E2F1 and E2F2 prevent replicative stress and subsequent p53-dependent organ involution

Running title: E2F-p53 regulatory axis in tissue homeostasis

Ainhoa Iglesias-Ara[1‡], Olatz Zenarruzabeitia[1‡!], Luis Buelta[2], Jesus Merino[2] and Ana M. Zubiaga[1*]

[‡]A Iglesias-Ara and O Zenarruzabeitia share first authorship

[1]Department of Genetics, Physical Anthropology and Animal Physiology, University of the Basque Country, UPV/EHU, Spain; [2]University of Cantabria-IDIVAL, Spain

Correspondence: Ana M. Zubiaga, Department of Genetics, Physical Anthropology and Animal Physiology, University of the Basque Country, UPV/EHU, 48080, Bilbao, Spain; Tel.: 34.94.601.2603; Fax: 34.94.601.3143; Email: ana.zubiaga@ehu.es

[!]Present address: BioCruces Health Research Institute, Barakaldo, Spain, and Basque Center for Transfusion and Human Tissues, Galdakao, Spain

ABSTRACT

Tissue homeostasis requires tight regulation of cellular proliferation, differentiation and apoptosis. E2F1 and E2F2 transcription factors share a critical role in tissue homeostasis, since their combined inactivation results in overall organ involution, specially affecting the pancreatic gland, which subsequently triggers diabetes. We have examined the mechanism by which these E2Fs regulate tissue homeostasis. We show that pancreas atrophy in E2F1/E2F2 double-knockout (DKO) mice is associated with mitochondrial apoptosis and activation of the p53 pathway in young animals, prior to the development of diabetes. A deregulated expression of E2F target genes was detected in pancreatic cells of young DKO animals, along with unscheduled DNA replication and activation of a DNA damage response. Importantly, suppression of DNA replication *in vivo* with aphidicolin led to a significant inhibition of the p53 pathway in DKO pancreas, implying a causal link between DNA replication stress and p53 activation in this model. We further show that activation of the p53 pathway plays a key role in the aberrant phenotype of DKO mice, since targeted inactivation of p53 gene abrogates cellular apoptosis and prevents organ involution and insulin-dependent diabetes in E2F1/E2F2-deficient mice. By contrast, we show that p21^{CIP1} is dispensable for the DKO phenotype. Remarkably, p53 inactivation unmasked tumorigenic features of E2F1/E2F2-deficient cells. Collectively, our data reveal a role for E2F1 and E2F2 as suppressors of replicative stress in differentiating cells, and uncover the existence of a robust E2F-p53 regulatory axis to enable tissue homeostasis. Cells undergoing replicative stress after E2F1/E2F2 loss are rapidly eliminated by p53-mediated apoptosis, thus preventing the accumulation of aberrant cells *in vivo* at the expense of tissue atrophy.

KEYWORDS

INTRODUCTION

The challenges that cells face to maintain a differentiated status within a tissue entail overcoming a number of obstacles, such as uncontrolled proliferation, damaged organelles, reactive oxygen species or DNA breaks. Aberrant cells can activate surveillance pathways to be eliminated, or they may, alternatively, accumulate. Lack of balance between these two decisions results in tissue degeneration or in tumorigenesis. Cancer cells commonly exhibit deregulation of a functional retinoblastoma protein (pRb) pathway[1]. Such deregulation can occur at multiple points of the pathway, including mutations or deletions of pRb gene itself, activation of cyclin/CDK kinases that phosphorylate and thereby functionally inactivate pRb, or inactivation of CDK inhibitors, such as p16[INK4A] (ref.2). The oncogenic stress induced by loss of pRb function is known to activate the other major tumor suppressor, p53 (ref.2). Activated p53 can, depending on the cellular context, induce cell cycle arrest, senescence or apoptosis as a failsafe mechanism against tumorigenesis, and the combined loss of pRb and p53 pathways leave cells prone to tumorigenesis. Conversely, hiperactivation of the p53 pathway upon oncogenic stress induced by pRb loss prevents tumor development, yet premature aging may arise leading to a reduction in lifespan[3].

An extensively characterized function of pRb involves its interaction with members of the E2F family of transcription factors, whereby hypophosphorylated pRb protein forms a complex with E2F members and blocks their transcriptional activity, preventing entry into the cell cycle[4]. Cyclin/CDK-mediated phosphorylation of pRb during G1 releases E2F, thus allowing gene transcription and cell cycle progression. Inactivation of the pRb pathway is thought to lead to inappropriate activation of E2F factors, followed by increased cellular proliferation rates and tumorigenesis[5]. Thus, inhibition of E2F activity has been proposed as a therapeutic strategy for the treatment of cancer[6]. Mammalian E2F comprises a family of eight transcription factors, originally discovered for their crucial role in the control of cell cycle progression through the regulation of a large set of responsive genes[7-9]. Subsequently, it was shown that E2Fs also play essential roles in a wide range of functions such as differentiation, apoptosis, and DNA repair, underlying their relevance in tissue homeostasis[10, 8-9].

E2F1 and E2F2 are known to function both as transcriptional activators and repressors of their target genes, including those coding for microRNAs[11-15]. Moreover, E2F1 and E2F2 perform positive as well as negative regulatory roles in cellular proliferation, which appear to be cell-specific and context-dependent. They have been reported to promote cell cycle entry and progression upon their ectopic expression[16]. By contrast, targeted inactivation of these E2Fs revealed their essential role in sustaining quiescence in hematopoietic cells[11], in supporting survival of progenitor cells[16] or in facilitating exit from the cell cycle in differentiating cells[13,18]. However, the mechanisms by which E2F1 and E2F2 accomplish such diverse functions remain unresolved.

The pancreas has emerged as an optimal system to unravel the role of E2F1 and E2F2 in the maintenance of tissue homeostasis. We and others have previously reported that combined inactivation of E2F1 and E2F2 transcription factors leads to exocrine and endocrine pancreatic dysfunction, causing diabetes and the collapse of the entire pancreas[19-20]. After a period of strong cell division cycles, which take place during embryonic development and finalize by the second week after birth, pancreatic tissue is virtually post-mitotic[21]. By contrast, pancreatic cells lacking E2F1 and E2F2 continue cycling aberrantly well beyond this age[20]. Strikingly, E2F1/E2F2 mutant pancreas exhibits an increased apoptotic rate and the organ degenerates rapidly[20]. Other organs in E2F1/E2F2 DKO mice also show a singular involution, particularly the hematopoietic system, salivary glands and testicles[20]. However, the mechanism involved in E2F1/E2F2-dependent tissue atrophy remains to be resolved.

Here we explore the mechanism by which E2F1 and E2F2 regulate tissue homeostasis. We show that inactivation of E2F1 and E2F2 results in accumulation of DNA replication mediators, activation of the DNA damage response and induction of p53-dependent apoptosis. Genetic elimination of p53 abrogates apoptosis and pancreatic gland involution, and rescues insulin-dependent diabetes. Importantly, the absence of p53 uncovers tumorigenic features of E2F1/E2F2-deficient cells. Collectively, our data stress the relevance of E2F1 and E2F2 as suppressors of replicative stress and regulators of cell cycle exit during differentiation, and reveal the existence of a robust E2F-p53 regulatory axis to ensure proper organ function and prevent tumorigenesis.

MATERIALS AND METHODS

Mouse strains

Colonies of E2F1^{-/-}, E2F2^{-/-}, and double knockout E2F1^{-/-}/E2F2^{-/-} mice have been described[22-23,20]. p53^{-/-} and p21[CIP1-/-] mice have also been described[24-25]. Double knockout E2F1^{-/-}/E2F2^{-/-} mice were bred to p53^{-/-} or p21[CIP1-/-] mice to generate triple knockout E2F1^{-/-}/E2F2^{-/-}/p53^{-/-} or E2F1^{-/-}/E2F2^{-/-}/p21[CIP1-/-] animals. All procedures were approved by the University of the Basque Country Animal Care and Use Committee.

Metabolic studies

Blood glucose levels were determined from blood taken from mouse-tails, using an automatic glucose monitor. Blood for insulin levels was collected from the submandibular vein. Insulin levels were measured in serum by ELISA (ALPCO Diagnostics, Windham, New Hampshire, USA), according to the manufacturer's instructions.

Amount of ROS in pancreas was determined in liquid nitrogen-frozen pancreases, using OxiSelect In Vitro ROS/RNS Assay Kit (Cell Biolabs, San Diego, CA) according to the protocol of the manufacturer.

Histology, immunohistochemistry and in vivo S-phase labeling

Freshly collected tissues were fixed with 10 % formalin in PBS and embedded in paraffin for staining with hematoxilin and eosin.

Immunohistochemical assays were performed on tissues fixed with 4 % paraformaldehyde in PBS using the indirect immunoperoxidase detection protocol Envision®FLEX (DAKO A/S, Glostrup, Denmark) in the automatic system DAKO autostainer link 48 (DAKO). The antibodies used were rabbit mAbs to glucagon (1:300; DAKO), Terminal Deoxynucleotidyl Transferase (TDT) (1:50; DAKO), γ-H2AX (1:2000; Millipore), and mouse mAbs to insulin (1:3,000), α-amylase (2 μg/ml) (Sigma-Aldrich, St. Louis, USA), CD3 (1:50; DAKO) and CD20 (1:400; DAKO). Control immunostainings using the secondary antibody in the absence of the primary antibody were routinely performed.

For S-phase labeling, mice were injected intraperitoneally (i.p.) with bromodeoxyuridine (BrdU; Sigma, St Louis, MO, USA) (50 μg/gr of body weight). Mouse organs were harvested eight hr later, and fixed in 10 % formalin. Tissue sections were processed for inmunohistochemistry using the rabbit monoclonal mAb to BrdU (1:100; DAKO). Images were captured using Olympus Cell B software.

WT and DKO mice were injected i.p. with 5-120 μg/g aphidicolin (Sigma). Controls received an equivalent volume of normal saline i.p. After 30 min, mice were injected i.p. with BrdU. Mouse organs were harvested 4 hr after the injection with aphidicolin. Freshly collected tissues were divided in two pieces and one set was fixed with 10 % formalin in PBS and embedded in paraffin, whereas the other set was placed immediately in liquid nitrogen to perform protein extraction.

Immunofluorescence of phospho-histone H3 (pH3) was performed on frozen tissue sections. Slides were microwaved at boiling for 30 min in 10 mM citric acid buffer, pH 6.0 and allowed to cool (30 min) prior to 5 min washes in 50 mM TBS, 0.1% Tween 20, pH 7.4 (TBS-T). Tissue sections were immunostained using primary antibody against pH3 (Ser15; Upstate) and Alexa Fluor 594 (Molecular Probes, Carlsbad, CA, USA) secondary antibody. DNA was counterstained with Hoechst 33288 dye and analysis was performed using an Olympus Fluoview FV500 microscope and Olympus Fluoview 1.7b software.

Image J Software from NIH was used to determine the percentage of BrdU-, γ-H2AX-, or pH3-positive nuclei in images of 3 randomly selected fields (20X magnification) from 2-4 pancreases per genotype.

Pancreatic cell isolation

Individual pancreatic cells were prepared essentially as previously described[26]. Briefly, 2.5 ml of 0.3 mg/ml collagenase solution (Clostridium histolyticum; Roche) in Krebs-Henseleit bicarbonate (KHB) buffer, pH 7.4 (118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.1 mM $MgCl_2$,, 2.5 mM $CaCl_2$, 1.2 mM $NaHPO_4$, and 14 mM glucose) was injected into the pancreas with a 27 gauge needle, and the gland was incubated at 37°C in a 15 ml polythene flask containing additional 2.5 ml of collagenase solution. After 20 min, the solution was replaced with KHB lacking Mg^{2+} and Ca^{2+} and containing 2 mM EDTA and incubated for 3 min. The solution was then replaced with fresh calcium-free KHB and the tissue

was incubated for a further 3 min. This was followed by a final incubation in collagenase in calcium-containing KHB for 20-30 min, until mostly single cells were present. The tissue was then further dispersed and filtered through 100 μm nylon meshes (Spectrum Laboratories).

Analysis of mitochondrial membrane potential, mitochondrial mass and apoptosis

Mitochondrial membrane potential was measured using the lipophilic cationic probe JC-1 as described[27]. JC-1 dye was purchased from Molecular Probes (Leiden, The Netherlands) and stored in DMSO (Sigma). Cells were adjusted to a density of $1x10^6$/ml and stained with 3μM JC-1 for 15 min at 37ºC. The mitochondrial uncoupler carbonyl cyanide m-chloro phenyl hydrazone (CCCP, Molecular Probes) was used at 50 μM for 5 min previous to incubation with JC-1 dye as a control in order to measure compromised mitochondrial membrane potential. Cells were then washed twice with pre-warmed KHB buffer and resuspended in KHB. Fluorescence was detected and analyzed using a FACSCalibur flow cytometer.

To detect mitochondrial mass, nonyl-acrydine-orange (NAO) was used as described elsewhere[28]. Briefly, cells were incubated at the concentration of $0.5x10^6$/ml with 5 μM NAO for 10 min at 37ºC, washed twice in cold KHB and analyzed using a FACSCalibur flow cytometer.

Apoptosis was measured in freshly isolated acinar cells with the Annexin V-FITC Apoptosis Detection KIT II (BD Pharmingen) and analyzed by flow cytometry.

Quantitative RT-PCR analysis

Total RNA was isolated from pancreatic tissue with Trizol reagent (Invitrogen), according to the manufacturer's instructions, purified using the RNeasy kit (Qiagen) and electrophoresed on a denaturing agarose gel to examine for RNA integrity. cDNA was synthesized from 2.5 μg of RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and all samples were diluted to the same final cDNA concentration. Real-time PCR was performed on several cDNA dilutions plus 1x SYBR green PCR Master Mix (Applied Biosystems) and 300 to 900 nM of primers for the analyzed genes (sequences in Supplementary Table). Reactions were carried out using an ABI Prism 7000 SDS

(Applied Biosystems) for 40 cycles (95ºC for 15 s and 60ºC for 1 min) after an initial 10-min incubation at 95ºC. Relative amounts of cDNA were normalized to the internal control *Gapdh*, whose levels were invariant in all the analyzed genotypes.

Protein extraction and western blot analysis

Total pancreatic homogenates were prepared as described[29] in liquid nitrogen in a mortar cooled in a bath of methanol with dry ice. The powder was transferred to a tube and proteins were solubilized in lysis buffer (1% SDS, 25 mM Tris pH 7.5, 1 mM EGTA pH 8, 1 mM EDTA pH 8, 2 mM pefabloc, 10µg/ml leupeptin, 10µg/ml pepstatin, 10µg/ml benzamidine, 10µg/ml aprotinin and 10 mM sodium orthovanadate). After vortexing, extracts were boiled for 15 min, centrifuged at 13000 r.p.m. for 5 min at 4°C, and the pellet was discarded. Western blots were performed with 20 µg of total protein extract per lane, using antibodies against Apex1 (Novus Biologicals), acetylated p53 (Lys 382), phosphorylated pRb (Ser 807/811 and Ser 780), Cyclin B1, phosphorylated Cdk1 (Tyr 15) (Cell Signalling), phospho-histone H2AX (Ser 139, Millipore), p53, p19, p21, pRb, Cdk1, Wee1, Cdc25c and β-actin (Santa Cruz). Semi-quantitative analysis was performed using Quantity One (Bio-Rad).

Statistical analysis

Data are given as mean±SD. Statistical analysis was performed using ANOVA and Fisher's test. Significance was defined by $p < 0.05$.

RESULTS

Apoptosis in pancreas lacking E2F1 and E2F2 is associated with p53 accumulation

E2F1/E2F2 double-knockout (DKO) pancreatic cells exhibit increased rates of apoptosis as early as postnatal day 15, prior to manifestations of pancreatic atrophy[20]. To characterize the apoptotic cell death, we isolated pancreatic cells from 2-week-old DKO and wild-type mice and measured changes in mitochondrial membrane potential by examining fluorescence of cells incubated with JC-1 (Fig. 1A). In live cells, JC-1 forms aggregates and emits mostly greenish orange fluorescence, while in dying cells with collapsed $\Delta\psi_m$, JC-1 emits mostly green fluorescence. The percentage of green fluorescence-positive cells was substantially higher in DKO samples compared to wild-type controls ($45.46\pm7.06\%$ *vs.* $15.51\pm1.07\%$), suggesting mitochondrial apoptosis. Consistent with this result, we detected a reduction of mitochondrial mass in DKO pancreatic cells, as evidenced by the marked difference in fluorescence intensity between DKO and wild-type samples treated with the cardiolipin-binding molecule Nonyl-Acrydine-Orange (NAO) (Fig. 1B). To get an insight into the underlying mechanism of apoptosis, we examined p53 protein expression. Accumulation of p53 was detected in the pancreas of DKO mice (Fig. 1C). Furthermore, typical p53 direct transcriptional target genes involved in intrinsic (*Bax*, *Puma*, *Apaf-1*, *Pidd*) and extrinsic (*Dr5*) pathways were significantly overexpressed in DKO samples (Fig. 1D). Taken together, these results suggest that E2F1/E2F2 double-deficient pancreatic cells undergo apoptosis as a result of p53 protein accumulation and activation.

The levels of p53 can be increased in cells by diverse mechanisms, some of which are mediated by E2F target genes, including p19[ARF], Sirt1 and Sirt3[30,17]. To assess their role in p53 accumulation in our model, we analyzed the expression of these genes in E2F1/E2F2-deficient cells. The expression of p19[ARF] was reduced in DKO pancreas, both at the mRNA and protein levels (Fig. 1E-F). Additionally, Sirt1 and Sirt3 mRNA levels remained unchanged in DKO samples compared to WT controls (Fig. 1E), and no p53 hyperacetylation was observed (Fig. 1F). These results rule out a role for p19[ARF] and Sirt proteins in the accumulation of p53 displayed by DKO pancreases.

We also examined levels of reactive oxygen species (ROS), which are known to activate p53 via Apex1[31]. However ROS or Apex1 protein accumulation were not detected in young DKO mice (Fig. 1F,G), and only older diabetic DKO mice showed elevated ROS accumulation (Fig. 1G), indicating that activation of p53 pathway in DKO pancreas is not ROS-dependent.

Deregulated E2F target gene expression and aberrant cell cycle control in pancreas of mice deficient in E2F1 and E2F2

To further characterize the phenotype observed in E2F1/E2F2-deficient pancreatic cells we analyzed DNA synthesis in pancreas of 2-week-old mice. By this age, wild-type pancreas is virtually post-mitotic. As expected, DNA synthesis was essentially undetectable in WT controls (Fig. 2A). In contrast, unscheduled DNA replication was detected in E2F1/E2F2 DKO cells. Consistent with these results, we detected an upregulated expression of E2F target genes that promote DNA synthesis and positively regulate G1/S transition and mitosis (Fig. 2B, upper panels).

We also examined the expression of E2F targets that encode CDK regulators. The levels of p16^{INK4A} and p27^{KIP1} were unchanged in DKO cells relative to WT samples. By contrast, the expression of p21^{CIP1} and Cdc25a was increased in DKO cells and the expression of the G1 CDKi p18^{INK4C} was significantly reduced (Fig. 2A lower panel).

Elevated levels of phosphorylated pRb (Ser807/811 and Ser780) were detected DKO pancreatic cells, suggesting hyperactivation of G1 cyclin/CDK function. Similarly, mitotic cyclin/CDK activity appeared significantly elevated in DKO cells, as evidenced by the near disappearance of the Cdk1 inhibitory Tyr15 phosphorylation, the increased levels of cyclin B1 and Cdc25c, and the nearly complete absence of Wee1 protein (Fig. 2C). An increased Cdk1 activity is consistent with the accumulation of the mitotic marker phospho-histone H3 (pH3) that was noted in DKO pancreas (Fig. 2A). Taken together, these findings suggest that terminally differentiating DKO pancreatic cells are progressing aberrantly through the cell cycle in the absence of repressive signals emanated from E2F1 and E2F2 to promote cell cycle exit.

p53 accumulation in the pancreas of DKO mice is linked to DNA replication stress

Given the aberrant cyclin/CDK activity observed in DKO samples, we examined DNA damage response (DDR) in pancreas by assessing accumulation of γ-H2AX, a marker for DNA breaks. Western blotting of pancreatic lysates from 7 day-old mice did not show global accumulation of γ-H2AX. However, the levels of γ-H2AX increased significantly in 15 day-old DKO mice and were dramatically higher in adult mice (Fig. 3A). Immunohistochemical analysis evidenced that over 6% of pancreatic cells were positive for γ-H2AX in 15 day-old DKO mice (Fig. 3B). Importantly, γ-H2AX positivity was not observed in organs that undergo scheduled DNA replication, such as the small intestine. Intestinal crypt cells from small intestine exhibited actively replicating DNA in both WT and DKO mice, but no γ-H2AX-positive cells were detected in either genotype (Suppl. Fig.1A). Taken together, our results suggest that the unscheduled DNA replication observed in pancreatic cells lacking E2F1/E2F2 may lead to a strong DNA damage response.

To examine the relationship between DNA replication stress and DDR in E2F1/E2F2 mutant pancreas, we treated DKO and WT mice (15 to 20 days-old) with the DNA polymerase inhibitor aphidicolin to inhibit DNA replication *in vivo*[32]. As a control, we showed that *in vivo* administration of aphidicolin was able to reduce DNA replication substantially in the small intestine of both WT and DKO mice (Suppl. Fig.1B). In the pancreas of DKO mice *in vivo* aphidicolin treatment blocked DNA replication, as evidenced by the reduction in BrdU uptake (Fig. 3C, left panels). Next, we examined the expression of the marker of DDR γ-H2AX by immunohistochemistry. Importantly, aphidicolin reduced the number of cells with γ-H2AX staining in DKO pancreas (Fig. 3C, right panels). Furthermore, Western analyses showed that inhibition of DNA replication by aphidicolin prevented the accumulation of p53 and p21^{CIP1} in a dose-dependent manner in DKO pancreatic cells (Fig. 3D). We conclude that the abnormal DNA replication and the resulting replication stress trigger DDR and subsequent activation of p53 pathway in DKO pancreas.

p21^{CIP1} is dispensable for the aberrant pancreatic phenotype developed by cells lacking E2F1/E2F2

We next analyzed the contribution of the pathways governed by p53 and its transcriptional target p21^{CIP1} to the atrophy of DKO pancreatic tissue. We first generated E2F1/E2F2/p21^{CIP1} triple knockout mice

and examined these mice in parallel with E2F1$^{-/-}$/E2F2$^{-/-}$, p21$^{-/-}$ and wild-type mice. Loss of p21^{CIP1} did not reverse the histological abnormalities previously described in adult DKO pancreases. In fact, these abnormalities were more severe in E2F1/E2F2/p21^{CIP1} triple knockout mice (Suppl. Fig.2). Consistently, loss of p21^{CIP1} did not prevent the overexpression of apoptosis markers, the pancreatic atrophy or the hyperglycemia in E2F1/E2F2 DKO mice (Suppl. Fig.3). Other organs that exhibit involution in DKO mice, such as, salivary glands, testicles and hematopoietic system remained atrophic in the triple knockout animals (data not shown). These findings suggest that the mechanism that dictates cell death induction and triggers organ involution in E2F1/E2F2 DKO mice is p21^{CIP1}-independent.

Disruption of p53 in E2F1/E2F2-deficient mice prevents apoptosis and restores a normal pancreatic phenotype

We next generated E2F1/E2F2/p53 triple knockout (TKO) mice and analyzed these mice in parallel with E2F1$^{-/-}$/E2F2$^{-/-}$, p53$^{-/-}$ and wild-type mice. Importantly, the expression of p53 target genes (*Bax*, *Puma*, *Apaf-1*, *Pidd and Dr5)* was down to normal in E2F1/E2F2/p53 TKO pancreases compared to E2F1$^{-/-}$/E2F2$^{-/-}$ samples (Fig. 4A). Consistent with this result, compromised mitochondrial membrane potential was completely rescued in pancreatic cells isolated from TKO animals (Fig. 4B), and elimination of p53 from E2F1/E2F2 DKO cells restored cell survival (Fig. 4C). By contrast, increased BrdU uptake and accumulation of γ-H2AX persisted in E2F1/E2F2/p53 TKO cells (Suppl. Fig.4). These findings place p53 downstream of DNA replication stress signals and upstream of apoptotic pathways after E2F1/2 loss.

Strikingly, elimination of p53 completely prevented loss of insulin- and glucagon-producing cells and restored the expression of α-amylase in E2F1/E2F2 DKO mice (Fig. 5). Histological and morphological abnormalities described in DKO pancreas such as loss of normal architecture, lower number of acini and increased number of ductal structures, were completely absent in E2F1/E2F2/p53 TKO mice (Fig. 6A). Furthermore, the acinar cell hypertrophy with marked karyomegaly characteristic of DKO pancreases was completely rescued in triple knockout animals (Fig. 6B). Indeed, mean size of the nuclei in TKO pancreatic acinar cells was clearly reduced compared to the mean size of nuclei in DKO cells

(3.17±0.72-fold over WT in DKO mice *vs.* 1.55±0.24-fold over WT in TKO mice). Endocrine pancreas, which was atrophic in DKO mice, appeared overtly normal when p53 was eliminated (Figs. 6, 7A). Importantly, glucose levels in serum of TKO mice were similar to the levels found in WTs (Fig. 7C), suggesting that insulin secreted by pancreas of E2F1/E2F2/p53 TKO mice is sufficient to maintain normoglycemia.

Histological as well as functional features of other organs that are severely affected in DKO mice, such as testicles and salivary glands, were also overtly normal in TKO mice, including their ability to generate offspring (Suppl. Fig.5 and data not shown). Taken together, these data indicate that p53 is the crucial mediator of the abnormal phenotype displayed by organs in mice lacking E2F1/E2F2.

Disruption of p53 in E2F1/E2F2 mutant mice shortens lifespan owing to the emergence of thymic lymphomas

Unexpectedly, lifespan of TKO mice was found to be significantly shorter than that of WT (Fig. 8A), even though TKO mice were normoglycemic and showed normal gland structure (Figs. 6, 7). While performing the necropsies we noted that the thymus was severely hypertrophic in E2F1/E2F2/p53 TKO mice. Relative thymus weight in TKO mice was 6-fold higher than that of WT or DKO mice (Fig. 8B and C), suggesting that the reduced survival rate of TKO animals could stem from a respiratory failure due to thymic tumor growth, as demonstrated for p53$^{-/-}$ mice[24]. Histological sections of TKO thymus confirmed the development of a T cell lymphoblastic leukemia/lymphoma in these mice (Fig. 8D and E), supporting the idea that pathways regulated by E2F1/E2F2 and p53 work in concert to impede cellular proliferation of aberrant cells.

DISCUSSION

Here we show that E2F1 and E2F2 are crucial players in an essential pathway involved in the maintenance of a post-mitotic state by limiting the capability of the DNA replication and cell cycle progression machinery. Failure of this control mechanism results in exacerbated CDK activity and inappropriate DNA replication that induces a DNA damage response pathway. Consequently, an apoptotic program that affects tissue integrity is activated.

In this work we have analyzed the pathway that leads to apoptosis in pancreas lacking E2F1 and E2F2. It is known that apoptotic cell death in pancreas may be induced as a consequence of diabetes, as aberrantly increased glucose levels have been found to trigger both extrinsic as well as intrinsic apoptotic pathways[33]. Conversely, apoptosis may also be the causal factor in diabetes development, as shown in Perk^{-/-} and Pdx-1^{-/-} mouse models of diabetes[34-37]. In E2F1/E2F2-deficient cells, altered expression of proapoptotic mediators and mitochondrial membrane depolarization preceded the onset of diabetes, suggesting that apoptosis is a triggering event for pancreatic degeneration and diabetes in our mouse model. Furthermore, our results demonstrate that apoptosis in mice lacking E2F1/E2F2 is exclusively dependent on p53, since targeted inactivation of p53 hinders apoptosis and restores normal pancreas phenotype. Additionally, the fact that elimination of p21[CIP1] does not rescue the phenotype of DKO cells suggests that the program activated by p53 in DKO mice is not primarily aimed to arrest cell cycle, but to activate apoptosis.

Several mechanisms could account for the accumulation and activation of p53 in E2F1/E2F2-deficient cells. For example, it is well established that the expression of p19[ARF] stabilizes p53 by inhibiting the ubiquitin ligase activity of Mdm2 (ref.38), and recently it has been shown that downregulated expression of Sirt1 upon E2F1-3 loss in retinal progenitor cells results in elevated levels of acetylated p53[17]. However, we failed to detect upregulation of p19[ARF]. Moreover, Sirt1 or Sirt3 levels were unchanged in DKO cells relative to WT, and no p53 hyperacetylation was detected in DKO pancreases, arguing that activation of p53 pathway in this scenario is not p19[ARF]- or sirtuin-dependent.

Our data strongly suggest that activation of p53 in DKO cells is associated with their aberrant DNA replication. Accumulating experimental evidence indicates that p53 can be activated as part of the DNA damage response pathway triggered by replicative stress[39]. Moreover, abnormal DNA replication leading to p53-dependent apoptosis and inner ear involution has been shown after concomitant inactivation of p19[ARF] and p21[CIP1] CDKis[40]. In line with these studies, our data showing that *in vivo* inhibition of DNA replication dose-dependently prevents activation of the DNA damage response and accumulation of p53 provide support for the notion that replication stress has a major causative role in p53 activation in cells lacking E2F1 and E2F2.

DKO pancreas is overtly normal until the second week of postnatal age[20], suggesting that E2F1 and E2F2 are dispensable for the timely cellular proliferation occurring up to this age in pancreas. During terminal differentiation, in contrast, they appear to play a major role as repressors of the cell cycle by promoting attenuation of DNA replication. A similar finding has been reported for differentiating monocyte and intestinal epithelial cells[13,18]. However, the cell fates in these cases are different from those observed in pancreatic tissue, as monocyte progenitor cells lacking E2F1/2 result in senescence, and intestinal progenitor cells lacking E2F1-3 lead to p53-independent apoptosis. The mechanism that leads to replication stress in DKO cells is still unknown, and it would be interesting to determine whether it involves illegitimate replication origin firing and fork stalling, as shown for replication stress caused by overexpression of Cdt1[41] or by aberrant CDK activity[42].

A controlled CDK activity is key to maintain genome integrity during S phase[43], and it has been reported that most of the DNA replication stress arises as a result of elevated CDK activity[44]. Interestingly, this phenotype can be alleviated by nucleotide addition[45, 42]. In E2F1^{-/-}/E2F2^{-/-} cells, overexpression of replication proteins such as Mcm3, Cdc6 and Orc1, together with the increased Cyclin/CDK activity, could be the source of the observed DNA damage. It remains, however, to be determined what the contributing role of CDK activity in unscheduled DNA replication is, and the role of intracellular nucleotide pools in DKO phenotype.

Many of the features exhibited by the E2F1/E2F2 mutant mouse model are similar to those exhibited by aging animals, including general tissue atrophy and degeneration [46]. Interestingly, animal models of progeria have also shown that the absence of p53 relieves some of the growth disadvantages of aging and enhances the lifespan of these animals, unless cancer arises[47]. Individuals with rare genetic diseases that accelerate aging, such as Werner or MDP (Mandibular hypoplasia, Deafness and Progeroid features) syndromes are also known to develop tissue degeneration that affects many organs including the pancreatic gland. Intriguingly, these two syndromes are the result of mutations in genes involved in DNA replication, WRN helicase and DNA polymerase δ catalytic subunit encoding gene *POLD1*, respectively[48-49], suggesting that DNA replication stress, such as that exhibited by E2F1/2-deficient cells may drive tissue involution and aging in some pathological conditions.

Curiously, constitutive inactivation of p53 in E2F1/E2F2-deficient mice results in tumor development in the thymus but not in other organs, possibly because thymus is the first organ to develop tumors in our p53$^{-/-}$ mouse strain[24]. It is possible that premature death of these animals as a result of lymphoma development might be preventing the development of tumors affecting other organs. In fact, inactivation of p53 in E2F1-3-deficient fibroblasts has been shown to result in transformation of this cell type[50], implying a general role for the E2F-p53 axis in malignancy. Thus, exacerbating DNA replication rates by either inactivating pRb[51], or, paradoxically, by inactivating E2F1/2 could lead to atrophy in a p53-competent context, but be tumorigenic in a p53-deficient context. These observations suggest that cells have more than one failsafe mechanism whereby deregulation of one arm of tumor suppression is not sufficient to initiate tumorigenesis, and instead leads to tissue atrophy. E2F1/2-deficient mice constitute a useful model in which to study the mechanisms regulating the control of malignant transformation as well as tissue degeneration in the context of aberrant DNA replication.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig. 1. Apoptosis in pancreas deficient in E2F1 and E2F2 is associated with p53 accumulation. **A)** FACS analysis on freshly isolated WT and DKO pancreatic cells (from 2-week-old mice), stained with JC-1 under basal conditions or after treatment with the mitochondrial uncoupler carbonyl cyanide m-chloro phenyl hydrazone (CCCP) as a control for loss of mitochondrial membrane potential. Cells with compromised mitochondrial membrane potential shift from greenish orange (upper panel) to green (lower panel). Numbers inside each panel represent the percentage of cells (mean±SD) from 3 independent experiments.*$p<0.002$ *vs*. basal WT. **B)** FACS analysis of mitochondrial mass on freshly isolated WT and DKO pancreatic cells stained with NAO. Results are expressed as fold over NAO fluorescence obtained in WT cells (mean±SD) from 3 independent experiments. *$p<0.05$. **C)** Representative Western blot analysis of p53 in extracts prepared from 2-week-old WT and DKO pancreas (2 mice/genotype). Expression of β-actin was used as loading control. **D)** Reverse transcription Q-PCR analysis of indicated genes in 1-month-old WT and DKO pancreas. *Gapdh* was used as normalization control. Results are expressed as fold over WT (mean±SD) from 3 mice per genotype.*$p<0.05$, **$p<0.01$. **E)** Reverse transcription Q-PCR analysis of of indicated genes in 2-week-old WT and DKO pancreas. *Gapdh* was used as normalization control. Results are expressed as fold over WT (mean±SD) from 3 mice per genotype. **$p<0.01$. **F)** Representative Western blot analysis of indicated proteins in extracts prepared from 2-week-old WT and DKO pancreas (2 mice/genotype). Cell extracts from cultured cells that express the analyzed proteins were used as positive controls (+ con) and were run along with the samples. Expression of β-actin was used as loading control. **G)** Concentration of ROS in pancreas of WT and DKO mice analyzed at different ages, as described in Materials and Methods. For each age and genotype 3 mice were analyzed. *$p<0.0001$.

Fig. 2. Pancreas of E2F1/E2F2 DKO mice exhibits evidence of inappropriate progression through the cell cycle and replicative stress. **A)** Immunohistochemical analyses to identify BrdU-positive

(upper panels) or pH3-positive (lower panels) nuclei in the pancreas of 2-week-old WT and DKO mice. As shown, DKO mice have an increased number of BrdU-positive as well as pH3-positive cells (x20). The percentages of positive nuclei are indicated (mean± SD). **B)** Reverse transcription Q-PCR expression analysis of indicated genes in 2-week-old WT and DKO pancreas. *Gapdh* was used as normalization control. Results are expressed as fold over WT (mean±SD) from 3 mice per genotype. *p<0.05, **p<0.01, ***p<0.0001. **C)** Representative Western blot analysis of the indicated proteins in extracts prepared from 2-week-old WT and DKO pancreas (2 mice/genotype). Expression of β-actin was used as loading control.

Fig. 3. Activation of DDR is triggered by unscheduled DNA replication in cells lacking E2F1 and E2F2. **A)** Western blot analysis of γ-H2AX in extracts prepared from WT and DKO pancreas collected at different ages. Expression of β-actin was used as loading control. Numbers below the blots represent normalized densitometric values expressed as fold over WT (mean± SD). **B)** Immunohistochemical analysis to identify γ-H2AX-positive nuclei in the pancreas of 2-week-old WT and DKO mice. As shown, DKO mice have an increased number of cells with pan-nuclear γ-H2AX staining (x20). **C)** *In vivo* BrdU labeling (left panels) and γ-H2AX accumulation (right panels) in pancreases derived from 2-week-old WT and DKO mice, after i.p. injection of vehicle (saline) or aphidicolin. As shown, treatment with aphidicolin (10μg/g) completely abolishes the incorporation of BrdU and reduces the number of cells with pan-nuclear γ-H2AX staining in DKO cells. The sections shown are representative examples from 3 mice per treatment (x40). The percentage of BrdU-positive nuclei and γ-H2AX -positive nuclei is indicated (mean± SD). **D)** Western blot analysis of γ-H2AX, p53 and p21^{CIP1} in extracts prepared from pancreas treated with the indicated concentrations of aphidicolin. Expression of β-actin was used as loading control. Numbers below the blots correspond to the densitometric values of a representative experiment, expressed as fold over WT.

Fig. 4. Disruption of p53 in E2F1/E2F2 mutant mice prevents apoptosis in pancreas. **A)** Reverse transcription Q-PCR analysis of indicated genes in WT, E2F1/E2F2 DKO, p53$^{-/-}$ and

E2F1/E2F2/p53 TKO pancreas. *Gapdh* was used as normalization control. Results are expressed as fold over 1 month-old WT (Mean±SD) from 3 mice per age and genotype. *$p<0.05$, **$p<0.01$, ***$p<0.0001$. **B)** FACS analysis on freshly isolated pancreatic cells derived from 15 day-old mice stained with JC-1. Numbers inside each panel represent the percentage of cells (mean±SD) with normal or compromised mitochondrial membrane potential from 3 independent experiments. *$p<0.05$ vs. WT, †$p<0.002$ vs. DKO. **C)** Apoptotic death in 15 day-old pancreatic cells assessed by Annexin V-FITC and PI staining followed by FACS analysis. Different stages of apoptosis were distinguished as follows: early apoptosis (Annexin V+/PI−); late apoptosis (Annexin V+/PI+); necrosis (Annexin V−/PI+) and viable cells (Annexin V−/PI−). In the table below results are expressed as % of cells (mean±SD) from 3 independent experiments. *$p<0.05$ vs. WT, **$p<0.0001$ vs. WT, † $p<0.001$ vs. DKO

Fig. 5. Elimination of p53 in pancreas of E2F1/E2F2 DKO mice restores expression of pancreas-specific protein markers. Representative pancreas sections of 2-month-old WT, E2F1/E2F2 DKO, p53$^{-/-}$ and E2F1/E2F2/p53 TKO mice immunostained with antibodies to insulin, glucagon and α-amylase. A light hematoxylin counterstaining was performed in all sections for contrast (x40). Similar results were obtained in more than 10 mice per genotype.

Fig. 6. Disruption of p53 in E2F1/E2F2 mutant mice restores normal pancreatic histological appearance. Representative sections of pancreas from 2-month-old WT, E2F1/E2F2 DKO, p53$^{-/-}$ and E2F1/E2F2/p53 TKO mice stained with hematoxylin/eosin (upper 4 panels, x20; lower 4 panels, x40). Note the presence of islets and acini in TKO pancreas with a morphology similar to that found in WT animals. The sections shown are representative examples of more than 10 mice per genotype.

Fig. 7. Disruption of p53 in E2F1/E2F2 mutant mice prevents pancreatic atrophy, insulin deficiency and hyperglycemia. **A)** Pancreas weight expressed as fraction of total body weight of 3 to 4 month-old animals. Shown are means ± SD for 3 to 5 mice per genotype. *$p<0.05$, **$p<0.0001$. **B)** Insulin levels in serum of 3-month-old male mice (n=5 per genotype, *$p<0.05$, **$p<0.005$). **C)**

Spot blood glucose levels were determined at the indicated times. Results are the means ± SD for 5-10 animals per sex group and genotype. *p<0.0001.

Fig. 8. Disruption of p53 in E2F1/E2F2 mutant mice promotes development of thymic lymphomas and compromises survival. **A**) Life spans of WT, E2F1/E2F2 DKO, p53$^{-/-}$ and E2F1/E2F2/p53 TKO mice (n=15/genotype) analyzed using a log-rank nonparametric test and expressed as Kaplan-Meier survival curves (E2F1/E2F2 DKO *vs*. WT, p<0.0001; E2F1/E2F2/p53 TKO *vs*. WT, p<0.0001; E2F1/E2F2/p53 TKO *vs*. E2F1/E2F2 DKO, p<0.0001; E2F1/E2F2/p53 TKO vs. p53$^{-/-}$, p<0.0001). **B**) Thymus weight expressed as fraction of total body weight in 4 to 6 month-old animals (n=5 per genotype, *p<0.0001). **C**) Representative thymus organs obtained from of 6-month-old WT, E2F1/E2F2 DKO and E2F1/E2F2/p53 TKO animals. **D**) Thymus sections obtained from 6-month-old WT and E2F1/E2F2/p53 TKO animals stained with hematoxylin/eosin (H/E, x40). Note that the typical structure of WT thymus divided in cortex and medulla is completely loss in TKO thymus. **E**) Immunohistochemical analysis of thymus sections obtained from 6-month-old E2F1/E2F2/p53 TKO animals to detect CD3, CD20 and TDT (x40). Note that E2F1/E2F2/p53 TKO mice exhibit positive staining for CD3 and TDT and negative staining for CD20, implying that TKO mice develop T-cell lymphoblastic leukemia/lymphoma.