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Mdm2 controls CREB-dependent transactivation and initiation of adipocyte differentiation

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The role of the E3 ubiquitin ligase murine double minute 2 (Mdm2) in regulating the stability of the p53 tumor suppressor is well documented. By contrast, relatively little is known about p53-independent activities of Mdm2 and the role of Mdm2 in cellular differentiation. Here we report a novel role for Mdm2 in the initiation of adipocyte differentiation that is independent of its ability to regulate p53. We show that Mdm2 is required for cAMP-mediated induction of CCAAT/enhancer-binding protein δ (C/EBPδ) expression by facilitating recruitment of the cAMP regulatory element-binding protein (CREB) coactivator, CREB-regulated transcription coactivator (Crtc2)/TORC2, to the *c/ebpδ* promoter. Our findings reveal an unexpected role for Mdm2 in the regulation of CREB-dependent transactivation during the initiation of adipogenesis. As Mdm2 is able to promote adipogenesis in the myoblast cell line C2C12, it is conceivable that Mdm2 acts as a switch in cell fate determination.
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Adipocytes originate from mesenchymal stem cells (MSCs) that also are precursors for muscle and bone cells. Studies of preadipocyte cell lines and embryonic fibroblasts have delineated a transcriptional cascade involving peroxisome proliferator-activated receptor γ (PPARγ) and three members of the CCAAT/enhancer-binding protein (C/EBP) family – C/EBPδ, C/EBPβ and C/EBPα – that are activated sequentially during adipogenesis.[1]

C/EBPβ and C/EBPδ are induced early and transiently during differentiation and are considered to play key roles during the initiation of the adipogenic program. The two C/EBPs act in concert with overlapping, but not identical, activities as indicated by the more severe adipose phenotype of mice lacking both C/EBPβ and C/EBPδ than that of mice lacking either of the two C/EBPs.[2] Activation of C/EBPβ and C/EBPδ leads to the induction of C/EBPα and PPARγ expression orchestrating terminal adipocyte differentiation.[3]

Besides PPARγ and the three C/EBPs, other transcription factors are reported to be required for adipocyte differentiation. Activation of the cAMP regulatory element-binding protein (CREB) at the onset of adipocyte differentiation is critical for adipogenesis.[4–6] The recent cloning and characterization of a CREB cofactor family, denoted as CREB-regulated transcription coactivator (Crtc/TORC), have revealed how CREB can induce expression of distinct target genes dependent on different stimuli.[7–9]

The murine double minute 2 (Mdm2) is an E3 ubiquitin ligase with oncogenic properties. Its importance in the control of p53 activity is underscored by the finding that knockout of *p53* rescues the embryonic lethality of mice lacking *mdm2*.[10,11] Deregulation of p53 activity in embryos lacking *mdm2* leads to widespread apoptosis and ensuing embryonic death.[12] Although Mdm2 plays a critical role in the regulation of p53 signaling, an increasing body of evidence indicates that Mdm2 may exert p53-independent functions.[13]

Amplification of the *mdm2* gene occurs in 10% of all human cancers.[14] Interestingly, *mdm2* is amplified in nearly all liposarcomas.[15] As the genetic aberration in a malignant transformation of an MSC was recently suggested to regulate the differentiation of the transformed cells,[16] the high prevalence of *mdm2* amplification in liposarcomas could indicate an involvement of *mdm2* in adipogenesis. Furthermore, the *mdm2* gene is amplified in the widely used preadipocyte cell line, 3T3-L1.[17] Still, the functional consequence of its amplification and the role of Mdm2 in adipogenesis have not been elucidated. Here we show that Mdm2 regulates adipogenesis by promoting cAMP-mediated transcriptional activation of CREB and induction of C/EBPδ expression by facilitating the recruitment of Crtc2 to a

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Abbreviations: Areg, amphiregulin; C/EBP, CCAAT/enhancer-binding protein; ChIP, chromatin immunoprecipitation; CRE, cAMP-response element; CREB, cAMP regulatory element-binding protein; CREM, cAMP-response element modulator; Crtc, CREB-regulated transcription coactivator; Dex, dexamethasone; Epac, exchange protein activated by cAMP; IBMX, 3-isobutyl-1-methylxanthine; Ins, insulin; KLF5, Krüppel-like factor 5; MAPK, mitogen-activated protein kinase; MDI, 3-isobutyl-1-methylxanthine, dexamethasone, insulin|Mdm2, murine double minute 2; MEF, mouse embryonic fibroblasts; MSC, mesenchymal stem cell; PKA, protein kinase A; PPARγ, peroxisome proliferator-activated receptor γ; Rosi, rosiglitazone; SIK, salt-inducible kinase; PMA, phorbol 12-myristate 13-acetate; XOR, xanthine oxidoreductase
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cAMP-response element (CRE) in the promoter of *c/ebpδ*. Our unexpected findings identify Mdm2 as a novel critical player in the intricate network of factors that regulate CREB-dependent transactivation and adipocyte differentiation.

Results

Mdm2 is required for adipogenesis *ex vivo*. The 3T3-L1 preadipocyte cell line and mouse embryonic fibroblasts (MEFs) have been instrumental for studying adipocyte differentiation. Although MEFs lacking Mdm2 cannot be established owing to the early embryonic lethality,[12] MEFs deficient for both p53 and Mdm2 can be obtained.[10,11]

When treating *p53*$^{-/-}$ and *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs with a standard hormonal cocktail (MDI) containing the cAMP-elevating compound, 3-isobutyl-1-methylxanthine (IBMX), the glucocorticoid receptor agonist, dexamethasone (Dex) and insulin (Ins) commonly used for the induction of adipocyte differentiation, we observed a dramatic reduction in the adipogenic potential of *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs compared with *p53*$^{-/-}$ MEFs as visualized by Oil-Red-O staining of triglycerides and marker gene expression (Figure 1a). Inclusion of the potent PPARγ ligand rosiglitazone during differentiation did not restore differentiation of *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs (Figure 1b).

To establish a causal link between lack of Mdm2 and impaired adipogenesis, we attempted to rescue adipogenesis by restoring Mdm2 expression using retroviral transduction of *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs. In accordance with a previous report,[18] cells expressing full-length Mdm2 did undergo cell cycle arrest (data not shown). This presumably relates to the use of the cDNA of *mdm2* for retroviral expression, as overexpression of Mdm2 using a genomic clone harboring the entire *mdm2* gene results in cell transformation.[19]

To circumvent the cell cycle arrest imposed by expressing full-length *mdm2* cDNA, we retrovirally expressed different portions of Mdm2 (Mdm2 aa 1–220 and Mdm2 aa 221–491) separately (Figures 1c and d). *P53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs expressing the N-terminal half of Mdm2 (Mdm2 aa 1–220) underwent adipocyte differentiation, whereas MEFs transduced with either empty vector or vector encoding Mdm2 aa 221–491 did not. Ectopic expression of full-length MdmX (or Mdm4), a protein that is structurally and functionally related to Mdm2, failed to restore adipogenesis in the *p53*$^{-/-}$*; mdm2*$^{-/-}$ MEFs (Supplementary Figure 1).

Collectively, these results indicate that Mdm2 regulates adipocyte differentiation, independent of its ubiquitin ligase activity and its ability to control p53 activity.

In an attempt to obtain evidence for the possible involvement of Mdm2 in adipogenesis in an *in vivo* setting, we used CT scanning to compare the amount of adipose tissue in mice harboring a missense mutation in p53 (*p53*$^{H/H}$)[20] with mice having mutated p53 and lacking *mdm2* (*p53*$^{H/H}$*mdm2*$^{-/-}$). Only a limited number of *p53*$^{H/H}$ and *p53*$^{H/H}$*mdm2*$^{-/-}$ mice were available ($n=4$ and 5, respectively). However, loss of Mdm2 did not affect bone mass, soft tissue and total adipose tissue ($P>0.05$) (Figure 1e), indicating that loss of Mdm2 *in vivo* may at least in part be counteracted by compensatory regulatory pathways.

Mdm2 promotes a switch from myogenesis to adipogenesis *ex vivo*. Adipocytes and myocytes are both derived from MSCs. Interestingly, in *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs induced to undergo adipocyte differentiation we observed the sporadic appearance of cells morphologically resembling myocytes (Figure 2a). Such cells were not seen in *p53*$^{-/-}$ MEF cultures. Expression of several myogenic markers was induced in *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs stimulated to undergo adipocyte differentiation. This was not the case in *p53*$^{-/-}$ MEFs (Figure 2b). This indicates that Mdm2 might act as a switch favoring adipogenesis over myogenesis.

Thayer and co-workers[21,22] have previously shown that amplification of the *mdm2* gene by microchromosomal transfer in the C2C12 myoblast cell line abrogates their ability to undergo myogenesis. We speculated if such C2C12 cells had increased propensity to undergo adipocyte differentiation. As expected, C2C12 cells in which *mdm2* had been amplified (Rh18-11) had increased Mdm2 protein levels compared with both normal C2C12 and C2C12 cells subjected to microchromosomal transfer of DNA that did not harbor the *mdm2* gene (Rh18-3) (Figure 2c). Interestingly, when adipogenesis was induced in these three cell lines, only Rh18-11 cells accumulated fat as shown by Oil-Red-O staining and induced robust expression of adipocyte marker genes (Figures 2d and e). These data indicate that Mdm2 regulates cellular fate by promoting adipogenesis at the expense of myogenesis.

Mdm2 is required for the cAMP-mediated induction of C/EBPδ. The inability of rosiglitazone to restore adipogenesis in MEFs lacking *mdm2* indicated that induction of PPARγ expression was perturbed in *mdm2*-null cells. Unlike in MEFs only lacking *p53*, neither PPARγ1, PPARγ2 nor C/EBPα mRNA levels were increased in *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs in response to MDI and rosiglitazone treatment (Figure 3a). However, retroviral-mediated restoration of PPARγ2 expression was sufficient to overcome the block in adipocyte differentiation of *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs (Supplementary Figure 2).

When we examined the expression pattern of C/EBPβ and C/EBPδ in *p53*$^{-/-}$ and *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs, we found that C/EBPβ was induced to comparable levels in the two MEF genotypes, whereas C/EBPδ induction was abrogated in *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs (Figure 3b). Intriguingly, upregulation of an immediate-early-induced gene, *Krox20*, that is required for adipogenesis and for C/EBPβ induction[23] was more robust in MEFs lacking *mdm2* (Figure 3b). Xanthine oxidoreductase (XOR) and Krüppel-like factor 5 (KLF5) are induced transiently in a C/EBPβ- and C/EBPδ-dependent manner, respectively, during adipocyte differentiation.[24,25] Consistent with the expression profiles of the two C/EBPs, XOR, but not KLF5, the expression was increased upon induction of differentiation in *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs (Figure 3b). Taken together, these results indicate that Mdm2 is required for the induction of C/EBPδ, but not C/EBPβ during adipogenesis.

To examine whether the failure to induce C/EBPδ expression is causally related to the impaired differentiation of *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs, we transduced *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs with a retrovirus expressing C/EBPδ. Forced expression of C/EBPδ at least partially restored adipocyte differentiation (Supplementary Figure 3).

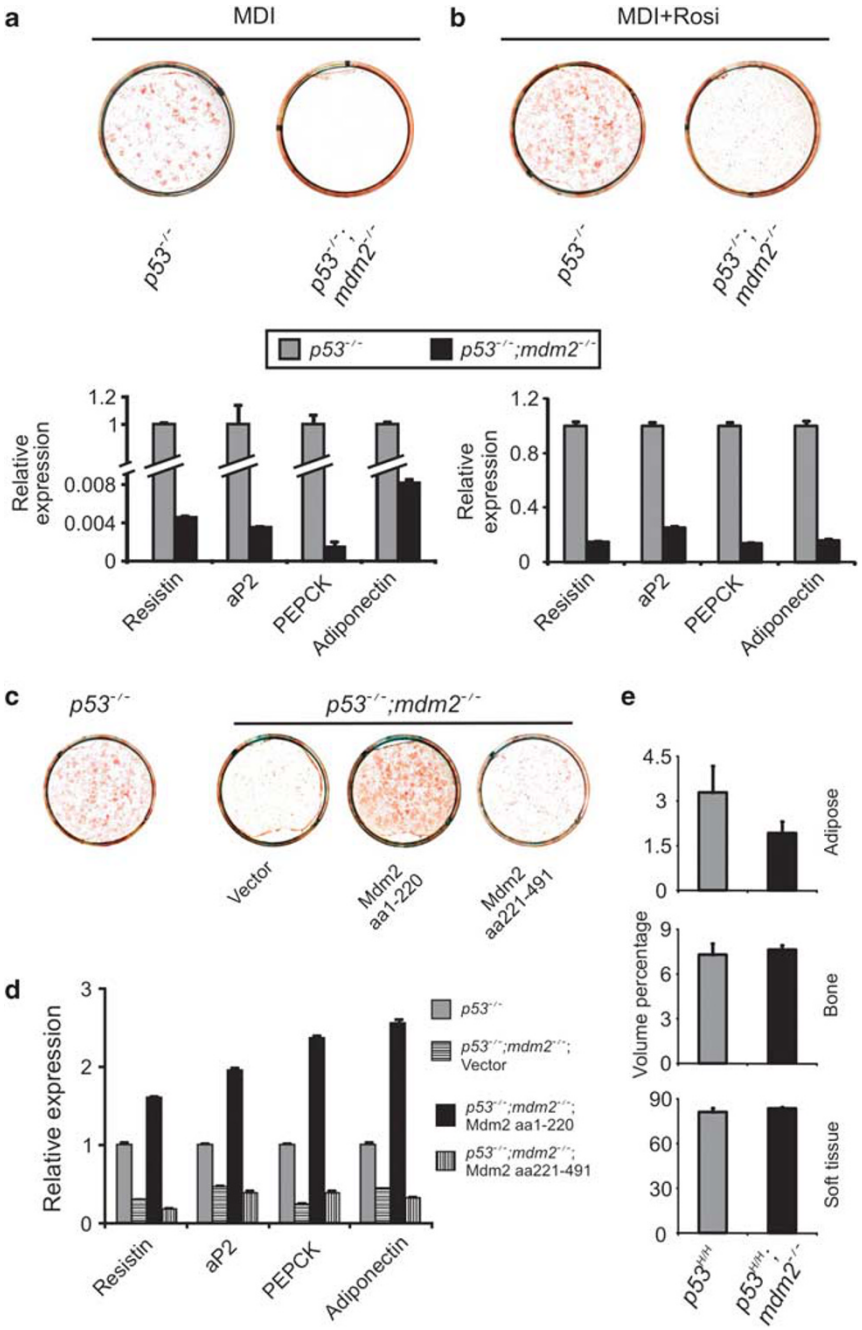

Figure 1 The N-terminal half of Mdm2 is required for adipogenesis. (**a** and **b**) *P53*$^{-/-}$ and *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs were induced to undergo adipocyte differentiation according to the MDI standard protocol in the absence (**a**) or presence (**b**) of rosiglitazone. Adipogenesis was assessed by Oil-Red-O staining of triglycerides (top) and expression of adipocyte marker genes was measured using real-time PCR and shown relative to *p53*$^{-/-}$ MEFs (bottom). (**c** and **d**) *P53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs were retrovirally transduced with empty vector, vector expressing the N-terminal half of Mdm2 (Mdm2 aa1–220) or vector expressing the C-terminal half of Mdm2 (Mdm2 aa 221–491). Transduced MEFs were induced to undergo adipogenesis according to the MDI standard protocol in the presence of rosiglitazone. Adipocyte differentiation was measured by Oil-Red-O staining of triglycerides (**c**) and adipocyte marker gene expression using real-time PCR (**d**). PEPCK, phosphoenolpyruvate carboxykinase. (**e**) *P53*$^{H/H}$ ($n=4$) and *p53*$^{H/H}$;*mdm2*$^{-/-}$ ($n=5$) mice were subjected to CT scanning. Volumes of total adipose, bone and soft tissue in general were scored. Error bars represent S.E.M.

As the expression of C/EBPδ is increased shortly after induction of differentiation, its expression may be regulated by individual components of the hormonal cocktail. C/EBPδ expression is normally considered to be induced by Dex both in preadipocytes and mature adipocytes.[26] C/EBPδ expression is, however, also known to be regulated by cAMP during adipogenesis.[27] When treating wild-type MEFs with the individual components of the adipogenic cocktail, we observed an increase in C/EBPδ expression upon treatment with Dex or IBMX alone, effects that were additive when both compounds were included (Figure 3c). Insulin or rosiglitazone did not affect C/EBPδ expression. We observed the same

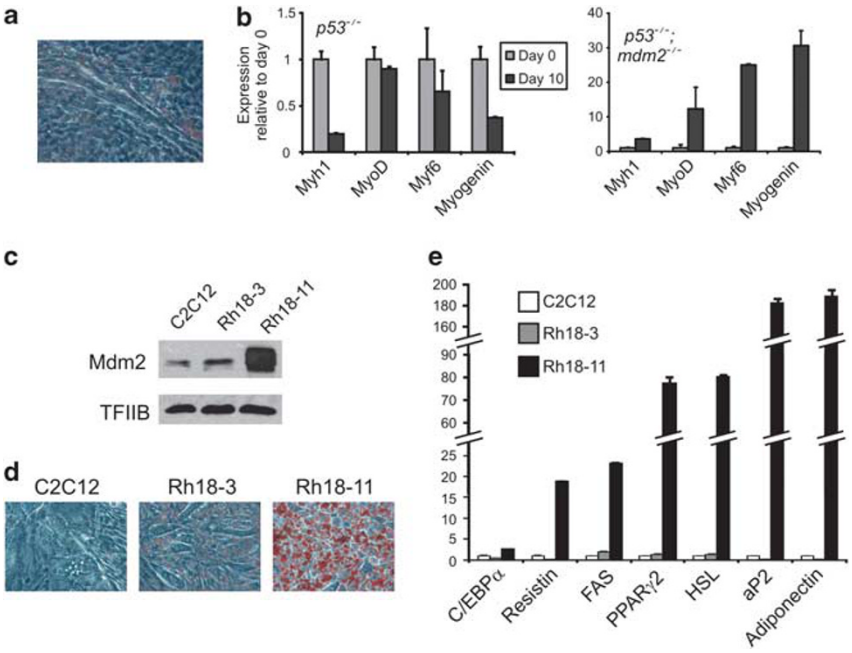

Figure 2 Mdm2 favors adipogenesis over myogenesis. (**a**) Myotube resemblance of *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs induced to undergo adipogenesis. *P53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs were induced to undergo adipocyte differentiation according to the standard MDI protocol and supplemented with rosiglitazone: × 400 magnification of *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs with myotube resemblance. (**b**) Expression of myocyte marker genes in *p53*$^{-/-}$ and *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs before and 10 days after induction of adipogenesis. *Myh1* (myosin heavy chain 1), *MyoD* (myogenic differentiation), *Myf6* (myogenic factor 6). (**c**) Western blot analysis of Mdm2 protein levels in C2C12, Rh18-3 and Rh18-11 at confluence. TFIIB was included as a loading control. (**d** and **e**) C2C12, Rh18-3 and Rh18-11 cells were induced to undergo adipogenesis according to the MDI standard protocols in the presence of rosiglitazone. Adipogenesis was scored by Oil-Red-O staining (**d**) and adipocyte marker gene expression (**e**). FAS, fatty acid synthase; HSL, hormone-sensitive lipase

pattern of C/EBPδ induction in 3T3-L1 cells (data not shown), showing that cAMP contributes to the induction of C/EBPδ expression at the onset of adipocyte differentiation.

We have recently shown that the adipogenic effect of elevated cAMP levels relies on the activation of both protein kinase A (PKA) and the exchange protein activated by cAMP (Epac).[28] Using selective activators of both PKA and Epac, we show that only the PKA activator augmented expression of C/EBPδ (Figure 3d).

As *p53*$^{-/-}$;*mdm2*$^{-/-}$ failed to increase C/EBPδ mRNA levels at the onset of adipogenesis, we speculated that this could be attributed to a failure to respond to either Dex or IBMX. As in wild-type MEFs, treating *p53*$^{-/-}$ MEFs with Dex, IBMX or a combination led to an induction of C/EBPδ expression. By contrast, treatment with Dex, but not IBMX, induced expression of C/EBPδ in *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs (Supplementary Figure 4). To ensure that the induction of C/EBPδ was an early effect downstream of elevated cAMP levels, C/EBPδ expression was measured 1 h after the addition of vehicle, IBMX or another cAMP-elevating compound, forskolin. In contrast to their effect in *p53*$^{-/-}$ MEFs, addition of IBMX or forskolin to *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs failed to increase C/EBPδ mRNA levels (Figure 3e).

IBMX elevates the level of cAMP by inhibiting cAMP-degrading phosphodiesterases. The recent association of Mdm2 with phosphodiesterase degradation[29] raised the possibility that IBMX treatment resulted in different levels of cAMP in *p53*$^{-/-}$ and *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs. Contrary to this possibility, we found that MEFs of both genotypes had equal levels of cAMP both before and after IBMX stimulation (Supplementary Figure 5). Collectively, these data suggest that Mdm2 is required for adipogenesis in a manner related to cAMP signaling.

Mdm2 is required for CREB activation. C/EBPδ was recently suggested to be a putative CREB-regulated gene in a global screen for CREB target genes.[30] The canonical transcriptional pathway downstream of PKA activation is mediated by direct serine-133 phosphorylation of CREB. In addition to PKA, several other kinases can also activate CREB by phosphorylating serine 133. In fact, it is the mitogen-activated protein kinase (MAPK) that is responsible for CREB phosphorylation during the initiation of adipocyte differentiation.[28] An important function of PKA during adipose conversion is inhibition of the Rho kinase[28] that in turn can inhibit adipogenesis by blocking the MAPK-mediated phosphorylation of CREB.[31] To ensure that the failure to induce C/EBPδ in the absence of Mdm2 was not a result of uncontrolled Rho-kinase activity, we concomitantly treated the *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs with IBMX and increasing doses of a Rho-kinase inhibitor. The inhibitor failed to restore IBMX-mediated induction of C/EBPδ in *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs (Supplementary Figure 6). In further support of normal Rho-kinase activity in the absence of Mdm2, we observed no differences in serine 133 phosphorylation of CREB in *p53*$^{-/-}$ and *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs upon induction of adipogenesis (Supplementary Figure 7).

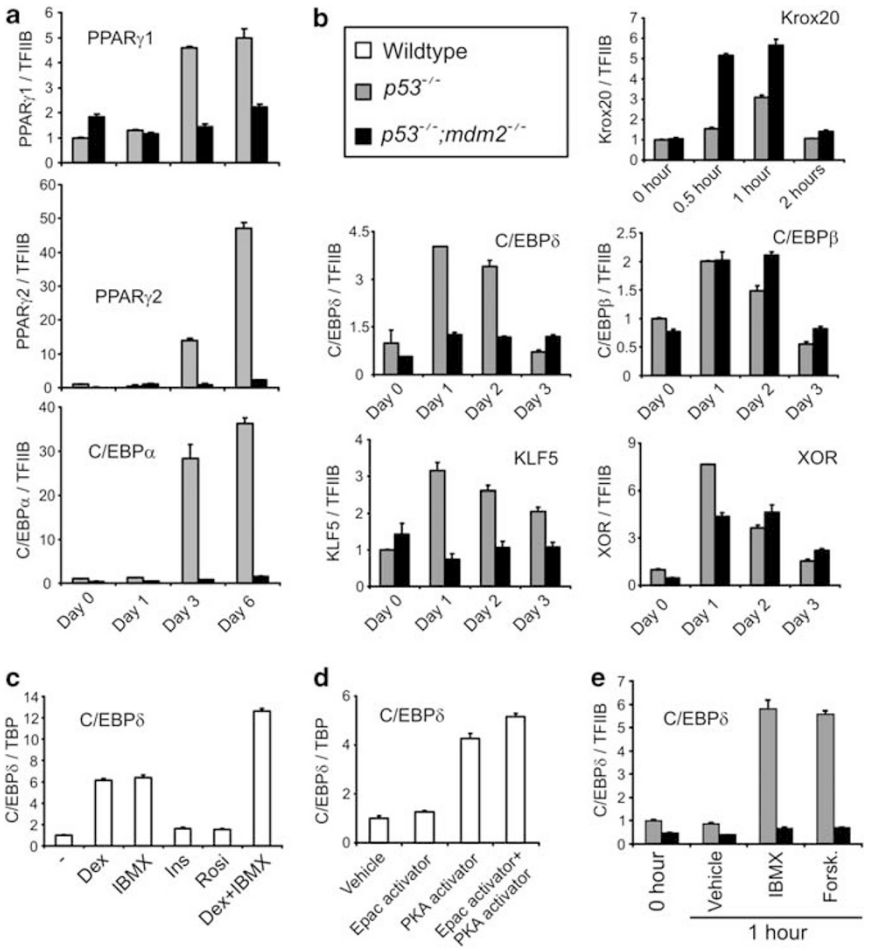

Figure 3 Mdm2 is required for cAMP-mediated induction of C/EBPδ. (**a** and **b**) *P53*$^{-/-}$ and *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs were induced to undergo adipogenesis according to the MDI standard protocol in the presence of rosiglitazone. mRNA levels of Krox20, C/EBPs, PPARγ, XOR and KLF5 were assessed using real-time PCR. (**c**) Wild-type MEFs were treated with Dex, IBMX, insulin or rosiglitazone. Expression of C/EBPδ was scored 24 h later using real-time PCR. (**d**) Wild-type MEFs were treated for 24 h with vehicle, PKA activator, Epac activator or both. mRNA levels of C/EBPδ were measured using real-time PCR. (**e**) *P53*$^{-/-}$ and *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs were treated with either vehicle, IBMX or forskolin for 1 h. Expression of C/EBPδ was assessed using real-time PCR

Although CREB phosphorylation was comparable in MEFs of both genotypes, the induction of several cAMP-responsive CREB target genes[8,30] was perturbed in *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs upon IBMX treatment (Figure 4a). We therefore speculated whether Mdm2 could be a direct modulator of CREB activity. We measured the transcriptional activity of CREB fused to the GAL4 DNA-binding domain in the absence and presence of increasing levels of Mdm2 in *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs. As shown in Figure 4b, Mdm2 enhanced the activity of the fusion protein markedly in the presence of forskolin.

CREB is not only activated by elevated cAMP levels, but also by several growth factors, hormones and by stress signals. A commonly used stress activator of CREB is the phorbol ester TPA/PMA (phorbol 12-myristate 13-acetate). cAMP and TPA elicit two distinct transcriptional programs through CREB.[9] To examine if the requirement for Mdm2 in CREB-dependent transactivation was restricted to cAMP-mediated activation, we treated *p53*$^{-/-}$ and *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs with vehicle or TPA. As shown in Figure 4c, TPA administration failed to induce the expression of examined CREB target genes in *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs. Collectively, these data suggest that Mdm2 is required for the activation of CREB in response to both stress and cAMP-elevating stimuli.

Mdm2 is required for recruitment of Crtc2/TORC2 to the *c/ebpδ* promoter. A family of transcriptional cofactors, denoted as CREB-regulated transcription coactivator (Crtcs or TORCs), has recently been implicated in the cAMP-mediated response of CREB. The Crtcs solely coactivate CREB bound to genes regulated by cAMP[9] by facilitating the recruitment of the two histone acetylases p300 and CBP to CREB.[8,9]

Under basal conditions, Crtc is localized to the cytoplasm as a result of a phosphorylation-dependent interaction with 14-3-3 proteins, mediated by members of the salt-inducible kinase (SIK) family. Upon elevated cAMP levels, PKA inhibits the SIKs, leading to dephosphorylation and translocation of Crtc to the nucleus and subsequent coactivation of CREB.[7]

If Crtcs are involved in the induction of C/EBPδ expression, repression of SIK should lead to an increase in the C/EBPδ

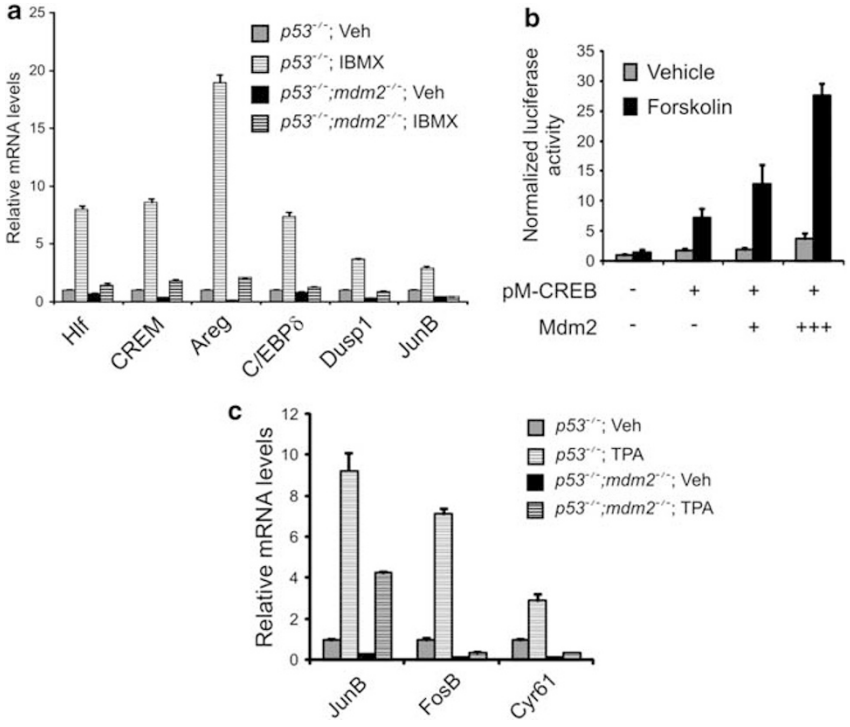

Figure 4 Mdm2 augments the activity of CREB. (**a**) *P53*$^{-/-}$ and *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs were treated with IBMX for 4 h. mRNA levels were measured using real-time PCR. Areg, amphiregulin; CREM, cAMP-response element modulator; Dusp1, dual-specificity phosphatase 1; Hlf, hepatic leukemia factor. (**b**) *P53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs were transfected with UAS-GAL luciferase reporter plasmid, CMV-β-galactosidase reporter plasmid, GAL4-CREB and increasing levels of a vector expressing Mdm2. Cells were treated overnight with vehicle or forskolin. Luciferase activity was normalized to β-galactosidase measurements. (**c**) *P53*$^{-/-}$ and *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs were treated with TPA for 1 h. mRNA levels were measured using real-time PCR

mRNA level. Although there is no specific chemical inhibitor of SIK, the general kinase inhibitor staurosporine has been used as it inhibits SIK at low concentrations.[32] We show that while staurosporine induced C/EBPδ expression in *p53*$^{-/-}$ MEFs, it did not change the level of C/EBPδ mRNA in *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs (Figure 5a).

The involvement of Crtc in regulating cAMP-mediated induction of C/EBPδ expression was underscored by the finding that ectopic expression of a dominant-negative Crtc (DN-Crtc) lowered the induction of C/EBPδ and other CREB target genes in *p53*$^{-/-}$ MEFs upon IBMX treatment (Figure 5b). Furthermore, ectopic expression of the DN-Crtc lowered the adipogenic potential of *p53*$^{-/-}$ MEFs as assessed by adipocyte marker gene expression (Figure 5c).

Of the three Crtcs, Crtc2 is the best described and has been reported to be expressed in adipose tissue.[33] We therefore examined if Mdm2 could interact directly with Crtc2. Using GST pull down, we were able to pull down *in vitro* translated Crtc2 with GST-Mdm2, but not with GST alone (Figure 5d). Interestingly, Crtc2 was able to interact separately with the two halves of Mdm2 albeit with lower affinity than full-length Mdm2 (Figure 5d).

The N-terminal half of Mdm2 harbor the nuclear localization and export signals. As the Crtcs are imported into the nucleus upon cAMP stimulation, we speculated if Mdm2 was required for this translocation. However, GFP-tagged Crtc2 localized to the nucleus upon cAMP stimulation in both *p53*$^{-/-}$ and *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs (Supplementary Figure 8), arguing that Mdm2 is dispensable for the nucleic import of Crtc2.

The TFSearch program[34] revealed the presence of two putative CREs in the 5 kb region upstream of the transcriptional start site of the murine *c/ebp*δ promoter (Supplementary Figure 9). Both elements deviate from the consensus at only one position. We found no putative CREs within or 5 kb downstream of the murine *c/ebp*δ gene. In contrast to mice, humans have four putative CREs within the 5 kb region upstream of the transcriptional start site.

Using chromatin immunoprecipitation (ChIP), we assessed differences in the recruitment of the active CREB transcriptional complex in response to increased cAMP levels. Phosphorylated CREB was recruited to the proximal CRE (CRE2) relative to the transcriptional start site in the *c/ebp*δ promoter upon IBMX treatment (Figure 5e). Interestingly, Mdm2 was required for the recruitment of Crtc2 to the same CRE. The inability to form a transcriptional active complex on the *c/ebp*δ promoter in the absence of Mdm2 was underscored by the finding that in contrast to *p53*$^{-/-}$ MEFs, binding of the two histone acetylases p300 and CBP was not increased in *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs upon IBMX treatment. Western blotting showed equal levels of P-CREB, Crtc2, p300 and CBP in *p53*$^{-/-}$ and *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs (Supplementary Figure 10).

Collectively, these data indicate that Mdm2 is required for CREB-mediated induction of C/EBPδ by facilitating the assembly of the transcriptional complex consisting of CREB, Crtc2 and p300/CBP on the *c/ebp*δ promoter.

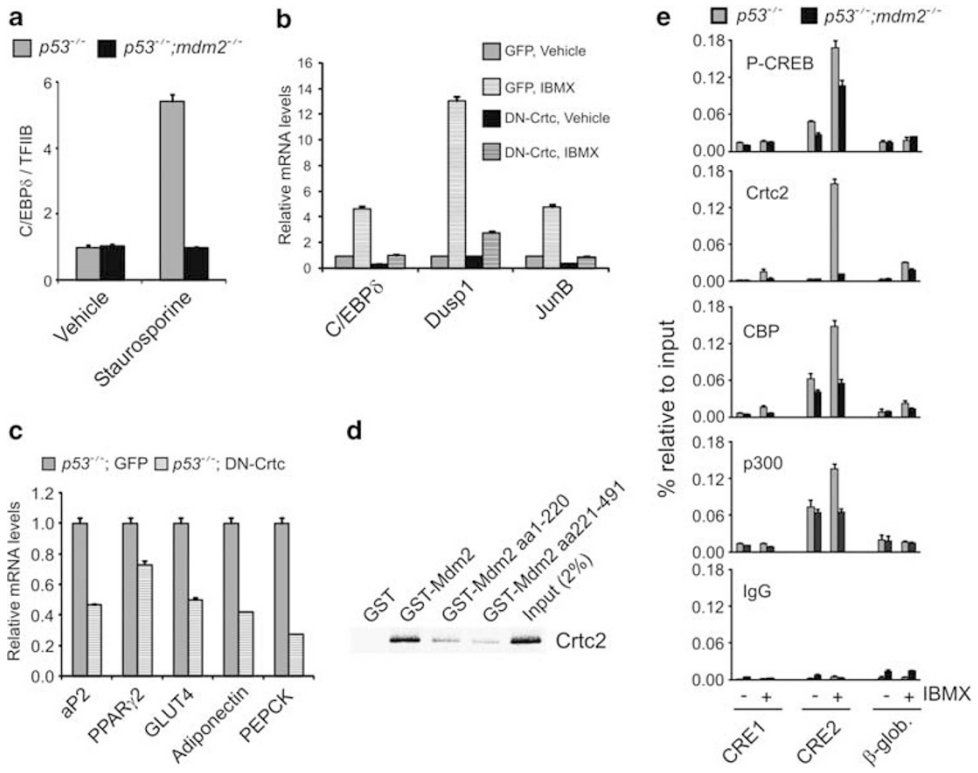

Figure 5 Mdm2 is required for the recruitment of Crtc2 and p300/CBP to CREs in the *c/ebpδ* promoter. (**a**) *P53*$^{-/-}$ and *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs were treated with vehicle or staurosporine for 4 h. C/EBPδ mRNA levels were measured using real-time PCR. (**b** and **c**) *P53*$^{-/-}$ MEFs were retrovirally transduced with vector expressing GFP or GFP-DN-Crtc. (**b**) Transduced MEFs were treated with vehicle or IBMX for 1 h. mRNA levels were determined using real-time PCR. (**c**) Transduced MEFs were induced to undergo adipogenesis according to the MDI standard protocol in the presence of rosiglitazone. Adipocyte differentiation was scored by adipocyte marker gene expression. GLUT4, glucose transporter 4. (**d**) *In vitro* translated Crtc2 was pulled down using Mdm2 or its halves fused to GST. (**e**) At 2 days postconfluence, *p53*$^{-/-}$ and *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs were left untreated or stimulated with IBMX for 30 min. Binding of P-CREB, Crtc2, p300 and CBP to the *c/ebpδ* promoter was assessed by chromatin immunoprecipitation. Non-specific IgG was included as control. β-Globin was used to assess background levels

Discussion

The transcription factor CREB plays a key role in adipocyte differentiation.[4–6] Our results show that Mdm2 enhances CREB activity and adipocyte differentiation by facilitating the recruitment of Crtc2 and p300/CBP to CREB bound to the *c/ebpδ* promoter. Conversely, MEFs lacking *mdm2* fail to undergo adipocyte differentiation.

Our *ex vivo* data revealed a striking dependency on Mdm2 for adipocyte differentiation by facilitating the induction of C/EBPδ. However, CT scans of mice harboring and lacking Mdm2 revealed no statistically significant difference in total adipose tissue ($P=0.08$). In their analyses of mice lacking C/EBPδ, C/EBPβ or both, Akira and co-workers[2] did not observe a decreased weight of adipose tissue in mice lacking either C/EBPδ or C/EBPβ. Only mice lacking both C/EBPs had a significant decrease in adipose mass. It is therefore conceivable that C/EBPδ is dispensable for adipogenesis in mice lacking Mdm2, and that C/EBPβ, the expression of which is unaffected by the absence of Mdm2, is sufficient to support adipogenesis *in vivo*.

Given the importance of Crtc2 in regulating a distinct transcriptional program through CREB downstream of cAMP stimulation, the modes of regulating the activity of the cofactor have been an area of intense research.

In resting cells, Crtc2 is localized to the cytoplasm. Upon cAMP stimulation Crtc2 translocates to the nucleus.[7] We observed no difference in the ability to translocate exogenously expressed Crtc2 in *p53*$^{-/-}$ and *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs upon cAMP elevation, arguing for normal translocation of Crtc2 in the absence of Mdm2.

Activation of Crtc2 is controlled by several serine phosphorylations, all exerting an inhibitory function on Crtc2 by either decreasing its coactivator activity or ablating nuclear translocation.[35] These phosphorylations can all be removed by treating cells with the general kinase inhibitor, staurosporine.[35] Intriguingly, although staurosporine was able to induce C/EBPδ expression in *p53*$^{-/-}$, the inhibitor was unable to induce C/EBPδ expression in *p53*$^{-/-}$;*mdm2*$^{-/-}$MEFs. This demonstrates that even removal of inhibitory phosphorylations on Crtc2 was unable to restore C/EBPδ induction in *p53*$^{-/-}$;*mdm2*$^{-/-}$ MEFs. Furthermore, IBMX treatment lead to disappearance of the upper, phosphorylated band in western blots of Crtc2 in MEFs of both genotypes (Supplementary Figures 10 and 11), demonstrating that deficient IBMX-mediated dephosphorylation was not the cause of abated Crtc2 activation upon cAMP stimulation in *mdm2*-deficient MEFs.

As Mdm2 is an ubiquitin ligase and regulates the stability of, for example, p53, it is of interest that the stability of Crtc2 has been reported to be regulated by ubiquitination.[36] However,

both the level and stability of Crtc2 were similar in *p53*$^{-/-}$ and *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs (Supplementary Figure 11).

Interestingly, the activity of Crtc2 is augmented by a p300-mediated acetylation.[36] Given our demonstration that Mdm2 can directly interact with Crtc2, and previous evidence that it can also interact with p300/CBP,[37] it is possible that Mdm2 serves as a scaffold for the interaction between p300/CBP and Crtc2 and thereby facilitates the acetylation and activation of the latter. Although both the C- and the N-terminal halves of Mdm2 can interact with Crtc2, the ability of the N-terminal part of Mdm2 to restore adipogenesis might reflect the potential of this half of Mdm2 to also bind p300/CBP.[37]

Besides the involvement of Mdm2 in the cAMP-stimulated activation of CREB, Mdm2 is also required for in stress-mediated stimulation of CREB. This was shown by the impaired induction of CREB-responsive genes in cells lacking Mdm2 after TPA treatment. As TPA-mediated activation of CREB is independent of Crtc2 recruitment, it is most likely that Mdm2 regulates CREB downstream of stress signals through a distinct mechanism.

Our data point to a p53-independent requirement of Mdm2 for adipocyte differentiation. Given the important role of Mdm2 in regulating p53 activity and the recently shown inhibitory effect of p53 on adipogenesis,[38] it is conceivable that Mdm2 exerts dual functions in relation to the regulation of adipocyte differentiation. Mdm2 could theoretically augment adipogenesis through potentiating of CREB-mediated transactivation and restriction of p53 activity. Further studies are required to determine the relative contribution of Mdm2 to these two functions. This could be assessed by comparing adipogenesis in wild-type and p53-deficient MEFs deficient for Mdm2 through Cre-mediated deletion of floxed-Mdm2 alleles or lowered expression through siRNA-mediated knockdown of Mdm2.

Earlier studies have shown that increased levels of Mdm2 block myocyte differentiation due to inhibition of Sp1 activity.[21,22] Here, we show that Mdm2 favors the differentiation of adipocytes at the expense of myogenesis. As myocytes and adipocytes originate from MSCs, it is possible that Mdm2 is involved in the determination of cell fate of MSCs, a notion underscored by the consistent amplification of Mdm2 in liposarcomas.

Materials and Methods

Cell culture and differentiation. Wild-type MEFs were a generous gift from Dr. Jiri Bartek. *P53*$^{-/-}$ and *p53*$^{-/-}$*;mdm2*$^{-/-}$ MEFs have been described previously.[39] MEFs were grown in AmnioMax basal medium (Invitrogen, Carlsbad, CA, USA) supplemented with 7.5% fetal bovine serum, 7.5% AmnioMax-C100 supplement (Invitrogen), 2 mM glutamine, 62.5 μg/ml penicillin and 100 μg/ml streptomycin (Lonza, Basel, Switzerland). The medium was changed every second day. For differentiation, 2-day postconfluent cells (day 0) were treated with growth medium containing 1 μM Dex (Cat. no. D1756), 0.5 mM IBMX (Cat. no. I7018), 1 μg/ml Ins (Cat. no. I6634) (all Sigma-Aldrich, St. Louis, MO, USA). From days 2 to 4, the medium contained Ins. Rosiglitazone (0.5 μM) (Cat no. 71740; Cayman, Ann Arbor, MI, USA) or vehicle (DMSO) (Cat no. D8418; Sigma-Aldrich) was added throughout differentiation. Forskolin (10 μM) (Cat. no. F3917) and TPA/PMA (20 nM) (Cat. no. P1585) were from Sigma-Aldrich; staurosporine (10 nM) (Cat. no. S-9300) from LC Laboratories (Woburn, MA, USA); Rho kinase inhibitor (10 μM) (Cat. no. 555550) from Calbiochem (San Diego, CA, USA); and specific PKA (100 μM) (Cat. no. M 003) and Epac (200 μM) (Cat. no. C 041) activators were from Biolog (Hayward, CA, USA). Cycloheximide (Cat. no. O1810; Sigma-Aldrich) was used in a concentration of 15 μg/ml.

Retroviral transduction. Phoenix cells were transfected with pBABE-based plasmids. At 2 days post-transfection, media were isolated, spun down at 1200 $\times$ *g* for 5 min to remove cellular debris, mixed 1 : 1 with standard media and added to cells. Polybrene was added to a final concentration of 6 μg/ml. Cells were selected for 2 days using puromycin (3 μg/ml).

Chromatin immunoprecipitation. ChIP was carried out essentially as described previously,[40] except that crosslinking was carried out by adding formaldehyde directly to the media to a final concentration of 1%, followed by incubation at 37°C for 20 min. Furthermore, 48 rounds of sonication were applied. Finally, DNA was purified by phenol–chloroform extraction. Antibodies used were P-CREB-1 (Ser133) (sc-7978), CBP (sc-369), p300 (sc-584) and Crtc2 (sc-46272) (all from Santa Cruz Technology, Santa Cruz, CA, USA). Primer sequences are CRE1, 5′-GTTCAGCTTCTGTGTTTAGAGG-3′ and 5′-CCCTCTCCTTCTGCTCCTCC-3′; CRE2, 5′-GCTGCGGAGCCTTGATCC-3′ and 5′-CACTCCTTGCCTTCCCTCC-3′; and β-globin, 5′-CCTGCCCTCTCTATCCTGTG-3′ and 5′-GCAAATGTGTTG CCAAAAAG-3′.

RNA purification, reverse transcription and real-time PCR. RNA was purified using TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed essentially as described elsewhere.[41] Quantitative PCR was performed in 20 μl reactions containing SYBR^{-} Green JumpStart Taq ReadyMix (Sigma-Aldrich), 1.5 μl of diluted cDNA and 300 nM of each primer. Reaction mixtures were preheated at 94°C for 2 min, followed by 40 cycles of melting at 94°C for 15 s, annealing at 60°C for 30 s and elongation at 72°C for 45 s. Reactions were ran on a Stratagene MX3000P and quantified using Stratagene MxPro. Primer sequences are included in supporting Materials and Methods.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)