E2F1 is crucial for E2F-dependent apoptosis

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Loss of the retinoblastoma protein, pRB, leads to apoptosis, and several results have suggested that this is dependent on the E2F transcription factors. However, so far, the ability of the different E2F family members to contribute to apoptosis is controversial. Here, we show that ectopic expression of E2F3 results in apoptosis in both primary mouse fibroblasts and transgenic mice. Apoptosis induced by E2F3 is associated with the accumulation of E2F1 and, strikingly, we found that E2F3-induced apoptosis is dependent on E2F1. On the basis of these results, we propose that the accumulation of crucial levels of E2F1 activity, and not total E2F activity, is essential for the induction of apoptosis in response to a deregulated pRB pathway. These results are consistent with previous findings that E2F1, but not other E2Fs, can have tumour-suppressing activities.

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INTRODUCTION

Deregulation of the retinoblastoma protein (pRB) pathway is one of the hallmarks of human cancers (Weinberg, 1995). Tumours harbouring genetic alterations in the pRB pathway often have additional mutations affecting the apoptotic response (Weinberg, 1995; Sherr, 1996). As these data suggest that apoptosis has a crucial role in tumour suppression, it is of considerable scientific interest to understand the molecular mechanisms triggering apoptosis. The hypophosphorylated form of pRB binds to, and negatively regulates, the 'activating' E2Fs (E2F1–3; Attwooll *et al*, 2004). Thus, loss of functional pRB leads to deregulation of the activating E2Fs and increased expression of E2F-regulated genes. The finding that loss of either *E2f1* or *E2f3* can rescue the apoptosis observed in pRB-deficient mouse embryos (Tsai *et al*, 1998; Ziebold *et al*, 2001) suggests that deregulation of E2F transcription factors is a key step for apoptosis induction after loss of pRB. E2F1

overexpression, both *in vivo* and in tissue culture, results in apoptosis (Qin *et al*, 1994; Shan & Lee, 1994; Holmberg *et al*, 1998; Pierce *et al*, 1998a,b, 1999). Still controversial, however, is whether other activating E2Fs can trigger apoptosis. On the basis of the observation that loss of both *E2f1* and *E2f3* rescues apoptosis in pRB-null embryos, Trimarchi & Lees (2002) proposed that apoptosis is triggered in cells when deregulated E2F activity reaches a threshold level. In contrast, tissue-culture-based experiments have supported the notion that induction of apoptosis is a specific function of E2F1 (DeGregori *et al*, 1997). Hallstrom & Nevins (2003) recently reported that the E2F1 marked box domain has a unique proapoptotic activity among E2Fs.

Contrary to previous reports, we have analysed the ability of E2F3 to trigger apoptosis *in vivo*. We show here that E2F3 deregulation triggers apoptosis and, interestingly, that E2F3-induced apoptosis is dependent on E2F1 and is associated with increased E2F1 levels. Taken together, our data suggest that loss of pRB, here exemplified by E2F3 overexpression, causes deregulation of the activating E2Fs. This deregulation results in the increased expression of E2F1, ultimately leading to apoptosis.

RESULTS

Deregulated E2F3 activity induces apoptosis *in vivo*

We have recently described the generation of a mouse model that expresses E2F3 fused to a modified version of the ligand-binding domain of the oestrogen receptor (ER–E2F3) in the intermediate lobe of the pituitary gland (ILP; Denchi *et al*, 2005). ER–E2F3 is expressed under the control of the tissue-specific promoter POMC and the fusion protein is inactive in the absence of the inducer 4-hydroxy tamoxifen (OHT). Activation of E2F3 leads to increased expression of E2F target genes and proliferation of the otherwise quiescent melanotrophs of the ILP (Fig 1A; Denchi *et al*, 2005). Sustained E2F3 activation results in hyperplasia of the ILP, but not in tumour formation, suggesting that a tumour suppression mechanism is capable of restraining proliferation induced by E2F3 deregulation (Denchi *et al*, 2005). Therefore, we analysed whether E2F3 deregulation leads to apoptosis and, consequently, whether apoptosis acts as a principal tumour suppression mechanism. Overexpression of E2F1 in tissue culture cells and in transgenic mice results in apoptosis (Qin *et al*, 1994; Holmberg *et al*, 1998; Pierce *et al*, 1998a,b, 1999) and suppression of this apoptosis can result in tumour formation (Pierce *et al*, 1998a,b). To assess whether deregulated E2F3 activity can result in apoptosis, a group of transgenic mice and a group of wild-type

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Fig 1 | E2F3 activation triggers apoptosis in transgenic mice. (A) 5-Bromodeoxyuridine (BrdU) incorporation assay (3 h pulse). Shown are melanotrophs of the intermediate lobe of the pituitary gland (ILP) derived from transgenic mice treated with tamoxifen (Tg + TAM) and controls, wild type treated with tamoxifen (WT + TAM) and transgenic nontreated (Tg). Right panel: quantification of percentage of BrdU-positive cells. Results represent standard deviation of the mean as described in the Methods. (B) TdT-mediated dUTP nick end-labelling assay (TUNEL; top panels) and immunohistochemical (IHC) analysis for activated caspase 3 (Ac. Casp3; bottom panels). Panels show ILP sections derived from wild-type (WT + TAM) and transgenic (Tg + TAM) mice treated with tamoxifen for 3 days. Right panels: quantification of TUNEL- and active caspase-3-positive cells in transgenic mice treated with tamoxifen (dashed bars) and in controls, wild type treated with tamoxifen (white bars). (C) IHC analysis for activated caspase 3 (Ac. Casp3; bottom panels) and TUNEL assay (top panels). Panels show ILP melanotrophs derived from wild-type ($p53^{+/+}$), transgenic ($p53^{+/+}$, Tg) and compound $p53^{-/-}$; transgenic mice ($p53^{-/-}$, Tg) treated with tamoxifen for 3 days. Graphs on the right: quantification of TUNEL- and activated caspase-3-positive cells of wild-type (white box, $p53^{+/+}$), transgenic (black box, $p53^{+/+}$, Tg) and compound $p53^{-/-}$; transgenic mice (dashed box, $p53^{-/-}$, Tg).

mice (five mice/genotype) were treated with tamoxifen for 3 days. As mentioned above, E2F3 activation results in S-phase entry (Fig 1A); we also observed that E2F3 induces apoptosis, as measured by active caspase-3 staining and TdT-mediated dUTP nick end-labelling (TUNEL) assay (Fig 1B). Importantly, no apoptosis was observed in control mice, either tamoxifen-treated wild type or nontreated transgenic (Fig 1B; data not shown). Notably, the observed difference in apoptosis between transgenic induced mice and controls is statistically significant (both assays $P < 0.005$; Fig 1B). Similar levels of apoptosis were also detected in animals treated for longer periods of time (data not shown). Therefore, we conclude that deregulation of E2F3 activity results in apoptosis *in vivo*.

E2F3 can induce p53-independent apoptosis

Next, we assessed whether E2F3-induced apoptosis in the ILP is mediated by p53. Strikingly, we found that loss of p53 had no effect on the ability of E2F3 to induce apoptosis, as measured by both TUNEL assay ($P = 0.47$) and activation of caspase 3 ($P = 0.29$; Fig 1C). These results are in agreement with previous published data showing that loss of pRB and ectopic expression of E2F1 can trigger p53-dependent and p53-independent apoptosis (Morgenbesser *et al*, 1994; Macleod *et al*, 1995; Holmberg *et al*, 1998). We therefore conclude that deregulated E2F3 activity induces apoptosis independently of p53 in the mouse pituitary gland.

E2F3 induces E2F1-dependent apoptosis in MEFs

We investigated alternative mechanisms by which E2F3 could induce apoptosis, and tested whether E2F1 could be involved. Among the E2F family members, E2F1 is the best characterized for its ability to induce apoptosis and, interestingly, it is an E2F-responsive gene, which is transactivated by the activating E2Fs (Hsiao *et al*, 1994; Johnson *et al*, 1994). We, therefore, speculated that activation of E2F3 could lead to an increase in E2F1 levels that could subsequently lead to apoptosis. We tested this hypothesis by expressing ER-E2F3 in mouse embryo fibroblasts (MEFs). Wild-type and *E2f1*^{-/-} MEFs were infected and, after selection, the viability of cells was assessed by Trypan blue exclusion in the presence or absence of OHT. As previously reported, the viability and growth potential of wild-type and *E2f1*^{-/-} infected MEFs are similar (Fig 2A). On activation of E2F3, the growth of wild-type cells is severely impaired and cells undergo apoptosis (Fig 2A). In contrast, E2F3 activation leads to an insignificant amount of apoptosis in MEFs lacking E2F1 and a consequential increase in viable cells (Fig 2A). Importantly, however, E2F3 activation still leads to a decrease in proliferation rate, which is probably caused by E2F3-induced senescence (Denchi *et al*, 2005). The observed difference in apoptosis is not because of the different expression levels of ER-E2F3 (Fig 2B) and, moreover, the functionality of ER-E2F3 is not impaired in *E2f1*^{-/-} cells, as E2F target genes, such as Cyclin E1 and PCNA (proliferating cell nuclear antigen), are induced to similar levels in the two cell lines (Fig 2B,F). We also excluded possible effects arising from either OHT treatment or ER expression, because HAER-expressing (ER fused to an HA tag) cells do not show any growth defects in the absence or presence of OHT (data not shown; Moroni *et al*, 2001). As an independent marker of apoptosis, we measured the levels of active caspase 3 in wild-type and *E2f1*^{-/-} cells. As shown in Fig 2C, activation of E2F3 leads to a significant increase in activated caspase 3 in wild-type

but not *E2f1*^{-/-} MEFs (Fig 2C). Interestingly, we found that increased E2F3 activity led to a strong upregulation of *E2f1* expression in wild-type MEFs (Fig 2C). As *E2f1* is a known E2F target gene, our results therefore suggest that activation of E2F3 leads to E2F1-dependent apoptosis through direct transactivation of the *E2f1* gene.

Next, we infected wild-type and *E2f1*^{-/-} MEFs with a retrovirus expressing ER-E2F1, ER-E2F3 or both. In agreement with previous reports (e.g. Qin *et al*, 1994), E2F1 activation results in robust induction of apoptosis in wild-type and *E2f1*^{-/-} MEFs despite a slight difference in the kinetics of the response (Fig 2D). These data show that *E2f1*^{-/-} MEFs do not have a general defect in the apoptotic machinery. In agreement with previous reports, we found that E2F1 triggers apoptosis more efficiently than E2F3, despite being expressed at similar levels (Fig 2E). Moreover, we analysed the effect of the concomitant induction of ER-E2F1 and ER-E2F3 in wild-type and *E2f1*^{-/-} MEFs. In both cell types, we found that E2F3 does not significantly increase apoptosis to a level higher than when only E2F1 is activated. These results support the hypothesis that E2F3-induced apoptosis is dependent on E2F1, and, mechanistically, that E2F3 activation leads to E2F1 accumulation, which results in apoptosis. This hypothesis is supported further by the fact that loss of p53 in MEFs, to a similar extent, reduces E2F1- and E2F3-induced apoptosis (supplementary Fig 1 online). To investigate whether a reciprocal dependency exists between E2F1 and E2F3 in inducing apoptosis, we infected MEFs containing floxed *E2f3* alleles (Wu *et al*, 2001). As shown in supplementary Fig 1 online, deletion of *E2f3* did not lead to a significant reduction of apoptosis. Taken together with the fact that overexpression of E2F1 induces apoptosis in *E2f1*^{-/-} MEFs, whereas E2F3 overexpression does not, these data suggest that the E2F1 levels rather than the overall levels of E2F activity are crucial for determining whether a cell will undergo apoptosis.

E2F3 induces E2F1-dependent apoptosis *in vivo*

Induction of E2F3 in the ILP results in an increase of E2F1 levels, suggesting that E2F1 could also have a role in E2F3-induced apoptosis *in vivo* (Fig 3A). Therefore, we generated transgenic mice expressing ER-E2F3 in an *E2f1*^{-/-} genetic background. Significantly, we found that E2F3 activation in the pituitary gland failed to induce apoptosis in *E2f1*^{-/-} mice, whereas wild-type siblings showed significant apoptosis, as measured by active caspase-3 staining and by TUNEL assay (both assays $P < 0.005$; Fig 3B). Significantly, loss of *E2f1* does not impair E2F3-induced activation of E2F target genes, as shown by PCNA staining (Fig 3B). Similarly, E2F3 activation resulted in unscheduled proliferation in both wild-type and *E2f1*^{-/-} mice, as shown by 5-bromodeoxyuridine (BrdU) incorporation and PCNA staining (Fig 3B). These data show that loss of *E2f1* does not impair E2F3 functionality and, moreover, that induction of apoptosis and induction of proliferation are distinct functions of E2F3. Thus, we conclude that activation of E2F3 triggers E2F1-dependent apoptosis *in vivo*.

E2F3-induced proliferation is enhanced in *E2f1*^{-/-} mice

As the activation of E2F3 in *E2f1*^{-/-} mice can induce unscheduled proliferation but not apoptosis, we tested the effect of sustained E2F3 activity in the absence of apoptosis. Mice (ten mice/genotype/treatment) were injected daily with tamoxifen, and pituitary homeostasis was monitored throughout a period of

Fig 2 | E2F3 results in E2F1-dependent apoptosis in mouse embryo fibroblasts (MEFs). (A) Early-passage MEFs derived from wild-type (*E2f1*^{+/+}) or *E2f1*^{-/-} littermate embryos were infected with pBabe-HAER-E2F3 (ER-E2F3). After selection, cells were either not treated or treated with 4-hydroxy tamoxifen (+OHT). Live cells (left panel) and apoptotic cells (right panel) were measured by Trypan blue exclusion at the indicated durations. (B) Top panel: expression of ER-E2F3 in *E2f1*^{-/-} and *E2f1*^{+/+} cells as detected with an anti-ER antibody. Bottom panel: ER-E2F3 fusion protein is functional in both *E2f1*^{-/-} and *E2f1*^{+/+} MEFs as shown by Cyclin E1 induction. As a loading control, blots were incubated with an anti-vinculin antibody. (C) Lysates were generated from MEFs collected at the indicated durations. Western blots were probed with anti-E2F1, anti-activated caspase-3 and vinculin antibodies. (D) MEFs from wild-type (*E2f1*^{+/+}) or *E2f1*^{-/-} littermate embryos were infected with HAER-E2F1, HAER-E2F3 or both. After selection, cells were induced with OHT and cell death was measured by Trypan blue exclusion at the indicated durations. (E) Top panel: expression of ER-E2F1 and ER-E2F3 in *E2f1*^{-/-} and *E2f1*^{+/+} cells, as detected with an HA (haemagglutinin) antibody. Bottom panel: ER-E2F1 expression relative to endogenous *E2f1* expression. As loading control, blots were incubated with an anti-vinculin antibody. n.i., not infected. (F) ER-E2F1 and ER-E2F3 proteins are equally functional in both *E2f1*^{-/-} and *E2f1*^{+/+} MEFs, as shown by PCNA (proliferating cell nuclear antigen) induction. As a loading control, blots were incubated with an anti-vinculin antibody.

6 months. As recently described (Denchi *et al*, 2005), increased E2F3 activity in the pituitary gland leads to hyperplasia. Significantly, this hyperplasia is enhanced in *E2f1*^{-/-} mice when

compared with *E2f1*^{+/+} mice (Fig 4A,B). Interestingly, however, we did not detect the formation of tumours in any of the mice monitored during the course of the experiments. On the basis of

Fig 3 | E2F3 induces E2F1-dependent apoptosis in transgenic mice. (A) Immunohistochemical (IHC) analysis for *E2f1* in melanotrophs of the intermediate lobe of the pituitary gland derived from wild-type (WT + TAM) and transgenic mice (Tg + TAM) induced with tamoxifen. (B) IHC analysis for activated caspase 3 (Ac. Casp3, top panels), TdT-mediated dUTP nick end-labelling assay (TUNEL, second panels from the top) and IHC analysis for PCNA (proliferating cell nuclear antigen; second panels from the bottom) and 5-bromodeoxyuridine (BrdU) (bottom panels). Panels show melanotrophs derived from wild-type (*E2f1* +/+), transgenic (*E2f1* +/+, Tg) and compound *E2f1* -/-; transgenic mice (*E2f1* -/-, Tg) treated with tamoxifen for 3 days. Panels on the left: quantification of activated caspase-3, TUNEL-, PCNA- and BrdU-positive melanotrophs of wild-type (white box, *E2f1* +/+), transgenic (black box, *E2f1* +/+, Tg) and compound *E2f1* -/-; transgenic mice (dashed box, *E2f1* -/-, Tg).

these results, we conclude that E2F1-mediated apoptosis contributes to pituitary homeostasis after E2F3 deregulation, but its suppression is not sufficient to result in tumour formation. These results are consistent with data showing that long-term activation of E2F3 in transgenic mice leads to senescent-like features, characterized by lack of proliferation, senescent-associated heterochromatin foci and increased expression of p19^{Arf} and p16^{Ink4a} (Denchi *et al*, 2005).

DISCUSSION

Here, we have shown that E2F3, like E2F1, can trigger apoptosis both *in vivo* and in MEFs. Our results show that E2F3-induced apoptosis is dependent on E2F1, and that the molecular mechanism involves increased expression of E2F1. Interestingly, loss of E2F1 affects the apoptotic response to E2F3 deregulation without affecting the ability of E2F3 to induce E2F target genes and unscheduled proliferation of melanotrophs. On the basis of this,

Fig 4 | E2F1 loss enhances E2F3-induced hyperplasia. (A) Haematoxylin and eosin staining of pituitaries derived from transgenic mice (Tg, *E2f1*^{+/+}) and compound *E2f1*^{-/-}; transgenic (*E2f1*^{-/-}, Tg) mice treated with tamoxifen for 3 months and pituitaries derived from nontreated *E2f1*^{-/-} mice. (B) Thickness of the intermediate lobe of the pituitary gland (ILP) relative to wild-type controls at the indicated time of tamoxifen treatment in *E2f1*^{-/-}, transgenic mice wild type for *E2f1* (*E2f1*^{+/+}, POMC-ER-E2F3) and transgenic mice null for *E2f1* (*E2f1*^{-/-}, POMC-ER-E2F3). Bars indicate the relative thickness of the ILP expressed as percentage relative to wild-type controls. (C) Model for E2F-induced apoptosis. The retinoblastoma protein represses the activity of the activating E2Fs, which regulate the expression of several genes involved in proliferation. E2F1 is required for E2F-induced apoptosis, and it regulates the expression of a number of genes and the activity of proteins involved in apoptosis.

we propose that it is E2F1 that directly triggers apoptosis, rather than a checkpoint activated by abnormal proliferation. Moreover, as primary cells lacking E2F1 undergo apoptosis like wild-type cells after activation of ectopic E2F1, but not E2F3, our data suggest that it is E2F1 activity rather than the total amount of E2F activity that triggers apoptosis. Thus, we propose that deregulation of the pRB pathway, here exemplified by E2F3 expression, leads to

accumulation of E2F1, which specifically leads to apoptosis (Fig 4C). On the basis of previous reports, we envision that increased E2F1 activity leads to apoptosis through an ARF-p53-dependent pathway as well as p53-independent pathways, which include the activation of E2F1 target genes such as *APAF1* and *p73* or of *CHK2* (Irwin *et al*, 2000; Moroni *et al*, 2001; Kang *et al*, 2002; Rogoff *et al*, 2004). Importantly, our data reconcile previous

published observations by demonstrating that the activating E2Fs can all induce apoptosis, but that this is dependent on E2F1, which specifically regulates the activation of proapoptotic genes.

METHODS

Mice and tamoxifen administration. The generation of the POMC-ER-E2F3 mice has been described previously (Denchi *et al*, 2005). POMC-ER-E2F3 transgenic mice, *E2f1* mutant mice (Yamasaki *et al*, 1998), *p53*^{-/-} mice (Jacks *et al*, 1994) and compound mice were all bred in a mixed genetic background (129 sv//C57 Bl/6). Tamoxifen (Sigma, St Louis, MO, USA) was resuspended in sunflower oil (Sigma) and injected daily (1 mg dose).

Western blotting and antibodies. Proteins were extracted using E1A lysis buffer (50mM Hepes pH 7.0, 250mM NaCl, 0.1% NP-40) buffer. Protein lysates were separated on an SDS-polyacrylamide gel electrophoresis gel and transferred to Immobilon-P (Millipore, Billerica, MA, USA) by electroblotting. Primary antibodies used were anti-PCNA (Pharmingen, Franklin Lakes, NJ, USA), anti-BrdU (Becton Dickinson, Franklin Lakes, NJ, USA), anti-E2F1 (C20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HA (haemagglutinin, HA.11; Babco, Denver, PA, USA), anti-activated caspase 3 (ASP175; Cell Signaling Technology, Beverly, MA, USA), anti-ER (MC-20; Santa Cruz) and anti-Cyclin E1 (M-20, Santa Cruz).

Immunohistochemistry. For all our analyses, at least five mice/genotype/treatment were analysed, two independent sections/animal were stained and sequentially different fields were counted to evaluate the fraction of positive cells/total. A total of six to ten independent counts (300 cells each) were performed. A two-tailed *t*-test was performed to evaluate statistical significance. All antibodies were used on paraffin-embedded sections. For detection, we used the Envision™ kit (DAKO, Glostrup, Denmark). TUNEL assay was performed using the Apoptag® Peroxidase Plus Kit (Chemicon, Temecula, CA, USA, S7101).

Virus-mediated gene transfer and cell counts. The pBabe-ER-E2F constructs have been described (Vigo *et al*, 1999; Müller *et al*, 2001). The retrovirus-mediated gene transfer was conducted, as described (Moroni *et al*, 2001). To activate ER-E2F3, OHT was added to the medium to a final concentration of 300 nM. The number of live cells is expressed as percentage relative to the number of cells at time 0. The number of dead cells is expressed as percentage relative to the number of live cells at the indicated time. Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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