Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. *Nat. Cell Biol.* **4**, 1–10.

- 10 D'Orazi G, Cecchinelli B, Bruno T, Manni I, Higashimoto Y, Saito S, Gostissa M, Coen S, Marchetti A, Del Sal G, Piaggio G, Fanciulli M, Appella E, Soddu S (2002) Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. *Nat. Cell Biol.* **4**, 11–19.
- 11 Kim EJ, Park JS, Um SJ (2002) Identification and characterization of HIPK2 interacting with p73 and modulating functions of the p53 family *in vivo*. *J. Biol. Chem.* **277**, 32020–32028.
- 12 Wang Y, Debatin KM, Hug H (2001) HIPK2 overexpression leads to stabilization of p53 protein and increased p53 transcriptional activity by decreasing Mdm2 protein levels. *BMC Mol. Biol.* **2**, 8–16.
- 13 Di Stefano V, Rinaldo C, Sacchi A, Soddu S, D'Orazi G (2004) Homeodomain-interacting protein kinase-2 activity and p53 phosphorylation are critical events for cisplatin-mediated apoptosis. *Exp. Cell Res.* **293**, 311–320.
- 14 Zhang Q, Yoshimatsu Y, Hildebrand J, Frisch SM, Goodman RH (2003) Homeodomain interacting protein kinase 2 promotes apoptosis by down-regulating the transcriptional corepressor CtBP. *Cell* **115**, 177–186.
- 15 Rui Y, Xu Z, Lin S, Li Q, Rui H, Luo W, Zhou HM, Cheung PY, Wu Z, Ye Z, Li P, Han J, Lin SC (2004) Axin stimulates p53 functions by activation of HIPK2 kinase through multimeric complex formation. *EMBO J.* **23**, 4583–4594.
- 16 Rinaldo C, Prodosmo A, Mancini F, Iacovelli S, Sacchi A, Moretti F, Soddu S (2007) MDM2-regulated degradation of HIPK2 prevents p53Ser46 phosphorylation and DNA damage-induced apoptosis. *Mol. Cell* **25**, 739–750.
- 17 Soddu S, Sacchi A (1997) P53 role in DNA repair and tumorigenesis. *J. Exp. Clin. Cancer Res.* **16**, 237–242.
- 18 Schmid P, Lorenz A, Hameister H, Montenarh M (1991) Expression of p53 during mouse embryogenesis. *Development* **113**, 857–865.
- 19 Soddu S, Blandino G, Scardigli R, Coen S, Marchetti A, Rizzo MG, Bossi G, Cimino L, Crescenzi M, Sacchi A (1996) Interference with p53 protein inhibits hematopoietic and muscle differentiation. *J. Cell Biol.* **134** (1), 193–204.
- 20 Almog N, Rotter V (1997) Involvement of p53 in cell differentiation and development. *Biochim. Biophys. Acta* **1333**, F1–F27.
- 21 Mazzaro G, Bossi G, Coen S, Sacchi A, Soddu S (1999) The role of wild-type p53 in the differentiation of primary hemopoietic and muscle cells. *Oncogene* **18** (42), 5831–5835.
- 22 Porrello A, Cerone MA, Coen S, Gurtner A, Fontemaggi G, Cimino L, Piaggio G, Sacchi A, Soddu S (2000) p53 regulates myogenesis by triggering the differentiation activity of pRb. *J. Cell Biol.* **151**, 1295–1303.
- 23 Cam H, Griesmann H, Beitzinger M, Hofmann L, Beinoraviciute-Kellner R, Sauer M, Hüttinger-Kirchhof N, Oswald C, Friedl P, Gattenlöhner S, Burek C, Rosenwald A, Stiewe T (2006) p53 family members in myogenic differentiation and rhabdomyosarcoma development. *Cancer Cell* **10** (4), 281–293.
- 24 Scardigli R, Bossi G, Blandino G, Crescenzi M, Soddu S, Sacchi A (1997) Expression of exogenous wt-p53 does not affect normal hematopoiesis: implications for bone marrow purging. *Gene Ther.* **4** (12), 1371–1378.
- 25 Ciardiello F, McGeady ML, Kim N, Basolo F, Hynes N, Langton BC, Yokozaki H, Saeki T, Elliott JW, Masui H (1990) Transforming growth factor- α expression is enhanced in human mammary epithelial cells transformed by an activated c-Ha-ras protooncogene but not by the c-neu protooncogene, and overexpression of the transforming growth factor- α complementary DNA leads to transformation. *Cell Growth Differ.* **1** (9), 407–420.
- 26 Karakas B, Weeraratna A, Abukhdeir A, Blair BG, Konishi H, Arena S, Becker K, Wood W III, Argani P, De Marzo AM, Bachman KE, Park BH (2006) Interleukin-1 α mediates the growth proliferative effects of transforming growth factor-beta in p21 null MCF-10A human mammary epithelial cells. *Oncogene* **25**, 5561–5569.
- 27 Cecchinelli B, Lavra L, Rinaldo C, Iacovelli S, Gurtner A, Gasbarri A, Ulivieri A, Del Prete F, Trovato M, Piaggio G, Bartolazzi A, Soddu S, Sciacchitano S (2006a) Repression of the antiapoptotic molecule galectin-3 by homeodomain-interacting protein kinase 2-activated p53 is required for p53-induced apoptosis. *Mol. Cell. Biol.* **26**, 4746–4757.
- 28 Cerone MA, Marchetti A, Bossi G, Blandino G, Sacchi A, Soddu S (2000) p53 is involved in the differentiation but not in the differentiation-associated apoptosis of myoblasts. *Cell Death Differ.* **7** (5), 506–508.
- 29 Halevy O, Novitch BG, Spicer BD, Skapek SX, Rhee J, Hannon GJ, Beach D, Lassar AB (1995) Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267**, 1018–1021.
- 30 Cecchinelli B, Porrello A, Lazzari C, Gradi A, Bossi G, D'Angelo M, Sacchi A, Soddu S (2006b) Ser58 of mouse p53 is the homologue of human Ser46 and is phosphorylated by HIPK2 in apoptosis. *Cell Death Differ.* **13**, 1994–1997.
- 31 Bossi G, Mazzaro G, Porrello A, Crescenzi M, Soddu S, Sacchi A (2004) Wild-type p53 gene transfer is not detrimental to normal cells *in vivo*: implications for tumor gene therapy. *Oncogene* **23**, 418–425.
- 32 Moller A, Sirma H, Hofmann TG, Rueffer S, Klimczak E, Dröge W, Schmitz ML (2003) PML is required for homeodomain-interacting protein kinase 2 (HIPK2)-mediated p53 phosphorylation and cell cycle arrest but is dispensable for the formation of HIPK domains. *Cancer Res.* **63**, 4310–4314.
- 33 Parker SB, Eichele G, Zhang P, Rawls A, Sands AT, Bradley A, Olson EN, Harper JW, Elledge SJ (1995) p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells. *Science* **267**, 1024–1027.
- 34 Cecchinelli B, Soddu S (2004) Physiology and regulation of p53: the role in differentiation and development. In Schmitz ML, Bacher S, eds. *Mechanisms of Signal Transduction and Inducible Gene Expression*, pp. 267–280. Kerala, India: Research Signpost.
- 35 Harada J, Kokura K, Kanei-Ishii C, Nomura T, Khan MM, Kim Y, Ishii S (2003) Requirement of the co-repressor homeodomain-interacting protein kinase 2 for ski-mediated inhibition of bone morphogenetic protein-induced transcriptional activation. *J. Biol. Chem.* **278**, 38998–39005.
- 36 Wei G, Ku S, Ma GK, Saito S, Tang AA, Zhang J, Mao JH, Appella E, Balmain A, Huang EJ (2007) HIPK2 represses beta-catenin-mediated transcription, epidermal stem cell expansion, and skin tumorigenesis. *Proc. Natl. Acad. Sci. USA* **104** (32), 13040–13045.
- 37 Taira N, Nihira K, Yamaguchi T, Miki Y, Yoshida K (2007) DYRK2 is targeted to the nucleus and controls p53 via Ser46 phosphorylation in the apoptotic response to DNA damage. *Mol. Cell* **25** (5), 725–738.
- 38 Shmueli A, Oren M (2007) Mdm2: p53's lifesaver? *Mol. Cell* **25** (6), 794–796.