OPEN

Citation: Cell Death and Disease (2013) 4, e737; doi:10.1038/cddis.2013.246

www.nature.com/cddis

A p38mapk-p53 cascade regulates mesodermal differentiation and neurogenesis of embryonic stem cells

Y Hadjal[1,2], O Hadadeh[1,2], CEl Yazidi[1,2], E Barruet[1,2] and B Binétruy*[,1,2]

Embryonic stem cells (ESCs) differentiate *in vivo* and *in vitro* into all cell lineages, and they have been proposed as cellular therapy for human diseases. However, the molecular mechanisms controlling ESC commitment toward specific lineages need to be specified. We previously found that the p38 mitogen-activated protein kinase (p38MAPK) pathway inhibits neurogenesis and is necessary to mesodermal formation during the critical first 5 days of mouse ESC commitment. This period corresponds to the expression of specific master genes that direct ESC into each of the three embryonic layers. By both chemical and genetic approaches, we found now that, during this phase, the p38MAPK pathway stabilizes the p53 protein level and that interfering directly with p53 mimics the effects of p38MAPK inhibition on ESC differentiation. Anti-p53 siRNA transient transfections stimulate Bcl2 and Pax6 gene expressions, leading to increased ESC neurogenesis compared with control transfections. Conversely, p53 downregulation leads to a strong inhibition of the mesodermal master genes *Brachyury* and *Mesp1* affecting cardiomyogenesis and skeletal myogenesis of ESCs. Similar results were found with $p53^{-/-}$ ESCs compared with their wild-type counterparts. In addition, knockout p53 ESCs show impaired smooth muscle cell and adipocyte formation. Use of anti-Nanog siRNAs demonstrates that certain of these regulations result partially to p53-dependent repression of Nanog gene expression. In addition to its well-known role in DNA-damage response, apoptosis, cell cycle control and tumor suppression, p53 has also been involved *in vivo* in embryonic development; our results show now that p53 mediates, at least for a large part, the p38MAPK control of the early commitment of ESCs toward mesodermal and neural lineages.
Cell Death and Disease (2013) **4,** e737; doi:10.1038/cddis.2013.246; published online 25 July 2013
Subject Category: Experimental Medicine

Because of their pluripotency and unlimited proliferation properties, embryonic stem cells (ESCs) have been proposed as cellular therapy for human diseases. However, this potential is impaired by the poor efficiency of ESC differentiation into mature differentiated cells and by the persistence of undifferentiated cells with a tumorigenic capacity within differentiated cultures. Therefore, there is a crucial need for a better understanding of molecular mechanisms governing ESC commitment into specific lineages. Self-renewal of mouse ESCs is dependent on the intracellular pathways involving a complex interplay between specific epigenetic processes, miRNAs and transcription factors, such as Oct4, Nanog or Sox2 (see for reviews[1,2]). *In vitro* ESC differentiation can be induced by various experimental protocols resulting in the commitment into a variety of mature differentiated cell types (see for reviews[3–5]). This process can be modulated between the 2nd and 5th day of differentiation by the potent morphogen retinoic acid (RA) that induces differentiation into neurons and adipocytes[6–8] and, conversely, inhibits cardiomyocyte and skeletal myotube formation.[6,9,10] Although ESC differentiation experimental models recapitulate the successive steps from pluripotency to well-differentiated cells, the molecular mechanisms underlying these processes are poorly understood.

p38 Mitogen-activated protein kinase (p38MAPK) is activated by phosphorylation in response to osmotic stress, UV and various cytokines involved in inflammatory responses and has been proposed to regulate several cellular processes, such as proliferation, cell survival and differentiation.[11,12] Deletion of the *p38α* gene leads to early embryonic lethality between 11.5 and 12.5 days,[13,14] demonstrating an essential role of p38MAPK in development. We previously found that the p38MAPK pathway is necessary at two successive early steps during the differentiation process of ESC commitment into cardiomyocyte, endothelial cells, smooth muscle and skeletal muscle cell lineages, whereas it inhibits neurogenesis.[10,15] Particularly, this p38MAPK control takes place during the critical first 5 days of ESC commitment, concomitantly with the expression of specific master genes that direct ESC into each of the three embryonic layers. To better understand the molecular mechanisms underlying these processes, we treated ESCs with a p38MAPK-specific

[1]INSERM U910, Faculté de Médecine, 27 Boulevard Jean Moulin, Marseille Cedex 5, France and [2]Faculté de Médecine, Aix-Marseille Université, 27 Boulevard Jean Moulin, Marseille Cedex 5, France
*Corresponding author: B Binétruy, INSERM U910, Faculté de Médecine La Timone, 27 Boulevard Jean Moulin, Marseille Cedex 5 13385, France. Tel: 33 04 9132 4886; Fax: 33 04 9182 6958; E-mail: Bernard.Binetruy@univ-amu.fr
Keywords: mouse embryonic stem cells; p53; p38MAPK; neurogenesis; cardiomyogenesis; skeletal myogenesis
Abbreviations: ERK, extracellular signal-regulated kinase; EB, embryoid body; ESC, embryonic stem cell; MAPK, mitogen-activated protein kinase; MyH1, myosin heavy chain-1; RA, retinoic acid; RT-qPCR, reverse-transcriptase quantitative PCR; PBS, phosphate-buffered saline
Received 31.5.13; revised 05.6.13; accepted 06.6.13; Edited by G Raschellà

inhibitor and performed microarray experiments on mRNAs extracted from treated *versus* untreated cells. Interestingly, one of the regulated targets is *Bcl2*, a well-known p53-inhibited gene.

The p53 protein is a short-lived, latent transcription factor, which is activated and stabilized in response to a wide range of cellular stresses; exacerbated p53 activation leads to dramatic cellular responses such as cell cycle arrest, apoptosis or senescence. Furthermore, p53 is a crucial tumor suppressor that is somatically mutated in about half of all the human cancers and has multiple roles in maintaining genomic stability in somatic cells (see for review[16]). Its role in ESC biology seems paradoxical, indeed undifferentiated ESCs fail to undergo p53-dependent cell cycle arrest, apoptosis and senescence after DNA damage. By contrast, p53 can induce ESC differentiation by directly suppressing the expression of Nanog, one of the master transcriptional factor maintaining ESC self-renewal (Lin *et al.*[17] and see for review[18]). However, its precise role in ESC commitment toward specific lineages is unknown.

We analyzed the role of p53 in ESC differentiation and found that p53 is an important mediator of p38MAPK-induced control of ESC commitment.

Results

Differential display experiments identify p53 as a potential mediator of the p38MAPK pathway. CGR8 mouse ESCs were induced to differentiate by forming embryoid bodies (EBs) and, at day 3, treated or not by the specific p38MAPK inhibitor PD169316, and mRNAs were extracted and analyzed by microarray experiments 48 h later. For each condition, three mRNA batches were hybridized on DNA chips spotted with 44 K mouse gene-specific sequences, and regulations of genes of interest were confirmed by reverse-transcriptase quantitative PCR (RT-qPCR) technique (data not shown). Among the identified targets, we focused our attention on one gene upregulated in our experiments, *Bcl2*, a well-known p53-inhibited gene.[19,20] Interestingly, *Bcl2* has already been described as a p38MAPK-inhibited gene and involved in ESC neurogenesis.[21] Altogether, these data suggested that p53 could mediate the p38MAPK control on ESC differentiation and prompted us to verify this hypothesis.

Chemical inhibition of p53 mimics the effects of p38MAPK inhibition on target genes and ESC commitment. CGR8 ESCs induced to differentiate were treated by either the p38MAPK inhibitor or pifithrin-α, a specific p53 chemical inhibitor,[22] and compared with untreated controls. As expected, p38MAPK inhibitor treatment induces Bcl2 gene expression and enhances neurogenesis, characterized by MAP2 gene expression; interestingly, p53 inhibition induces similar effects (Figures 1a and b). Conversely, cardiomyocyte formation was repressed by these treatments; as judged by Mesp1 expression (Figure 1c), a cardiac mesodermal master gene involved in ESC cardiomyogenesis[23] and TroponinT2

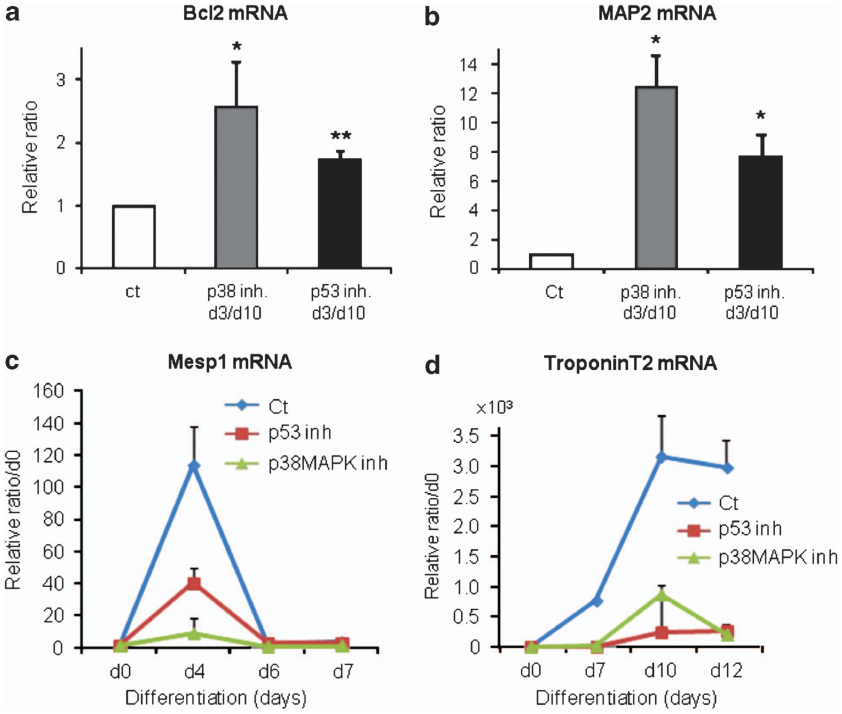

Figure 1 Chemical inhibition of p53 mimics the effects of p38MAPK inhibition. EBs from wild-type CGR8 ESCs at day 0 were induced to differentiate and treated or not, between day 3 and 10, by either the PD169316 p38MAPK inhibitor or the pifithrin-α p53 inhibitor. mRNAs from EBs were extracted at day 10 (**a** and **b**) or at various time as indicated (**c** and **d**) and analyzed by real-time RT-PCR for the expression of Bcl2 (**a**), MAP2 (**b**), Mesp1 (**c**) and TroponinT2 (**d**) gene expression. Results are expressed in arbitrary units, with the values of untreated CGR8 control cells (Ct) taken as 1, and are the mean ± S.E.M. of at least three independent experiments. Significance to Ct values is given as: $^{*}P<0.05$ and $^{**}P<0.01$

expression (Figure 1d), a cardiomyocyte-specific marker. These data suggest that the p38MAPK role in ESC differentiation could indeed be mediated by a p38MAPK-p53 cascade.

The p38 MAPK pathway regulates the stability of endogenous p53 proteins at day 3 of differentiation. In response to osmotic stress, p53 proteins have been shown to be directly phosphorylated by p38MAPK on several serine residues,[24,25] and, in addition, p53 acetylation has been involved in human ESC differentiation.[26] Therefore, we analyzed the consequences of p38MAPK inhibition on p53 protein expression and post-translational regulation. We could not find any significant effect of p38MAPK inhibition neither on phosphorylation of p53 serine residues 15 and 392 or on p53 acetylation (data not shown). As already shown,[17] we found that the p53 protein level decreases rapidly as ESCs differentiated (Figure 2a); however, at day 3 of differentiation, a stronger decrease is systematically observed 24 h after p38MAPK inhibition (Figure 2b). Noteworthy, this effect is not detectable after 4 h of inhibition. This regulation is not at the p53 transcriptional level, neither at day 3 (Figure 2b, lower panel) or during the first 5 days of differentiation (Figure 2c), and is not associated to a control of the cytoplasmic/nucleic localization of p53 proteins (Figure 2d). Finally, we found that this effect is not mediated by a p38MAPK-dependent regulation of the proteasome activity. Indeed, although inhibition of the proteasome efficiently stabilizes p53 proteins, the destabilization of p53 proteins induced by p38MAPK inhibition is still present after treatment with the proteasome inhibitor MG132 (Figure 2e). Although additional works are necessary to understand the molecular mechanism by which the p38MAPK pathway augments p53 protein stability, the experiments shown suggest that p38MAPK inhibition leads to a shorter half-life of p53 protein. Furthermore, the fact that this regulation necessitates 24 h (Figure 2b) strongly suggests that it corresponds to an indirect mechanism involving intermediate partner(s).

To precise the role of p53 in ESC differentiation and to avoid potential unspecific effects of chemical compounds, we then

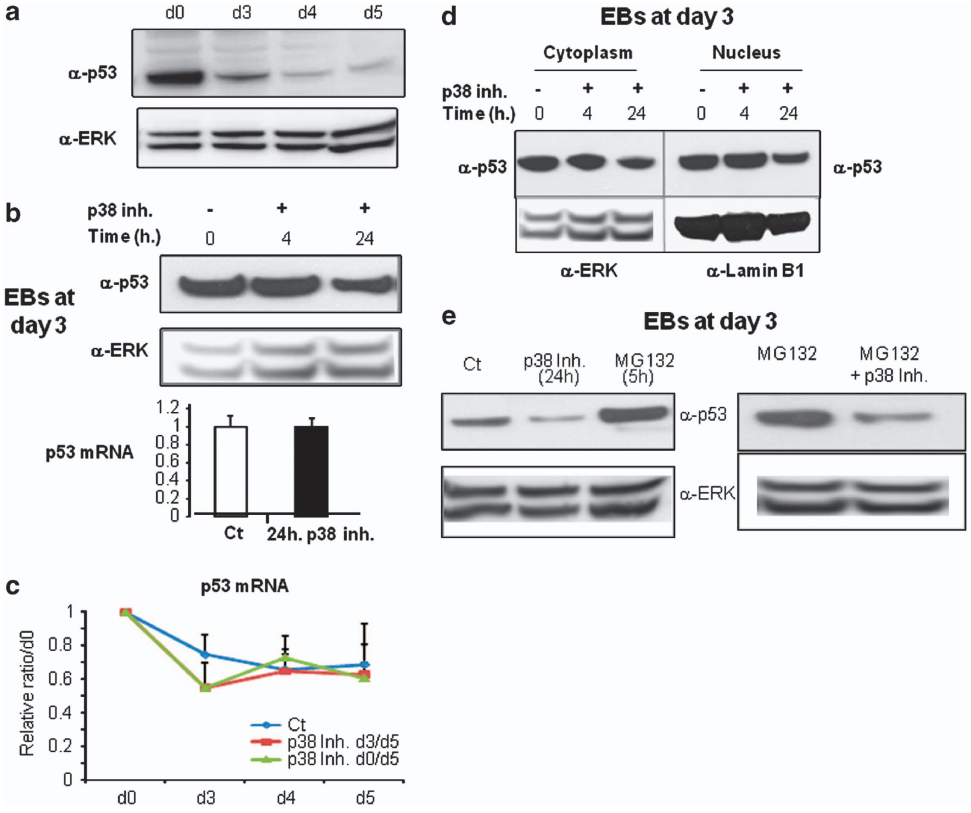

Figure 2 The p38MAPK regulates the stability of p53 proteins in CGR8 ESCs. (**a**) ESCs were induced to differentiate, and total cellular proteins were extracted at various time from day 0 to 5, as indicated, and analyzed by western blotting with either α-p53 antibodies or α-extracellular signal-regulated kinase (α-ERK) antibodies as loading control. (**b**) ESCs were induced to differentiate and, at day 3, treated or not, as indicated, by the p38MAPK inhibitor. Total cellular proteins or mRNAs were extracted 0, 4 or 24 h later and analyzed either by western blotting with α-p53 antibodies or α-ERK antibodies as loading control (upper panels) or by real-time RT-PCR for the expression of *p53* gene (lower panel). (**c**) ESCs were induced to differentiate and treated or not, as indicated, by the p38MAPK inhibitor either from day 0 to 5 or from day 3 to 5. mRNAs were extracted at various time, as indicated, and analyzed by real-time RT-PCR for the expression of *p53* gene. Results are expressed in arbitrary units, with the values of untreated CGR8 control cells at day 0 taken as 1, and are the means ± S.E.M. of at least three independent experiments. (**d**) ESCs were induced to differentiate and, at day 3, treated or not, as indicated, by the p38MAPK inhibitor. Cytoplasmic and nuclear cellular proteins were extracted 0, 4 or 24 h later and analyzed by western blotting with either α-p53 antibodies or α-ERK antibodies as loading control for cytoplasmic extracts, or α-LaminB1 as loading control for nuclear extracts. (**e**) ESCs were induced to differentiate until day 3 and treated or not either by the p38MAPK inhibitor for 24 h or by 20μM of proteasome inhibitor MG132 for 5 h (left panel); in parallel experiments, EBs were treated either by the proteasome inhibitor alone for 5 h or by the p38MAPK inhibitor for 24 h and then by MG132 for 5 h (right panel). Total cellular proteins were extracted and analyzed by western blotting with either α-p53 antibodies or α-ERK antibodies as loading control

used two genetic approaches: transient transfections of anti-p53 siRNAs and analysis of differentiation capacities of ESC knockout for the *p53* gene.[27]

siRNAs against p53 efficiently inhibit p53 expression and activity in ESCs. Anti-mouse-p53 siRNAs (siRNA SMART pool; Dharmacon, Waltham, MA, USA) were transiently transfected into undifferentiated CGR8 ESCs and p53 mRNA expression analyzed by RT-qPCR 24 and 48 h later. A strong decrease in expression is consistently observed compared with cells transfected with control siRNAs, with only 8.5–10% of p53 mRNA left (Figure 3a). This p53 inhibition is also detected at the protein level, as shown by western blot experiment (Figure 3b). We then analyzed the p53 repression during differentiation; undifferentiated ESCs were transfected with control or anti-p53 siRNAs, the day after (day 0) ESCs were induced to differentiate, and p53 mRNAs were analyzed at days 0, 3, 4, 5, 7 and 12 of differentiation. A significant p53 repression persists until day 5 of the protocol, and is only lost at day 7 (Figure 3c). Importantly, this inhibitory effect last long enough to cover the first 5 days of critical period for ESC commitment. We then investigated the effects of anti-p53 siRNA transfections on p53 activity. As expected, compared with control cells, transfections of anti-p53 siRNAs in ESCs lead to a 3–4-fold increase in Bcl2 expression (Figure 3d), whereas the expression of *p21* gene, a positively regulated p53 target, is decreased by 70% at 24 h and 60% at 48 h (Figure 3e).

siRNA-mediated inhibition or homozygote gene knockout of p53 interfere with ESC differentiation. Differentiation capacities of CGR8 ESCs transfected by anti-p53 siRNAs compared with control siRNAs transfection and those of $p53^{-/-}$ ESCs compared with their wild-type counterparts were then analyzed. Anti-p53 siRNA-transfected CGR8 ESCs and $p53^{-/-}$ ESCs display, on the one hand, augmented neurogenesis (Figure 4a) and, on the other hand, a strong inhibition of cardiomyogenesis, illustrated by the decrease in gene expression of both the cardiac transcription factor Nkx2.5 (Supplementary Figure S1A) and the cardiac marker TroponinT2 (Figure 4b) compared with controls. This cardiac inhibition is also clearly detected by immunofluorescence experiments using α-troponinT2 antibodies (Figures 4c and d). In addition to ESC neurogenesis and cardiomyogenesis, the p38MAPK pathway controls also endothelial cell, smooth and skeletal muscle cell[10] and adipocyte[28] lineage formation. Similarly to p38MAPK inhibition, we found that the two p53-inhibition approaches induce a strong inhibition of skeletal myogenesis compared with controls; as judged by the decrease in myosin heavy chain-1 (MyH1) mRNA (Figure 5a) and protein (Figure 5b) expression and by immunofluorescence experiments using α-MyH1 antibodies (Figures 5c and d). By contrast, although p38MAPK inhibition leads to a decrease in endothelial and smooth muscle cell formation and to an increase in adipogenesis, these three cell lineages are not significantly affected by anti-p53 siRNA transfections. These lineages were characterized by gene expression of the following specific markers: Tie2 and Flk1 for endothelial differentiation (Supplementary Figures S1B and C, left parts); α-smooth muscle actin for smooth muscle cells (Figure 6a, left part); and peroxisome-proliferative-activated receptor gamma and adipocyte-specific lipid-binding protein for adipocytes (Figures 6b and c, left parts). Similarly, endothelial differentiation is not modified in $p53^{-/-}$ ESCs compared with their wild-type counterparts (Supplementary Figures S1B and C, right parts). Surprisingly, smooth muscle

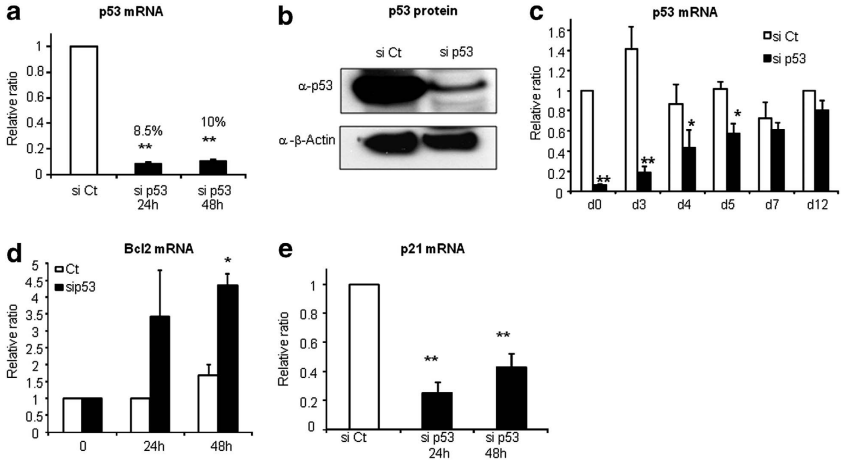

Figure 3 siRNAs against p53 inhibit p53 expression and activity in CGR8 ESCs. (**a** and **b**) Undifferentiated ESCs were transfected overnight with control siRNAs (si Ct) or anti-p53 siRNAs (si p53), kept in undifferentiated conditions. mRNAs (**a**) or proteins (**b**) were extracted after either 24 or 48 h, as indicated, and analyzed by real-time RT-PCR or western blotting for the expression of *p53* gene. (**a**) Results are expressed in arbitrary units, with the values of untreated CGR8 control cells taken as 1, and are the means ± S.E.M. of at least three independent experiments; residual p53 mRNA expression is indicated in %. (**b**) Total proteins were analyzed with either α-p53 antibodies or α-actin antibodies as loading control. (**c**) Undifferentiated ESCs were transfected overnight with si Ct or si p53 and induced to differentiate. mRNAs were extracted at various time from day 0 to 12, as indicated, and analyzed by real-time RT-PCR for the expression of *p53* gene. Results are expressed in arbitrary units, with the values of control siRNA-transfected control cells at day 0 taken as 1, and are the means ± S.E.M. of at least three independent experiments. (**d** and **e**) Undifferentiated ESCs were transfected overnight with si Ct or si p53 and kept in undifferentiated conditions. mRNAs were extracted 24 or 48 h later and analyzed by real-time RT-PCR for the expression of *Bcl2* (**d**) or *p21* (**e**) genes. Results are expressed in arbitrary units, with the values of control siRNA-transfected control cells taken as 1, and are the means ± S.E.M. of at least three independent experiments

cell formation and adipogenesis are strongly inhibited in $p53^{-/-}$ ESCs (Figures 6a–c, right parts). These data reveal different biological effects of transient p53 inhibition by siRNAs *versus* p53 homozygote gene knockout, suggesting variable successive roles for this gene during ESC differentiation. Altogether, our results also suggest the existence of other mediators than p53 for the p38MAPK pathway to account for all its effects on ESC differentiation.

To precise the p53 role in ESC differentiation, we analyzed the effects of p53 inhibition on expression of master genes important during embryonic development and ESC commitment. We focused our analysis on master genes whose gene expression peaked during the day 0–5 period of ESC differentiation, and therefore susceptible to be regulated by siRNA transient transfection experiments. Consistent with neurogenesis regulation, the peak expression of the *Pax6* ectodermal master gene is augmented by anti-p53 siRNA transfections (Figure 7a). We also investigated the expression of three master genes relevant to mesodermal lineages: *Brachyury*, *Mesp1* and *TBX1*. Gene expressions of Brachyury and Mesp1, which peak at day 5, are almost completely abolished by p53 inhibition, either after anti-p53 siRNA transfections or in $p53^{-/-}$ cells (Figures 7b–e). These inhibitions could account for the p53 effects on mesodermal-derived differentiations. By contrast, although TBX1 is not significantly affected by anti-p53 siRNAs (Figure 7f), expression of this gene is strongly downregulated in knockout cells (Supplementary Figure S2A), suggesting for this gene a complex regulation by p53. Noteworthy, the peak of expression of TBX1 appears at day 7, later than Brachyury and Mesp1, which could explain the lack of regulation by anti-p53 siRNAs.

p53-dependent Nanog repression contributes partially to the p53 effects on ESC differentiation. ESC differentiation is accompanied by the rapid downregulation of Nanog gene expression, a crucial gene involved in ESC self-renewal. It has been shown that this regulation is mediated by p53, which, upon differentiation, gets activated and represses Nanog expression by direct binding to its promoter.[17] We tested the hypothesis that this p53-dependent Nanog repression would contribute to p53 regulation of ESC differentiation. Consequently, Nanog expression should be augmented in $p53^{-/-}$ ESCs, and interfering with this expression should affect their differentiation capacities. Indeed, we found that a significant percentage of the level of Nanog expression in undifferentiated cells persists until day 12 of the differentiation of $p53^{-/-}$ ESCs, whereas this

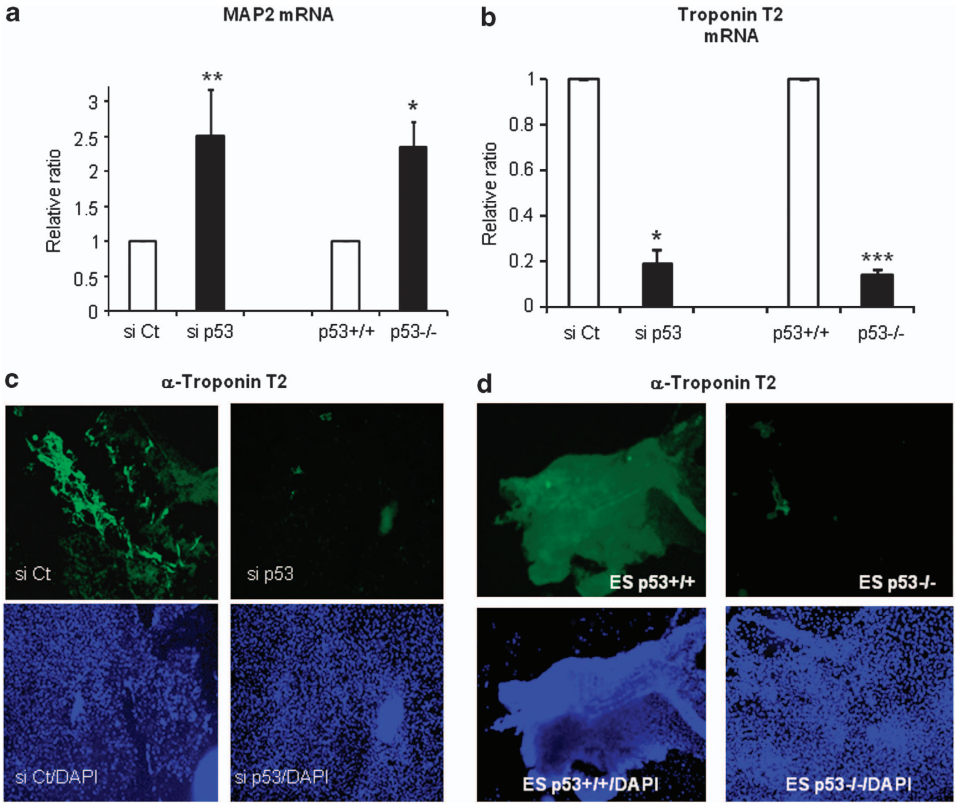

Figure 4 Effects of anti-p53 siRNAs or homozygote gene knockout of p53 on ESC neurogenesis and cardiomyogenesis. Undifferentiated CGR8 ESCs were transfected overnight with control siRNAs (si Ct) or anti-p53 siRNAs (si p53). The day after, day 0, these cells and $p53^{-/-}$ ESCs ($p53^{-/-}$) and their wild-type counterparts ($p53^{+/+}$) were induced to differentiate, cultivated until day 7 and replated on petri dishes until day 12 for analysis of neurogenesis (**a**) or cardiomyogenesis (**b**–**d**). mRNAs were extracted and analyzed by real-time RT-PCR for MAP2 (**a**) or TroponinT2 (**b**) gene expression. Results are expressed in arbitrary units, with the values of untransfected CGR8 control cells or wild-type control ESCs taken as 1, and are the means ± S.E.M. of at least three independent experiments. Significance to Ct values is given as: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. For immunofluorescence analysis, differentiated cell cultures were fixed and incubated with α-TroponinT2 (**c** and **d**) antibodies (upper panels), and nuclei were counterstained with DAPI (lower panels); representative fields are shown

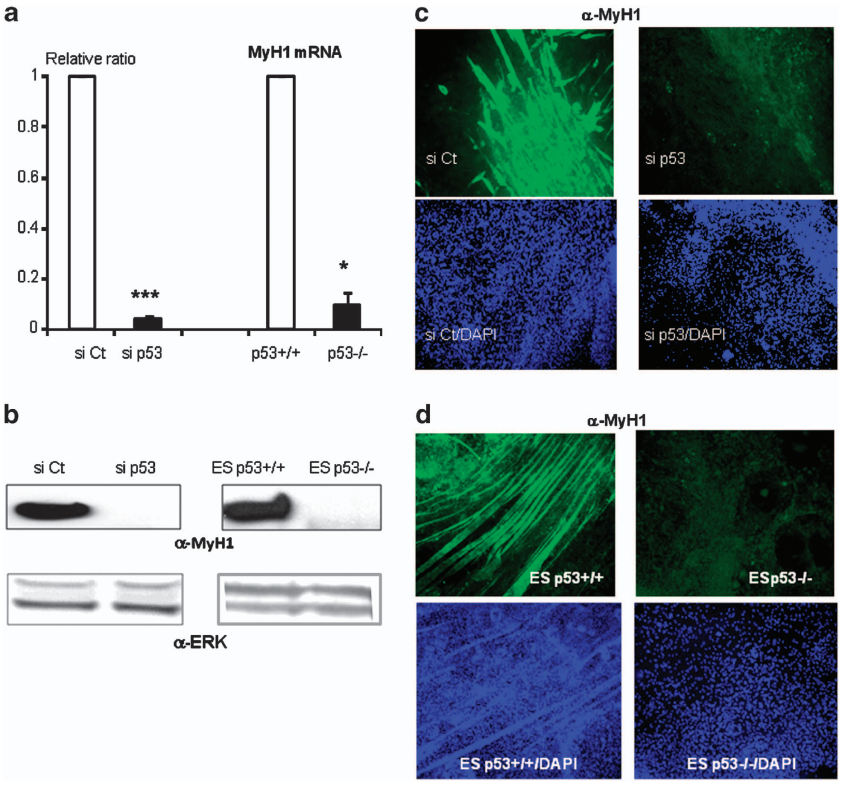

Figure 5 Effects of anti-p53 siRNAs or homozygote gene knockout of p53 on ESC skeletal myogenesis. Undifferentiated CGR8 ESCs were transfected overnight with control siRNAs (si Ct) or anti-p53 siRNAs (si p53). The day after, day 0, these cells and p53$^{-/-}$ ESCs (p53$^{-/-}$) and their wild-type counterparts (p53$^{+/+}$) were induced to differentiate, cultivated until day 7 and replated on petri dishes until day 26 for skeletal myotube formation. (**a**) mRNAs were extracted and analyzed by real-time RT-PCR for MyH1 gene expression. Results are expressed in arbitrary units, with the values of untransfected CGR8 control cells or wild-type control ESCs taken as 1, and are the means ± S.E.M. of at least three independent experiments. Significance to Ct values is given as: *$P<0.05$, **$P<0.01$ and ***$P<0.001$. (**b**) Total cellular proteins were extracted and analyzed by western blotting with either α-MyH1 antibodies or α-extracellular signal-regulated kinase (α-ERK) antibodies as loading control. (**c** and **d**) For immunofluorescence analysis, differentiated cell cultures were fixed and incubated with α-MyH1 antibodies (upper panels), and nuclei were counterstained with DAPI (lower panels); representative fields are shown

expression is barely detected after day 5 into their wild-type counterparts (Figure 8a). This persistence of Nanog expression seems to profoundly perturb the commitment of p53$^{-/-}$ ESCs. In addition to Nanog, the expression of Oct4, another key self-renewal transcription factor, is also persisting during differentiation instead of decreasing rapidly (Supplementary Figure S2B). This result is most likely due to the known capacity of these transcription factors to activate each other.[1] To test the role of Nanog during p53$^{-/-}$ ESC differentiation, we performed transient transfections of anti-Nanog siRNAs at day 0 and found that Nanog mRNA expression is inhibited by 70% (Figure 8b). This Nanog inhibition is sufficient to restore significant Brachyury and Mesp1 mRNA expressions, at days 5 (Figure 8c) and 7 (Figure 8d), respectively, of the differentiation process; by contrast, it has no effect on Pax6, Bcl2 (Supplementary Figures S3A and B) or TBX1 (data not shown) gene expressions. Subsequently, we observed the restoration of p53$^{-/-}$ ESC differentiation capacities toward cardiomyocyte and smooth muscle cell formation (Figures 8e and f and Supplementary Figure S3C). However, it is worth to note that this restoration is partial, reaching only 30–50% of the level of their wild-type counterparts. Importantly, in these experiments, anti-Nanog siRNAs display no significant effect on neurogenesis (Figure 8g), which correlates with the lack of effect on Bcl2 and Pax6 gene expression. Likewise, although activating Brachyury expression, anti-Nanog siRNAs do not restore skeletal myogenesis (Figure 8h), showing that Brachyury regulation is not sufficient for this lineage. Altogether, our results suggest that p53-dependent Nanog repression is involved in p53 activation of cardiomyogenesis and smooth muscle formation, whereas neurogenesis inhibition and skeletal myogenesis activation are Nanog independent.

Discussion

We previously described a role for p38MAPK as an early switch of ESC commitment into mesodermal lineages (p38MAPK on) or neurons (p38MAPK off).[10,15] This control takes place during the critical first 5 days of ESC commitment, concomitantly with the expression of specific master genes that direct ESC into each of the three embryonic layers. We found now that this effect is partially mediated by the transcription factor p53, unveiling an important p38MAPK-p53 cascade regulating the early commitment of ESCs. During the ESC differentiation process, p38MAPK induces a stabilization of p53 protein and increases its transcriptional activity. This cascade, regulating the expression of specific

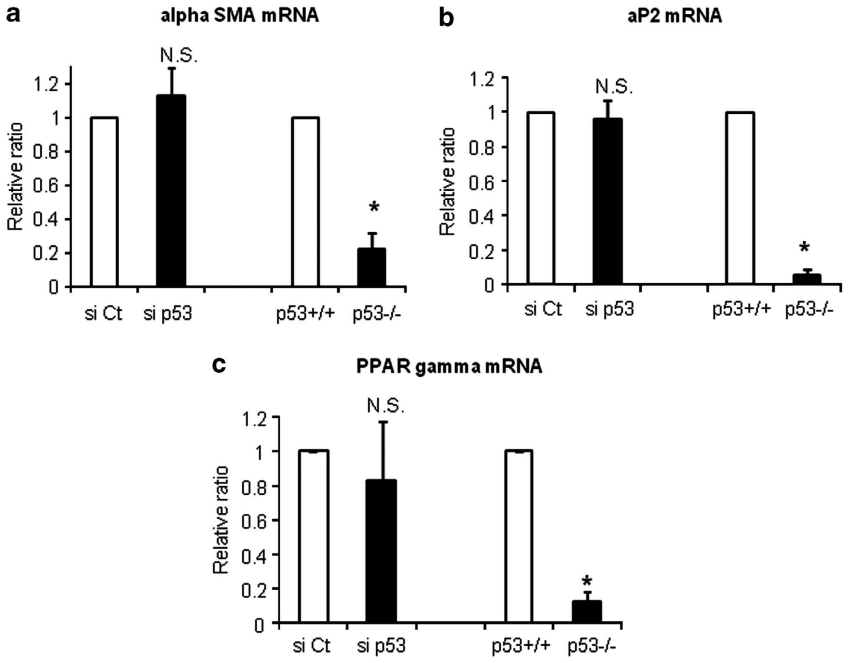

Figure 6 Effects of anti-p53 siRNAs or homozygote gene knockout of p53 on ESC smooth muscle cell and adipocyte formation. Undifferentiated CGR8 ESCs were transfected overnight with control siRNAs (si Ct) or anti-p53 siRNAs (si p53). The day after, day 0, these cells and $p53^{-/-}$ ESCs ($p53^{-/-}$) and their wild-type counterparts ($p53^{+/+}$) were induced to differentiate, cultivated until day 7 and replated on petri dishes until either day 12 for smooth muscle analysis (**a**) or day 26 for adipocyte analysis (**b** and **c**). mRNAs were extracted and analyzed by real-time RT-PCR for α-smooth muscle actin (α-SMA) (**a**), adipocyte-specific lipid-binding protein (aP2) (**b**) and peroxisome-proliferative-activated receptor gamma (PPARγ) (**c**) gene expression. Results are expressed in arbitrary units, with the values of untransfected CGR8 control cells or wild-type control ESCs taken as 1, and are the means ± S.E.M. of at least three independent experiments. Significance to Ct values is given as: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$

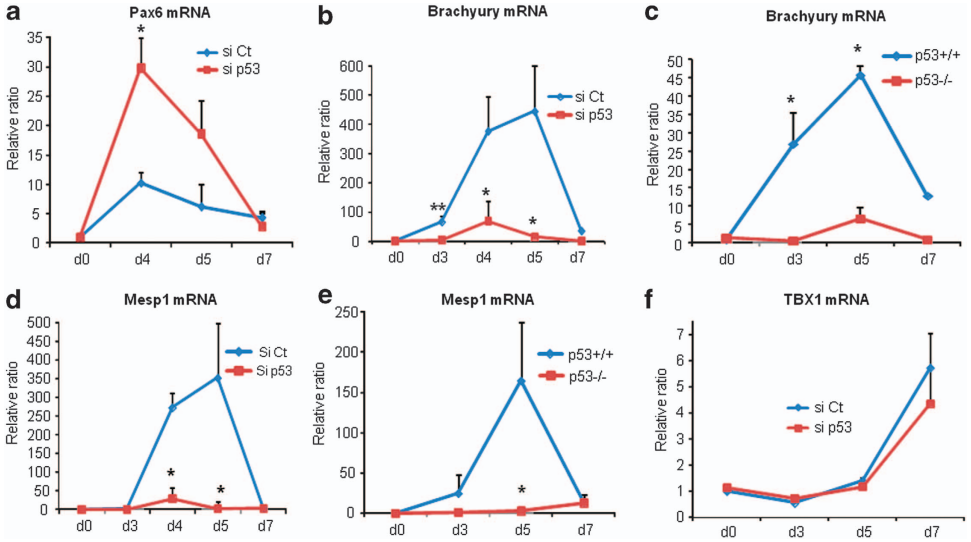

Figure 7 Effects of anti-p53 siRNAs or homozygote gene knockout of p53 on the expression of master genes of ESC commitment. Undifferentiated CGR8 ESCs were transfected overnight with control siRNAs (si Ct) or anti-p53 siRNAs (si p53). The day after, day 0, these cells and $p53^{-/-}$ ESCs ($p53^{-/-}$) and their wild-type counterparts ($p53^{+/+}$) were induced to differentiate. mRNAs were extracted at various time from day 0 to 7, as indicated, and analyzed by real-time RT-PCR for the expression of *Pax6* (**a**), *Brachyury* (**b** and **c**), *Mesp1* (**d** and **e**) and *TBX1* (**f**) genes. Results are expressed in arbitrary units, with the values of untransfected CGR8 control cells (**a**, **b**, **d** and **f**) or wild-type control ESCs (**c**–**e**) taken as 1, and are the means ± S.E.M. of at least three independent experiments. Significance to Ct values is given as: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$

master genes of ESC commitment, inhibits ectodermal and, conversely, activates several mesodermal lineages.

Homozygous p53-knockout mice were found to survive to birth, but then to succumb rapidly to tumors,[29] suggesting, at first, that the *p53* gene is dispensable during the embryonic development but crucial as tumor-suppressor gene during the adult life. However, a high rate of developmental abnormalities is associated with p53 deficiency, which result to defects

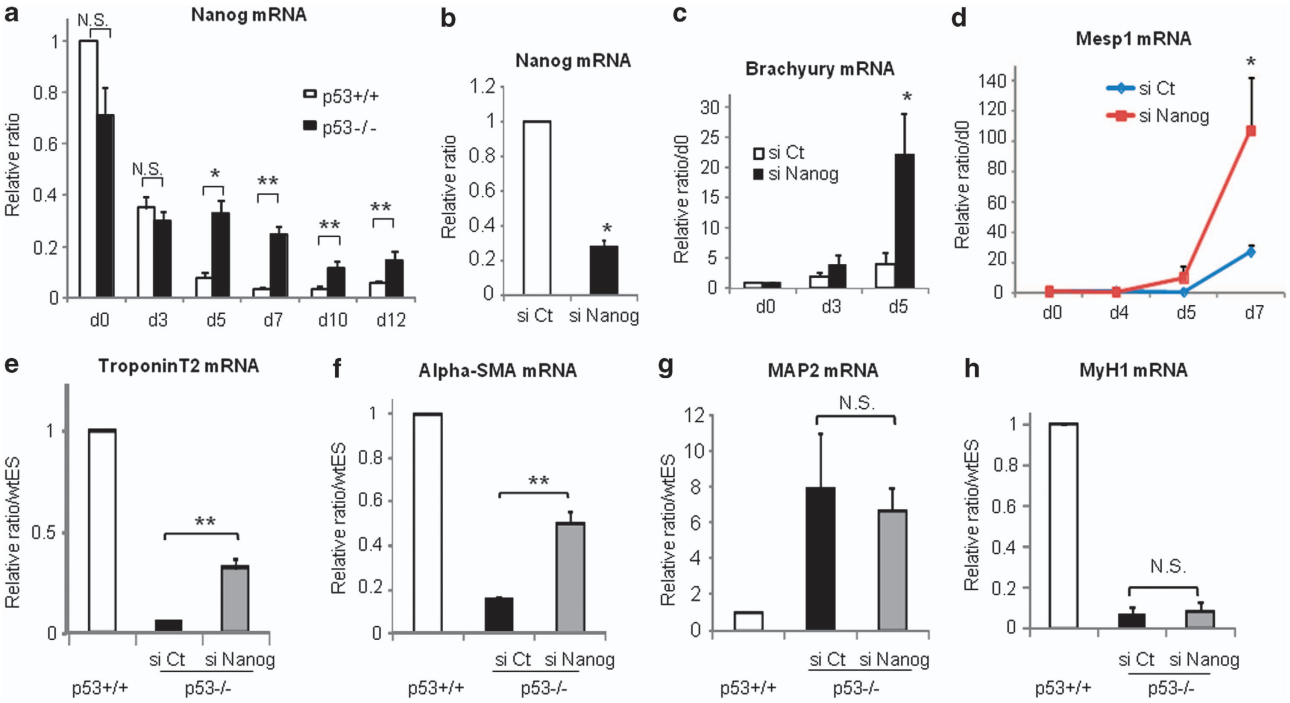

Figure 8 Role of p53-dependent Nanog repression on ESC differentiation. (**a**) $p53^{-/-}$ ESCs ($p53^{-/-}$) and their wild-type counterparts ($p53^{+/+}$) were induced to differentiate until day 12 and analyzed for endogenous Nanog expression at various time from day 0 to 12, as indicated. mRNAs were extracted and analyzed by real-time RT-PCR. Results are expressed in arbitrary units, with the values of wild-type control ESCs at day 0 taken as 1, and are the means ± S.E.M. of at least three independent experiments. (**b–h**) Undifferentiated $p53^{-/-}$ ESCs were transfected overnight with control siRNAs (si Ct) or anti-Nanog siRNAs (si Nanog) and were kept in undifferentiated conditions (**b**), or induced to differentiate until day 5 (**c**), day 7 (**d**), day 12 (**e–g**) or day 26 (**h**) and compared with differentiated $p53^{+/+}$ ESC counterparts. mRNAs were extracted 24 h later (**b**), at days 0, 3, 5 and 7 (**c** and **d**), or after differentiation (**e–h**), and analyzed by real-time RT-PCR for Nanog (**b**), Brachyury (**c**), Mesp1 (**d**), TroponinT2 (**e**), Alpha-SMA (**f**), MAP2 (**g**) and MyH1 (**h**) gene expression. Results are expressed in arbitrary units, with the values of si Ct control ESCs taken as 1 (**b–d**), or with the values of wild-type control ESCs taken as 1 (**e–h**), and are the means ± S.E.M. of at least three independent experiments. Significance to values obtained with $p53^{+/+}$ cells or siCt conditions is given as: $^{*}P<0.05$ and $^{**}P<0.01$

in neural tube closure, overgrowth of neural tissues and exencephaly, therefore suggesting a role for p53 in development.[30,31] This apparent discrepancy could be due to compensation by the p53 family members p63 and p73, which are expressed in early mouse embryos, resulting in the incomplete penetrance of the developmental phenotype observed. Furthermore, *in vitro*, depending on the cellular model analyzed, p53 can either facilitate or suppress cell differentiation.[32] In agreement with the *in vivo* negative role of p53 on neurogenesis, we found that this transcription factor is a potent early inhibitor of *in vitro* ESC neurogenesis and that this effect can be explained, at least partially, by the transcriptional repression of two genes *Bcl2* and *Pax6*. Indeed, Bcl2 is known to be targeted by p38MAPK and is critical for neuronal commitment of ESCs,[21] and Pax6 is a transcription factor long known to be essential for cortical development. Interestingly, it has been shown recently that $Pax6^{-/-}$ ESCs displayed impaired neurogenesis.[33] Therefore, we hypothesize that, in our experiments, p53 inhibition leads to Bcl2 and Pax6 overexpressions, which in turn augment ESC neurogenesis. These regulations seem independent of the p53-induced repression of Nanog gene expression observed during differentiation, suggesting that the p53 effect is mediated by another mechanism.

p53 is necessary for myotube formation in C2C12 myoblast[34] and mesenchymal stem cell[35] cellular models. Here we found a p53 requirement in skeletal myogenesis early on during ESC commitment, demonstrating a p53 control all along this process. This effect is associated to the control of the mesodermal master gene *Brachyury*, and we found that the p53-dependent repression of Nanog participates in this regulation. However, even if anti-Nanog siRNA transfections are capable to restore Brachyury expression, they do not restore skeletal myogenesis of $p53^{-/-}$ ESCs. This result could be due to a partial Nanog downregulation or, alternatively, the involvement of additional molecular mechanisms corresponding to later roles of p53.

Not much was known about p53 and *in vitro* cardiomyocyte formation; however, it has been shown recently that the pharmacological compound icariin augments ESC cardiomyogenesis via activation of both p53[36] and p38MAPK.[37] Our results show that the p38MAPK-p53 cascade is required for cardiomyocte formation from mouse ESCs. Furthermore, inhibition of Nanog gene expression in differentiated $p53^{-/-}$ ESCs restore both a cardiac phenotype and the expression of two master genes important for cardiac development, *Brachyury* and *Mesp1*.

In conclusion, our results identify a p38MAPK-p53-Nanog cascade important for the ESC early commitment toward several lineages. However, Nanog repression does not account for all the effects of p53, and, likewise, the p53 activation is not sufficient to account for all the effects of the

p38MAPK signal transduction pathway on ESC differentiation, suggesting the existence of additional molecular mechanisms for these processes.

Materials and Methods

Differentiation of ESCs. Mouse ESCs CGR8[38] and p53$^{-/-}$ and their wild-type counterpart ESCs[27] were grown on gelatin-coated plates and were induced to differentiate as described previously.[38] Briefly, 1×10^3 ESCs were aggregated without leukemia-inhibitory factor to form EBs at day 0, cultivated in suspension until day 7, treated (for neurogenesis and adipogenesis) or not (for cardiomyogenesis, endothelial differentiation, smooth and skeletal myogenesis) with 10^{-7} M all-*trans* RA (Sigma, Saint-Quentin-Fallavier, France) daily between day 3 and 5, plated at day 7 on gelatin-coated six-well plate and treated with 85 nM insulin (Sigma) and 2 nM 3,3′,5-tri-iodothyronine (Sigma) to induce terminal differentiation. Fresh medium containing the inducers was applied every 2 days. Neurons, cardiomyocytes, endothelial and smooth muscle cells were analyzed at day 12, and skeletal myotubes and adipocytes were analyzed at day 26.[10]

During EB differentiation, 10 μmol/l of the p38 inhibitor PD169316 (Sigma) or 10 μmol/l of pifithrin-α (Sigma) were added to the culture media as indicated.

RNA analysis. Total RNA was prepared using trizol reagent (Invitrogen, Life Technologies, Saint-Aubier, France). Real-time RT-PCR was performed with the ABI Prism7300 (Applied Biosystems, Life Technologies) and Taqman or Mesa Green MasterMix (Eurogentec, Angers, France). The relative amounts of the different mRNAs were quantified by using the comparative *CT* method ($2^{-(\Delta\Delta CT)}$). 36B4 was used as housekeeper transcripts, and gene expressions were normalized using 36B4 RNA levels. Primer sequences (Eurogentec) are given in Supplementary Table 1.

siRNAs experiments. ESCs grown without antibiotics were incubated at day 0 with siRNAs against either total p53 SMART pool (Dharmacon), Nanog SMART pool (Dharmacon) or control siRNAs (Dharmacon). Transfection of siRNAs was performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 30 μl of lipofectamine was incubated for 5 min in 1.5 ml of antibiotic-free cell culture medium plus serum, and 1 nmol of siRNA was added to the mix and incubated for 15 min; the mix was then added to 1×10^6 cells. p53 knockdown was determined either by immunoblotting with anti-p53 antibody (Cell Signaling, Ozyme, Saint-Quentin en Yvelines, France) or by quantitative real-time RT-PCR.

Western blot analysis. For p53 protein analysis, EBs were lysed as described.[38] Samples (40 μg) were separated by SDS-polyacrylamide gel electrophoresis on a 10% gel (Invitrogen) and transferred onto polyvinylidene difluoride membranes (Millipore, Molsheim, France). Membranes were incubated with antibodies against either mouse p53 (Cell Signaling), Lamin B1 (BioLegend, Ozyme) or ERK2 (Santa Cruz Biotechnology, Clinisciences, Nanterre, France).

Immunofluorescence staining. Differentiated EBs were washed and fixed at day 7 in 3% paraformaldehyde for 15 min at room temperature (RT). Cell membranes were permeabilized in phosphate-buffered saline (PBS)-0.1% Triton X-100 for 15min at RT and incubated with an anti-Troponin T (CT3) or an anti-MHC (MF2Ds) (DSHB, Iowa City, IA, USA) in PBS-10% fetal bovine serum for 1 h. After three washes in PBS, cells were incubated with anti-mouse ALEXA488 or ALEXA546 (Invitrogen) for 1 h at RT. Cell imaging was performed using a Nikon microscope Eclipse 90I. Z-stack pictures were obtained by using a Nikon Digital Sight DS-1QM camera controlled by NIS element AR software (Nikon, Champigny sur Marne, France).

Statistical analyses. All experiments were performed at least three times. All data are expressed as the mean ± S.E.M. Treatments were compared with their respective controls, and significant differences among the groups were determined using unpaired Student's *t*-test. A value of $P < 0.05$ was taken as an indication of statistical significance.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We thank Dr. Blattner (Karlsruhe, Germany) for the gift of p53 wild-type and knockout ESC lines.

1. Young RA. Control of the embryonic stem cell state. *Cell* 2011; **144**: 940–954.
2. Kashyap V, Rezende N, Scotland K, Shaffer S, Persson J, Gudas L *et al.* Regulation of stem cell pluripotency and differentiation involves a mutual regulatory circuit of the NANOG, OCT4, and SOX2 pluripotency transcription factors with polycomb repressive complexes and stem cell microRNAs. *Stem Cell Dev* 2009; **18**: 1093–1108.
3. Wobus AM. Potential of embryonic stem cells. *Mol Aspects Med* 2001; **22**: 149–164.
4. Wobus AM, Boheler KR. Embryonic stem cells: prospects for developmental biology and cell therapy. *Physiol Rev* 2005; **85**: 635–678.
5. Wobus AM, Guan K, Yang HT, Boheler KR. Embryonic stem cells as a model to study cardiac, skeletal muscle, and vascular smooth muscle cell differentiation. *Methods Mol Biol* 2002; **185**: 127–156.
6. Rohwedel J, Guan K, Wobus AM. Induction of cellular differentiation by retinoic acid in vitro. *Cells Tissues Organs* 1999; **165**: 190–202.
7. Ying Q-L, Stavridis M, Griffiths D, Li M, Smith A. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat Biotechnol* 2003; **21**: 183–186.
8. Dani C, Smith AG, Dessolin S, Leroy P, Staccini L, Villageois P *et al.* Differentiation of embryonic stem cells into adipocytes *in vitro*. *J Cell Sci* 1997; **110** (Pt 11): 1279–1285.
9. Hescheler J, Fleischmann BK, Wartenberg M, Bloch W, Kolossov E, Ji G *et al.* Establishment of ionic channels and signalling cascades in the embryonic stem cell-derived primitive endoderm and cardiovascular system. *Cells Tissues Organs* 1999; **165**: 153–164.
10. Barruet E, Hadadeh O, Peiretti F, Renault VM, Hadjal Y, Bernot D *et al.* p38 Mitogen activated protein kinase controls two successive-steps during the early mesodermal commitment of embryonic stem cells. *Stem Cells Dev* 2011; **20**: 1233–1246.
11. Cuenda A, Cohen P. Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. *J Biol Chem* 1999; **274**: 4341–4346.
12. Cuenda A, Rousseau S. p38 MAP-Kinases pathway regulation, function and role in human diseases. *Biochimic Biophys Acta* 2007; **1773**: 1358–1375.
13. Tamura K, Sudo T, Senftleben U, Dadak AM, Johnson R, Karin M. Requirement for p38alpha in erythropoietin expression: a role for stress kinases in erythropoiesis. *Cell* 2000; **102**: 221–231.
14. Adams RH, Porras A, Alonso G, Jones M, Vintersten K, Panelli S *et al.* Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. *Mol Cell* 2000; **6**: 109–116.
15. Aouadi M, Bost F, Caron L, Laurent K, Le Marchand Brustel Y, Binetruy B. p38 Mitogen-activated protein kinase activity commits embryonic stem cells to either neurogenesis or cardiomyogenesis. *Stem Cells* 2006; **24**: 1399–1406.
16. Vousden KH, Prives C. Blinded by the light: the growing complexity of p53. *Cell* 2009; **137**: 413–431.
17. Lin T, Chao C, Saito S, Mazur SJ, Murphy ME, Appella E *et al.* p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat Cell Biol* 2005; **7**: 165–171.
18. Zhao T, Xu Y. p53 and stem cells: new developments and new concerns. *Trends Cell Biol* 2010; **20**: 170–175.
19. Miyashita T, Harigai M, Hanada M, Reed JC. Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res* 1994; **54**: 3131–3135.
20. Wu Y, Mehew JW, Heckman CA, Arcinas M, Boxer LM. Negative regulation of bcl-2 expression by p53 in hematopoietic cells. *Oncogene* 2001; **20**: 240–251.
21. Trouillas M, Saucourt C, Duval D, Gauthereau X, Thibault C, Dembele D *et al.* Bcl2, a transcriptional target of p38 alpha, is critical for neuronal commitment of mouse embryonic stem cells. *Cell Death Differ* 2008; **15**: 1450–1459.
22. Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV *et al.* A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* 1999; **285**: 1733–1737.
23. Bondue A, Lapouge G, Paulissen C, Semeraro C, Iacovino M, Kyba M *et al.* Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. *Cell Stem Cell* 2008; **3**: 69–84.
24. Kishi H, Nakagawa K, Matsumoto M, Suga M, Ando M, Taya Y *et al.* Osmotic shock induces G1 arrest through p53 phosphorylation at Ser33 by activated p38MAPK without phosphorylation at Ser15 and Ser20. *J Biol Chem* 2001; **276**: 39115–39122.
25. Huang C, Ma WY, Maxiner A, Sun Y, Dong Z. p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. *J Biol Chem* 1999; **274**: 12229–12235.
26. Jain AK, Allton K, Iacovino M, Mahen E, Milczarek RJ, Zwaka TP *et al.* p53 regulates cell cycle and microRNAs to promote differentiation of human embryonic stem cells. *PLoS Biol* 2012; **10**: e1001268.
27. Solozobova V, Rolletschek A, Blattner C. Nuclear accumulation and activation of p53 in embryonic stem cells after DNA damage. *BMC Cell Biology* 2009; **10**: 46.
28. Aouadi M, Laurent K, Prot M, Le Marchand-Brustel Y, Binetruy B, Bost F. Inhibition of p38MAPK increases adipogenesis from embryonic to adult stages. *Diabetes* **55**: 281–289.

29. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr., Butel JS *et al.* Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 1992; **356**: 215–221.
30. Armstrong JF, Kaufman MH, Harrison DJ, Clarke AR. High-frequency developmental abnormalities in p53-deficient mice. *Curr Biol* 1995; **5**: 931–936.
31. Sah VP, Attardi LD, Mulligan GJ, Williams BO, Bronson RT, Jacks T. A subset of p53-deficient embryos exhibit exencephaly. *Nat Genet* 1995; **10**: 175–180.
32. Almog N, Rotter V. Involvement of p53 in cell differentiation and development. *Biochim Biophys Acta* 1997; **1333**: F1–27.
33. Nikoletopoulou V, Plachta N, Allen ND, Pinto L, Gotz M, Barde YA. Neurotrophin receptor-mediated death of misspecified neurons generated from embryonic stem cells lacking Pax6. *Cell Stem Cell* 2007; **1**: 529–540.
34. Porrello A, Cerone MA, Coen S, Gurtner A, Fontemaggi G, Cimino L *et al.* p53 regulates myogenesis by triggering the differentiation activity of pRb. *J Cell Biol* 2000; **151**: 1295–1304.
35. Molchadsky A, Shats I, Goldfinger N, Pevsner-Fischer M, Olson M, Rinon A *et al.* p53 plays a role in mesenchymal differentiation programs, in a cell fate dependent manner. *PLoS One* 2008; **3**: e3707.
36. Zhu D, Qu L, Zhang X, Lou Y. Icariin-mediated modulation of cell cycle and p53 during cardiomyocyte differentiation in embryonic stem cells. *Eur J Pharmacol* 2005; **514**: 99–110.
37. Ding L, Liang XG, Hu Y, Zhu DY, Lou YJ. Involvement of p38MAPK and reactive oxygen species in icariin-induced cardiomyocyte differentiation of murine embryonic stem cells in vitro. *Stem Cells Dev* 2008; **17**: 751–760.
38. Bost F, Caron L, Marchetti I, Dani C, Le Marchand-Brustel Y, Binetruy B. Retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage. *Biochem J* 2002; **361**(Pt 3): 621–627.

Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)