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Accelerated DNA replication in E2F1- and E2F2-deficient macrophages leads to induction of the DNA damage response and p21^{CIP1}-dependent senescence

Running title: Loss of E2F1 and E2F2 leads to DDR and senescence

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ABSTRACT

E2F1-3 proteins appear to play distinct roles in progenitor cells and in differentiating cells undergoing cell cycle exit. However, the function of these proteins in paradigms of terminal differentiation that involve continued cell division has not been examined. Using compound E2F1/E2F2-deficient mice we have examined the effects of E2F1 and E2F2 loss on the differentiation and simultaneous proliferation of bone marrow-derived cells toward the macrophage lineage. We show that E2F1/E2F2 deficiency results in accelerated DNA replication and cellular division during the initial cell division cycles of bone marrow-derived cells, arguing that E2F1/E2F2 are required to restrain proliferation of pro-monocyte progenitors during their differentiation into macrophages, without promoting their cell cycle exit. Accelerated proliferation is accompanied by early expression of DNA replication and cell cycle regulators. Remarkably, rapid proliferation of E2F1/E2F2 compound mutant cultures is temporally followed by induction of a DNA damage response and the implementation of a $p21^{CIP1}$-dependent senescence. We further show that differentiating E2F1/E2F2-knockout macrophages do not trigger a DNA damage response pathway in the absence of DNA replication. These findings underscore the relevance of E2F1 and E2F2 as suppressors of hematopoietic progenitor expansion. Our data indicate that their absence in differentiating macrophages initiates a senescence program that results from enforcement of a DNA damage response triggered by DNA hyper-replication.

Abstract: 212 words

KEYWORDS

E2F, macrophage, DNA damage response, senescence, DNA hyperreplication

INTRODUCTION

Mammalian E2F activity is composed of eight family members that have been traditionally classified into different subsets based on their structural and biochemical characteristics (Dimova and Dyson, 2005). E2F1-3 are typically known as transcriptional activators, and E2F4-8 as transcriptional repressors, although there is increasing evidence suggesting that this division is not as clear-cut as initially proposed. Ectopic expression of E2F1-3 not only leads to transcriptional activation, but also to the repression of numerous genes (Aslanian *et al*., 2004; Morris *et al*., 2008, Young *et al*., 2003). More convincingly, knockout of E2F2 or compound knockout of E2F1 and E2F2 leads to increased expression of E2F target genes involved in DNA replication and cell cycle progression (Iglesias *et al*., 2004; Infante *et al*., 2008; Pusapati *et al*., 2010). Additionally, knockout of E2F4 leads to decreased expression of target genes such as Ccna2 in erythroid cells (Kinross *et al*., 2006). Collectively, these results argue that each individual E2F may be capable of both activating and repressing transcription, through mechanisms that are poorly defined.

Gain-of-function as well as loss-of-function experiments reveal that the functions of the E2Fs are complex. In line with the original model of E2F function, overexpression of E2F1 in immortalized quiescent rodent fibroblasts results in increased DNA replication and cell proliferation (Lukas *et al*., 1996), and overexpression of E2F2 in thymic epithelial cells results in the development of thymomas (Scheijen *et al*., 2004). Furthermore, knockout of E2F1 in mouse embryo fibroblasts (MEFs) or in T lymphocytes modestly slows progression from quiescence to S phase (Murga *et al*., 2001; Wang *et al*., 1998), and $E2F2^{-/-}$ and $E2F1/E2F2^{-/-}$ mice exhibit impaired haematopoiesis resulting from defective S phase progression in progenitor populations (Li *et al*., 2003). The combined inactivation of E2F1, 2 and 3 completely abolishes entry into S phase, and proliferation, along with reduced expression of E2F target genes necessary for these processes (Wu *et al*., 2001). These results argue for a positive role for E2F1-3 in cell cycle progression. By contrast, we and others have shown that loss of E2F2, or more dramatically, the combined loss of E2F1 and E2F2 impairs cellular quiescence and leads to accelerated replication of DNA of several cell types, implying a negative role for these proteins in proliferation

control (Murga *et al*., 2001; Zhu *et al*., 2001; Infante *et al*., 2008; Pusapati *et al*., 2010). Moreover, loss of E2F leads to deficient erythropoiesis, which has been associated with increased DNA double-stranded breaks and cell-cycle arrest (Dirlam *et al*., 2007), although the source of the DNA damage has not been identified.

Differences in cellular context are likely to modulate the physiological role of E2F1-3. For instance, it has been recently demonstrated that E2F1-3 are dispensable for proliferation but necessary for cell survival in normally dividing progenitor cells. By contrast, they function as repressors in non-proliferating cells that are undergoing differentiation (Chong *et al*., 2009). However, the relative contribution of E2F1-3 in cell survival or in repression has not been determined for cellular contexts where terminal differentiation is not coupled to cell-cycle exit, but is instead concurrent with continued proliferation. Such is the case of several lineages of hematopoietic cells, including macrophages, which differentiate from bone marrow-derived monocytic progenitors while undergoing cellular divisions (Liu *et al*., 1999).

To explore the role of E2F1 and E2F2 in proliferating/differentiating cells, we have made use of hematopoietic progenitor cells lacking E2F1 and E2F2 during their differentiation toward the macrophage lineage in response to macrophage-colony stimulating factor (M-CSF). We show that loss of E2F1 and E2F2 results in accelerated DNA replication and cellular division during the initial cell division cycles of bone marrow-derived cells, arguing that E2F1/E2F2 are not dispensable for normal proliferation of pro-monocyte progenitors during their differentiation into macrophages. Instead, they appear to have a repressor role in this process. Remarkably, rapid proliferation is temporally followed by severe senescence of E2F1/E2F2 compound mutant cultures without appreciable loss of cellular viability. We further show that accelerated DNA replication triggers a DNA damage response pathway in differentiating E2F1/E2F2-knockout macrophages, thus implementing a $p21^{CIP1}$-dependent senescent program. These results add a new layer of complexity to our understanding of E2F1 and E2F2 function, by showing that in some cellular contexts these proteins limit the extent of proliferation without promoting the typical G1 arrest of differentiating cells.

RESULTS

Accelerated proliferation of bone marrow-derived differentiating macrophages deficient in E2F1 and E2F2

Undifferentiated monocyte progenitor cells derived from the bone marrow constitute a homogeneous population of non-transformed quiescent cells. In response to M-CSF, these cells undergo cell cycle entry and subsequent cell division during their differentiation into bone marrow-derived macrophages (BMDM) (Liu *et al*., 1999), providing a good model for cell cycle studies in mammals. Macrophage maturation is accompanied by the early induction of the differentiation marker CD11b (Mac1) (Brackman *et al*., 1995). To determine the role of E2F1 and E2F2 in this process, we studied cells derived from the bone marrow of mice lacking E2F1, E2F2 or both. The percentage of cells expressing high levels of CD11b after 6 days in the presence of M-CSF was close to 80% in both wild-type and mutant cultures (Figure 1A and data not shown), indicating that E2F1 and E2F2 are dispensable for expression of macrophage differentiation markers.

To examine proliferation in E2F1$^{-/-}$, E2F2$^{-/-}$ or compound E2F1/E2F2$^{-/-}$ bone marrow-derived differentiating macrophages we used the vital fluorescence dye CFSE, which is diluted approximately two-fold with each cell division. Cells were counterstained with an antibody against CD11b to specifically detect macrophages, and were analyzed by flow cytometry. As the population of CD11b^{hi} cells increased over time after M-CSF stimulation, these cells gradually lost their CFSE fluorescence (Supplementary figure 1), confirming that the population undergoing proliferation consisted of differentiating macrophages. Remarkably, a comparison between WT and compound E2F1/E2F2$^{-/-}$ BMDM showed a higher proliferation rate in mutant cells during the initial stages of culture (Figure 1B, supplementary figure 1 and supplementary table 1). Consistent with this finding, the incorporation of [^{3}H]thymidine 1.5 days after the stimulation with M-CSF was significantly higher in E2F1/E2F2$^{-/-}$ cells compared to wild-type controls (Figure 1C). Singly mutated E2F1$^{-/-}$ or E2F2$^{-/-}$ cells behaved in an

intermediate manner (Figure 1B and supplementary table 1), indicating that E2F1 and E2F2 function redundantly to restrain cell cycle progression of cells that undergo differentiation concomitantly with proliferation.

Increased proliferation is followed by cell cycle arrest of E2F1/E2F2$^{-/-}$ BMDM cells

The number of wild-type cells obtained in the BMDM cultures treated with M-CSF increased exponentially after an initial lag of two days (Figure 2A). By contrast, E2F1/E2F2$^{-/-}$ BMDM behaved very differently. They proliferated rapidly during the initial 3 days of culture with M-CSF compared to WT cultures, consistent with the CFSE and [^{3}H]thymidine results. However, cell number plateaud by day 4, and thereafter cell number remained constant (Figure 2A). Upon reaching the plateau on day 4.5 the incorporation of [^{3}H]thymidine was reduced by 75% compared to wild-type controls (Figure 2B). The rate of apoptosis, however, remained low throughout the experiment in both WT and mutant cultures (Figure 2C). Therefore, we conclude that the plateau in cell number results from proliferation arrest in the E2F1/E2F2$^{-/-}$ BMDM.

E2F1/E2F2$^{-/-}$ BMDM cells enter premature senescence

The proliferation arrest of E2F1/E2F2$^{-/-}$ BMDM, without increased apoptosis, raised the possibility that they had entered premature senescence. We measured metabolic activity using the MTT assay in BMDM cells after 5 days in culture with M-CSF. We found that E2F1/E2F2$^{-/-}$ cells remained fully viable and metabolically active (Figure 2D). We further examined the morphology of E2F1/E2F2$^{-/-}$ BMDM and found that they were large and flattened relative to wild-type control cells, also suggestive of premature senescence. Finally, we stained the cells for senescence associated-β-galactosidase (SA-β-gal) activity. E2F1/E2F2$^{-/-}$ BMDM, but not wild-type, E2F1$^{-/-}$, nor E2F2$^{-/-}$ BMDM, stained positive for SA-β-gal (Figure 2E). All these findings indicate that E2F1/E2F2$^{-/-}$ BMDM enter senescence after completing a strong proliferative burst.

We hypothesized that cell cycle and/or DNA replication defects during the accelerated proliferation of E2F1/E2F2$^{-/-}$ BMDM might be responsible for the premature senescence. BrdU incorporation analysis showed a higher percentage of cells in S phase in freshly isolated bone marrow cells of E2F1/E2F2$^{-/-}$ mice relative to wild-type controls (Figure 3B). The percentage of wild-type bone-marrow cells that incorporated BrdU after stimulation with M-CSF increased over time, reaching the peak at day 4 and maintaining similar levels through the end of the experiment on day 6. Interestingly, E2F1/E2F2$^{-/-}$ cultures treated with M-CSF behaved quite differently. At early time points the percentage of BrdU-positive cells was significantly higher than wild-type cells (Figures 3A and 3B). This increase was consistent with the M-CSF-dependent accelerated proliferation exhibited by E2F1/E2F2$^{-/-}$ cells during the initial days of cell culture (Figures 1 and 2A). However, we observed that E2F1/E2F2$^{-/-}$ cells displayed a marked defect in incorporation of BrdU after day 5 or 6 of stimulation (Figures 3A and 3B). Therefore, the E2F1/E2F2$^{-/-}$ BMDM had undergone cell cycle arrest. Flow cytometry to compare BrdU incorporation to DNA content revealed that these cells had arrested with a DNA content intermediate between 2N and 4N, suggesting arrest within S-phase, or with a 4N DNA content, suggesting arrest at G2/M (Figures 3A, 3C and 3D). Thus, the reduced cellular proliferation of DKO BMDM after the initial burst appears to result from an arrest of the cells at several points of the cell cycle, notably the S and the G2/M phases.

Altered expression of cell cycle regulators in E2F1/E2F2$^{-/-}$ BMDM

To define the mechanisms by which E2F1 and E2F2 regulate proliferation of BMDM, we monitored the expression of known E2F target genes implicated in cell cycle regulation. Bone-marrow cells freshly isolated from wild-type or from compound E2F1/E2F2$^{-/-}$ mice exhibited similarly low levels of cyclin expression. However, upon differentiation in response to M-CSF, E2F1/E2F2$^{-/-}$ BMDM demonstrated accelerated accumulation of cyclins, in particular cyclin D2 and cyclin A2, compared to wild-type controls (Figure 4A; see also figure 6), coincident with accelerated cell cycle progression. At later time-

points, E2F1/E2F2$^{-/-}$ BMDM displayed dramatically reduced expression of the cyclins, particularly cyclins A2 and B1 (Figure 4A), coincident with proliferative arrest.

We next examined the expression of E2F target genes implicated in the regulation of DNA replication. Freshly harvested bone marrow cells from E2F1/E2F2$^{-/-}$ mice exhibited significantly higher mRNA levels of the DNA replication proteins Mcm3, Cdc6 and Tk relative to freshly harvested wild-type bone marrow cells (Figure 4B, left panel). One day after M-CSF treatment, high levels of the proteins encoded by these messages were observed in E2F1/E2F2$^{-/-}$ BMDM (See figure 6). At day 7 of M-CSF treatment, however, mRNA levels of these DNA replication proteins were drastically reduced in E2F1/E2F2$^{-/-}$ cells compared to wild-type or single mutant cells (Figure 4B, right panel).

It has been reported that overexpression of E2F3a is able to induce abnormally increased proliferation, followed by premature senescence (Lazzerini Denchi *et al.*, 2005). Therefore, we tested whether the E2F1/E2F2$^{-/-}$ BMDM exhibit increased expression of E2F3a. However, expression of E2F3a was similar in wild-type and E2F1/E2F2$^{-/-}$ BMDM (Figure 4C).

CDK inhibitors have been implicated in the induction of cell cycle arrest and cellular senescence (Serrano et al., 1997). Therefore we also examined the expression of p16^{INK4A}, p19^{ARF} and p21^{CIP1} in E2F1/E2F2$^{-/-}$ bone marrow cells (Figure 4D). Upon differentiation of wild-type macrophages, p16^{INK4A} and p19^{ARF} maintained basal levels, whereas the levels of p21^{CIP1} were induced significantly, consistently with previously published data (Braun *et al.*, 1998). However, p21^{CIP1} accumulation was significantly higher in E2F1/E2F2$^{-/-}$ BMDM (Figure 4D).

Loss of p21^{CIP1} rescues E2F1/E2F2$^{-/-}$ cells from cellular senescence and restores cell cycle entry and progression

The results presented above raised the possibility that p21^{CIP1} may be responsible for triggering senescence in E2F1/E2F2$^{-/-}$ cells. To test this hypothesis, we crossed E2F1/E2F2$^{-/-}$ mice with p21$^{CIP1-/-}$ mice to generate triple knockout mice. BMDM were harvested from the triple knockout mice and cultured in the presence of M-CSF in parallel with BMDM from E2F1/E2F2$^{-/-}$, p21$^{CIP1-/-}$, and wild-type

mice. First, we examined the proliferation of these BMDM cultured in the presence of M-CSF by counting cell number over time (Figure 5A). Both the E2F1/E2F2$^{-/-}$ and E2F1/E2F2/p21$^{CIP1-/-}$ cells demonstrated accelerated proliferation at the early time point. At subsequent time-points we observed that E2F1/E2F2$^{-/-}$ cell number had plateaud, as observed previously. However, mutation of p21^{CIP1} in the context of E2F1/E2F2$^{-/-}$ eliminated this block in proliferation, allowing an increase in cell number through day 14, proliferating even faster than the wild-type cells. These results were confirmed by [^{3}H]thymidine pulse analysis (Figure 5B). Nevertheless, young triple knockout mice do not show an increased hematopoietic cellularity (Iglesias *et al.*, unpublished observation), suggesting that additional mechanisms operate in these mice to prevent aberrantly proliferating cells from expanding *in vivo*. Furthermore, mice lacking the expression of E2F1/E2F2/p21^{CIP1} develop non-autoimmune diabetes at an early age, and die within 5 months of age, similarly to E2F1/E2F2 DKO mice (Iglesias *et al.*, 2004 and unpublished observation), hindering analysis of the effect of p21^{CIP1} inactivation in older DKO mice.

We further examined S phase specific gene expression at day 7 in triple-mutant cells, measuring the levels of Mcm3, Cdc6, and Tk after 7 days in culture (Figure 5C). Again, E2F1/E2F2$^{-/-}$ cells expressed markedly decreased levels of these genes, but additional inactivation of p21^{CIP1} restored the expression of these genes. The abnormal large, flat morphology, and SA-β-gal activity observed for E2F1/E2F2$^{-/-}$ was also prevented by loss of p21^{CIP1} (Figure 5D), arguing that mutation of p21^{CIP1} rescues E2F1/E2F2$^{-/-}$ BMDM from premature senescence. Thus, E2F1/E2F2 loss induces a p21^{CIP1}-dependent senescence program.

DNA damage response activation in E2F1/E2F2$^{-/-}$ cells

Given the altered features exhibited by proliferating E2F1/E2F2$^{-/-}$ cells, we considered whether they undergo premature cell cycle arrest and cellular senescence as a consequence of activation of the DNA damage response (DDR). We examined a marker of the DDR, specifically the accumulation of histone H2AX phosphorylated at serine 139 (referred to as γ-H2AX). We performed immunofluorescence with an antibody to γ-H2AX in wild-type and E2F1/E2F2$^{-/-}$ BMDM 2 days after differentiation in the

presence of M-CSF. Approximately 8% of cells exhibited positive γ-H2AX staining in E2F1/E2F2$^{-/-}$ cultures compared to only 0.67% of cells in wild-type cultures. Moreover, approximately 20 nuclear foci were detected in E2F1/E2F2$^{-/-}$ cells exhibiting positive γ-H2AX staining, compared to only 1 nuclear foci per γ-H2AX-positive cell in wild-type cultures (Figure 6A). Likewise, western blotting of cell lysates prepared as early as one day after culture with M-CSF revealed a dramatic increase in γ-H2AX levels in E2F1/E2F2$^{-/-}$ BMDM (Figure 6B). DDR activation initiates a signaling cascade in cells undergoing DNA repair processes, resulting in the stabilization and transcriptional activation of p53 (Shieh *et al*., 1997). E2F1/E2F2$^{-/-}$ cultures exhibited accumulation of p53 as early as 1 day after M-CSF treatment (Figure 6B). Activation of p53 was observed by an increased expression of the p53 target protein p21^{CIP1} in E2F1/E2F2$^{-/-}$ cells (Figure 6B). Accumulation of γ-H2AX and p53 still occurred when p21^{CIP1} was deleted (Figures 6A and 6C), suggesting that activation of DDR is independent of the status of p21^{CIP1}. Interestingly, accumulation of g-H2AX, p53, and p21^{CIP1} correlated with the deregulated accumulation of Mcm2 and cyclin A (Figures 6B and 6C), proteins that function during S phase, suggesting that aberrant S phase entry and progression might have triggered DDR in E2F1/E2F2$^{-/-}$ cells.

Deregulated DNA replication in E2F1/E2F2$^{-/-}$ cells triggers DDR and induction of p21^{CIP1}

To address if deregulated DNA replication triggered DDR in E2F1/E2F2$^{-/-}$ cells, we stimulated bone marrow-derived cells with M-CSF and treated with aphidicolin to inhibit DNA replication (Xaus *et al*., 1999). We first confirmed that aphidicolin treatment blocked DNA replication in a dose-dependent manner (Figure 7A). Next, we examined the expression of markers of DDR, including γ-H2AX, p53, and p21^{CIP1}, as well as the expression of the S phase markers Mcm2 and cyclin A2. As expected, all these genes were expressed in E2F1/E2F2$^{-/-}$ BMDM (but not in wild-type) one day after culture with M-CSF. However, inhibition of DNA replication by aphidicolin prevented the expression of each of these markers in a dose-dependent manner (Figure 7B) that is parallel to the inhibition of DNA synthesis. This data suggests that the DDR exhibited by E2F1/E2F2$^{-/-}$ BMDM is a consequence of abnormal DNA replication. Taken together, our data demonstrate that deregulated DNA replication occurring after E2F1

and E2F2 inactivation triggers a DNA damage response pathway that implements a $p21^{CIP1}$-dependent program of senescence.

DISCUSSION

Our results demonstrate that loss of E2F1 and E2F2 produces BMDM that undergo accelerated DNA replication and cell proliferation, but that this consequently leads to activation of the DNA damage response and the induction of cellular senescence. Exacerbated DNA replication could account for the increased cell cycle arrest and DNA damage observed in B cell progenitors and erythroblasts of E2F2- and E2F1/E2F2-deficient mice, which results in impaired hematopoiesis in these mice (Li *et al*., 2003; Dirlam *et al*., 2007). The accelerated initial proliferation of E2F1/E2F2$^{-/-}$ BMDM indicates that normally E2F1 and E2F2 function to suppress proliferation of this cell type. The increased expression in E2F1/E2F2$^{-/-}$ BMDM of E2F target genes that function in DNA replication and cell cycle progression is the apparent cause of accelerated DNA replication, similar to previous findings in T lymphocytes (Infante *et al*., 2008). Furthermore, our results imply that the repressor role of E2F1/E2F2 may not be limited to post-mitotic differentiating cells, a function that has been recently demonstrated for E2F1-3 (Chong *et al*., 2009; Chen *et al*., 2009). Instead, they are critical for restraining cellular division in monocyte progenitors during their concomitant differentiation into macrophages. A similar mechanism may be operating in proliferating T cells and keratinocytes lacking E2F2 or E2F1/E2F2 (Murga *et al*., 2001; Infante et al., 2008; Pusapati et al., 2010). The role of E2F1/E2F2, and possibly also E2F3, as activators could be limited to ES or early progenitor cells, in which the levels of phosphorylated Rb may be sufficiently high to prevent transcriptional repressor complex recruitment to E2F targets.

A parallel can be drawn between our results and those reported in cancers driven by many oncogenes. It has been suggested that the high levels of DNA replication proteins and G1 cyclins that are found frequently in cancer may contribute to tumorigenesis in part through aberrant S phase progression (Ekholm-Reed *et al*., 2004; Hwang *et al*., 2005). Alternatively, the aberrant S phase progression may actually be tumor suppressive, in that it leads to senescence by activating the DNA damage response. Very rarely, a pre-cancerous cell with aberrant DNA replication could evade this tumor suppressive barrier, such as by mutating p53 (Di Micco *et al*., 2006). Activation of the DNA damage response is common in cells and tissues that overexpress oncogenic regulators of cellular

proliferation, such as Ras, Braf, mos, cyclinE or E2F3 (Bartkova *et al*., 2006, Di Micco *et al*., 2006, Paulson *et al*., 2008). It has been suggested that oncogenes cause DNA damage by increasing the production of reactive oxygen species, by telomere attrition, or through the vague concept of replicative stress. Our finding that aphidicolin can block the activation of DDR in E2F1/E2F2$^{-/-}$ cells has some similarity to other reports implicating aberrant DNA replication in oncogene-induced DNA damage (Bartkova *et al*., 2006, Di Micco *et al*., 2006, Paulson *et al*., 2008). It is possible that overexpression of the replication proteins Mcm2, Mcm3 and Cdc6 in E2F1/E2F2$^{-/-}$ cells shown in this study may cause DNA damage by promoting illegitimate replication origin firing, which could, in turn, result in head-to-tail fork collision and checkpoint activation, similarly to what has been shown in cell extracts overexpressing the replication protein Cdt1 (Davidson *et al*., 2006).

Cell cycle arrest in E2F1/E2F2 double knockout cells differs from the classical G1 block that has been shown for cells undergoing senescence (Serrano *et al*., 1997). A similar block in the G2 or S-phases of the cell cycle has been described in cells that undergo senescence upon oncogenic activation (Di Micco *et al*., 2006, Olsen et al., 2002). Embryonic fibroblasts carrying inactivating mutations for E2F1-3 also arrest at various phases of the cell cycle, concomitant with induction of p21 and p53 (Timmers *et al*., 2007). In these cultures, however, no early proliferative burst or overexpression of E2F target genes was reported. Thus, more that one p53-activating pathways could be induced after single or multiple inactivation of E2F1-3. Our results clearly show that p53 activation in bone marrow-derived E2F1/E2F2$^{-/-}$ hematopoietic cells is triggered by the accelerated DNA replication that occurs during their differentiation into macrophages.

Induction of senescence is thought to involve the p16^{INK4A}/Rb and p53/p21^{CIP1} axes, although the relative contribution of these pathways may vary depending on the cell type or the senescence-triggering signal (Campisi, 2005). Oncogene-induced senescence, the form of senescence that is most likely to be associated with precancerous lesions (Collado *et al*., 2005) has been traditionally linked to increased expression of the cell-cycle inhibitors p16^{INK4A} and p19^{ARF} in a DDR-independent manner (Lowe *et al*., 2004). However, recent evidence form several labs indicates that oncogene overexpression is associated with a DNA replication stress that includes the activation of an ATM- and p53-dependent DNA double-

stranded break checkpoint pathway that promotes senescence (Di Micco *et al*., 2006; Herbig *et al*., 2004). Acute loss of Pten or Rb tumor suppressors also induces a p53-dependent senescence pathway (Chen *et al*., 2005). The induction of p53-dependent senescence by loss of Rb may be particularly relevant since E2F1 and E2F2 function to tether Rb to DNA to repress transcription. By this model of E2F1 and E2F2 function, we would predict that E2F1/E2F2-/- cells would exhibit increased transcription of E2F target genes, and p53-dependent senescence. In fact, we observe that E2F1/E2F2$^{-/-}$ cells have increased expression of E2F target genes and accumulate p53 and p21^{CIP1}, suggesting induction of senescence via the p53- and p21-dependent pathway.

Our data suggest that in bone marrow derived macrophages E2F1 and E2F2 function under normal physiological conditions to restrict cellular proliferation. Their inactivation renders cells prone to aberrant proliferation depending on the status of p21^{CIP1}. In cells with an intact p21^{CIP1} pathway, this aberrant proliferation results in cell cycle arrest and DDR-induced senescence. However, in the absence of p21^{CIP1} these cells proliferate without control *in vitro*. From the pharmacological point of view, an aberrant cell with an intact p21^{CIP1} pathway could benefit from treatments leading to E2F1/2 inactivation, which would push the cells towards inappropriate DNA replication and DDR-induced senescence. However, in situations where the p21^{CIP1}–dependent pathway is mutated, we observe that inactivation of E2F1/2 leads to more rapid cell proliferation. Our results suggest that the status of p21^{CIP} should be taken into account before considering therapies that inactivate the E2F pathway.

MATERIALS AND METHODS

Mouse strains

Colonies of E2F1$^{-/-}$, E2F2$^{-/-}$, and double knockout E2F1/E2F2$^{-/-}$mice have been described (Field *et al*., 1996; Iglesias *et al*., 2004; Murga *et al*., 2001). p21$^{CIP1-/-}$ mice (Balomenos *et al*., 2000) were a kind gift from Dimitrios Balomenos. Double knockout E2F1/E2F2$^{-/-}$ mice were bred to p21$^{CIP1-/-}$ mice to generate triple knockout E2F1/E2F2/p21$^{CIP1-/-}$ animals. All procedures were approved by the University of the Basque Country Animal Care and Use Committee.

Cell culture and treatments

Primary bone-marrow progenitor cells were isolated from tibias and femurs of 4-to-6 week-old mice. Proliferation concomitant to macrophagic differentiation was induced by culture of bone-marrow derived cells (10^6/ml) in medium (DMEM with 10% FCS, 1X Pen/Strep) supplemented with 30% L-cell conditioned medium as source of macrophage colony stimulating factor (Celada *et al*., 1996). We referred to "30% L-cell conditioned medium" as "M-CSF", which is equivalent to 10 ng/ml of recombinant M-CSF. Where indicated, cells were treated with aphidicolin (0.25-1 μg/ml; Sigma).

S-phase entry, proliferation, apoptosis and cell cycle distribution analyses

Bone marrow-derived cells (10^6/ml) or differentiated macrophages (10^5/ml) were cultured in medium alone or medium supplemented with M-CSF. Twenty-four hours later, [^{3}H]thymidine (1 μCi/100 μl) was added and S-phase entry was measured after 16 hours.

For carboxyfluorescein diacetate succinimidyl ester (CFSE) staining, bone marrow-derived cells were incubated as described (Infante *et al*., 2008), and cultured (10^6/ml) in medium supplemented with M-CSF. After 1 or 2 days, early differentiating macrophages were detected with cell surface staining, performed using anti-CD11b (Mac1) antibody conjugated to phycoerythrin (Becton & Dickinson). Fluorescence was detected and analyzed using a FACSCalibur (Becton & Dickinson) flow cytometer.

For cell cycle distribution analysis, cells were pulse-labeled with 10 μM BrdU for the last 30 min of cell culture, washed in ice-cold PBS, and fixed in ice-cold 70% ethanol. Cells were analyzed by flow cytometry as described (Infante *et al*., 2008).

Apoptosis of bone marrow-derived cells (10^6/ml) cultured in medium supplemented with M-CSF for three or five days was measured with the Annexin V-FITC Apoptosis Detection KIT II (BD Pharmingen) and analyzed by flow cytometry.

Metabolic activity was assayed using the 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based kit (Sigma) according to the manufacturer's suggested protocol.

Protein and mRNA analyses

Protein extracts were prepared as described (Infante *et al*., 2008). Antibodies against the following proteins were used in Western blots: Cyclin D2, Cyclin E, Cyclin A2, CDK1, Mcm2, p53, $p21^{CIP1}$ (Santa Cruz), Cyclin B1 (Cell Signalling), phospho-histone H2AX (Ser 139, Upstate), β-actin (Sigma).

Total RNA was prepared and processed as described (Infante *et al*., 2008). 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 *Mcm3*, *Cdc6*, *Tk*, $p21^{CIP1}$, *p16* and *p19* (sequences in Supplementary table2). *E2F3a* expression was determined with a custom-made TaqMan gene expression assay following the manufacturer´s instructions (Applied Biosystems). Reactions were carried out using an ABI Prism 7900 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*.

Senescence-associated β-galactosidase (SA-β-gal) staining

Equal numbers of wild-type, $E2F1^{-/-}$, $E2F2^{-/-}$, $E2F1/E2F2^{-/-}$, $p21^{CIP1-/-}$ or $E2F1/E2F2/p21^{CIP1-/-}$ bone-marrow derived cells were cultured in medium supplemented with M-CSF. After 5 days, cellular senescence was detected by staining for acidic (pH 6.0) β-galactosidase activity.

Immunofluorescence

Bone-marrow derived cells were cultured in medium supplemented with M-CSF on coverslips. After 2 days, early differentiating macrophages were fixed in 10% paraformaldehyde, permeabilized with 0.2% Triton X-100, and immunostained using 5 μg/ml primary antibody against phospho-histone H2AX (Ser 139, Upstate) and Alexa Fluor 488 (Molecular Probes) secondary antibody. DNA was counterstained with Hoechst 33288 dye and image analysis was performed using an Olympus Fluoview FV500 microscope and Olympus Flouview 1.7b software.

Statistical analysis

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

CONFLICT OF INTEREST

Authors declare no competing financial interests in relation to the work described.

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FIGURE LEGENDS

Fig. 1. Accelerated proliferation of BMDM deficient in E2F1 and E2F2. **A)** Percentage of $CD11b^{hi}$ cells in WT and $E2F1/E2F2^{-/-}$ bone marrow cells treated with M-CSF. Cells were harvested before and 6 days after stimulation. Results are expressed as percentage of $CD11b^{hi}$ cells (Mean±SD) from 6 independent experiments. **B)** Analysis of proliferation from representative WT, $E2F1^{-/-}$, $E2F2^{-/-}$ and $E2F1/E2F2^{-/-}$ bone-marrow derived cells stimulated with M-CSF. Bone-marrow cells were stained with CFSE, cultured with M-CSF for 1 day (left panel) or 2 days (right panel) and then stained with anti-CD11b. Cells were harvested and CFSE and anti-CD11b fluorescence was determined by flow cytometry. Proliferation Wizard software was used to identify CD11b positive cells in different generations as indicated. Note that the parental population (unshaded) is the brightest population, with subsequent cell divisions showing reduced CFSE signal. **C)** Proliferation was measured by $[^3H]$thymidine incorporation in bone-marrow derived cells 1.5 days after stimulation with M-CSF. Results are expressed as fold over untreated (UT) cells (Mean±SD) from 4 independent experiments. *$p<0.0001$ vs. WT.

Fig. 2. Hyperproliferation is followed by cell cycle arrest of $E2F1/E2F2^{-/-}$ BMDM cells. **A)** Growth curves of WT and $E2F1/E2F2^{-/-}$ bone-marrow derived cells treated with M-CSF. Cells were plated at equal cell numbers and treated with M-CSF. Macrophages were counted 1 to 6 days after stimulation. Averages and standard deviation of 6 independent experiments are shown. *$p<0.005$ vs. WT, **$p<0.0001$ vs. WT. **B)** Proliferation was measured by $[^3H]$thymidine incorporation in WT and $E2F1/E2F2^{-/-}$ bone-marrow derived cells 4.5 days after stimulation with M-CSF. Results are expressed as fold over untreated (UT) cells (Mean±SD) from 4 independent experiments. *$p<0.0001$ vs. WT. **C)** Apoptosis was measured with anti-Annexin V-FITC and propidium iodide staining in WT and $E2F1/E2F2^{-/-}$ bone-marrow derived cells 3 and 5 days after stimulation with M-CSF. Results are expressed as percentage of apoptotic cells (Mean±SD) from 6 independent experiments. **D)** Cellular metabolic activity was measured by MTT assay 0 and 5 days after stimulation with M-CSF. **E)** WT, $E2F1^{-/-}$, $E2F2^{-/-}$ and $E2F1/E2F2^{-}$

$^{/-}$ bone-marrow derived cells were stimulated with M-CSF for 5 days and then stained for SA-β-gal marker to establish the status of cellular senescence.

Fig. 3. Analysis of cell cycle distribution from WT and E2F1/E2F2 mutant bone-marrow derived cells stimulated with M-CSF. Bone-marrow cells were cultured with (or without) M-CSF for the indicated times, pulse labeled with BrdU for 30 min, harvested and fixed. DNA content was assessed by staining with propidium iodide, and DNA synthesis was assessed by staining with an antibody to BrdU, and measeured by flow cytometry. **A)** Cell cycle distribution analysis of representative WT, E2F1$^{-/-}$, E2F2$^{-/-}$ and E2F1/E2F2$^{-/-}$ bone-marrow derived cells stimulated with MCSF at the indicated times, **B)** Percentages of BrdU-positive cells, **C)** BrdU-negative cells with 2N to 4N DNA content, **D)** BrdU-negative cells with 4C DNA content. Results are the Mean±SD of four independent experiments. *p<0.005 vs. WT, **p<0.0001 vs. WT.

Fig. 4. Effect of E2F1 and E2F2 on cell cycle regulators and DNA replication proteins. **A)** Western blot analysis of cyclin D2, E, A2, B1 and CDK1 in extracts prepared from WT, E2F1$^{-/-}$, E2F2$^{-/-}$ and E2F1/E2F2$^{-/-}$ bone-marrow derived cells stimulated with M-CSF during the indicated times. In each line, 20 μg of total protein extract was loaded. Expression of β-actin was used as loading control. These data are representative of at least three independent experiments. **B**) Reverse transcription Q-PCR analysis of Mcm3, Cdc6 and Tk expression before and 7 days after stimulation with M-CSF. *Gapdh* was used as housekeeping control gene. Results are expressed as fold over respective WT (Mean±SD) from 6 independent experiments.*p<0.005 vs. WT, **p<0.0001 vs. respective WT. **C)** Reverse transcription Q-PCR analysis of E2F3a expression before and 7 days after stimulation with M-CSF. *Gapdh* was used as housekeeping control gene. Results are expressed as fold over respective WT (mean±SD) from 3 independent experiments. **D)** Reverse transcription Q-PCR analysis of $p21^{CIP1}$, $p16^{ARF}$ and $p19^{INK4A}$ before and 5 days after stimulation with M-CSF. *Gapdh* was used as housekeeping control gene. Averages and standard deviation of 6 individual experiments are shown. *p<0.0001 vs. WT.

Fig. 5. Disruption of $p21^{CIP1}$ in E2F1/E2F2 mutant cells prevents cellular senescence and restores expression of DNA replication proteins and cell cycle entry and progression. **A)** Proliferation of WT, $E2F1/E2F2^{-/-}$, $p21^{CIP1-/-}$ and $E2F1/E2F2/p21^{CIP1-/-}$ bone-marrow derived cells treated with M-CSF. Cells were plated at equal cell numbers and stimulated with M-CSF continuously for 14 days. Macrophages were re-stimulated at day 3 and 7, and the total numbers were counted and mean population doublings (MPD) were determined. Results are expressed as millions of cells (Mean±SD) from 6 independent experiments. *$p<0.02$, **$p<0.0001$. **B**) Cell cycle entry was measured by [^{3}H]thymidine incorporation 1.5, 4.5, 8.5 and 15.5 days after stimulation with M-CSF. Results are expressed as fold over untreated (UT) cells (Mean±SD) from 3 independent experiments. *$p<0.005$, **$p<0.0001$. **C**) Reverse transcription Q-PCR analysis of Mcm3, Cdc6 and Tk expression 7 days after stimulation with M-CSF. *Gapdh* was used as housekeeping control gene. Results are expressed as fold over respective WT (Mean±SD) from 3 independent experiments. *$p<0.005$, **$p<0.0001$. **D)** 5 days after M-CSF stimulation cells were stained for SA-β-gal marker to establish the status of cellular senescence.

Fig. 6. DNA damage response activation in E2F1/E2F2 mutant cells. **A)** WT, $E2F1/E2F2^{-/-}$ and $E2F1/E2F2/p21^{CIP-/-}$ bone-marrow derived cells were stimulated with M-CSF for 2 days, processed for immunofluorescence by using γ-H2AX antibody and stained with Hoechst to visualize DNA. The numbers of γ-H2AX positive foci per cell are indicated (mean±SD, $p<0.02$). These data is representative of three experiments. 100 cells were counted in each experiment. **B)** The increased levels of γ-H2AX, p53 and $p21^{CIP1}$ correlates with the accumulation of S-phase proteins in E2F1/E2F2 mutant cells. WT and $E2F1/E2F2^{-/-}$ bone-marrow derived cells were stimulated with M-CSF. Cells were harvested before and 1 day after stimulation and proteins were analyzed by immunobloting with the indicated antibodies. Expression of β-actin was used as loading control. These data are representative of at least three experiments. **C)** The increased levels of Mcm2 and p53 are independent of the status of $p21^{CIP1}$. WT, $E2F1/E2F2^{-/-}$ and $E2F1/E2F2/p21^{CIP1-/-}$ bone-marrow derived cells were stimulated with M-CSF. Cells were harvested before and 1 day after stimulation and proteins were analyzed by

immunobloting with the indicated antibodies. Expression of β-actin was used as loading control. These data are representative of at least three experiments.

Fig. 7. Activation of DNA damage response is triggered by DNA hyper-replication in cells with E2F1 and E2F2 inactivated. WT and E2F1/E2F2$^{-/-}$ bone-marrow derived cells were stimulated with M-CSF in the absence or presence of aphidicolin. **A)** DNA replication rates were measured by [^{3}H]thymidine incorporation 1.5 days after M-CSF stimulation. Results are expressed as fold over untreated (UT) cells (mean±SD) from 4 independent experiments. *$p<0.0001$ vs. WT cells not treated with aphidicolin, †$p<0.0001$ E2F1/E2F2$^{-/-}$ cells treated vs. not treated with aphidicolin. **B)** Cells were harvested 1 day after M-CSF stimulation and proteins were analyzed by immunobloting with the indicated antibodies. Expression of β-actin was used as loading control. Data are representative of at least three experiments.

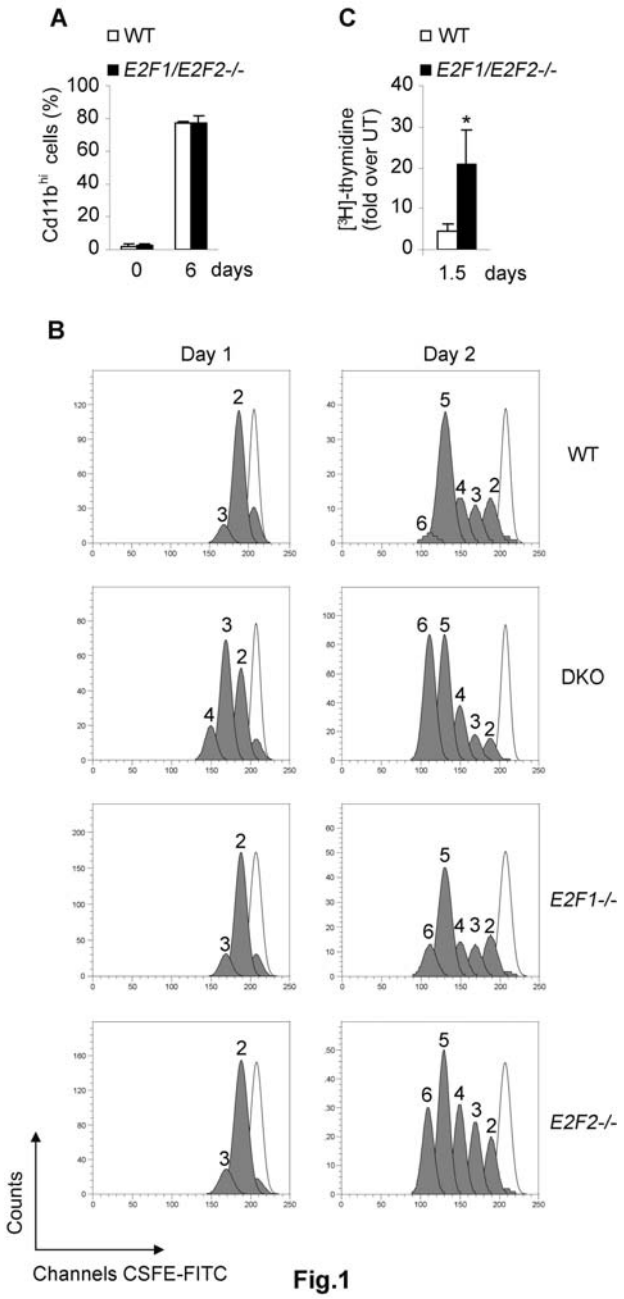
A
WT
E2F1/E2F2-/-
Cd11b^hi cells (%)
100
80
60
40
20
0
0
6 days
C
WT
E2F1/E2F2-/-
[3H]-thymidine (fold over UT)
40
30
20
10
0
*
1.5 days
B
Day 1
Day 2
WT
DKO
E2F1-/-
E2F2-/-
Counts
Channels CSFE-FITC

Fig.1

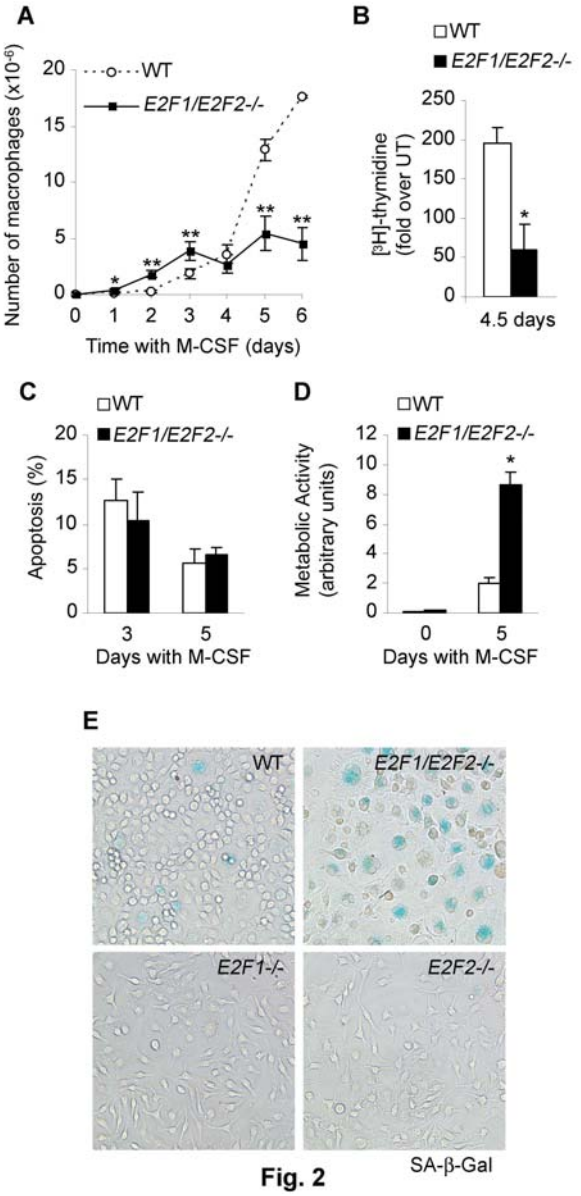
A
Number of macrophages (x10⁻⁶)
WT
E2F1/E2F2-/-
Time with M-CSF (days)
B
WT
E2F1/E2F2-/-
[³H]-thymidine (fold over UT)
4.5 days
C
WT
E2F1/E2F2-/-
Apoptosis (%)
Days with M-CSF
D
WT
E2F1/E2F2-/-
Metabolic Activity (arbitrary units)
Days with M-CSF
E
WT
E2F1/E2F2-/-
E2F1-/-
E2F2-/-
SA-β-Gal

Fig. 2

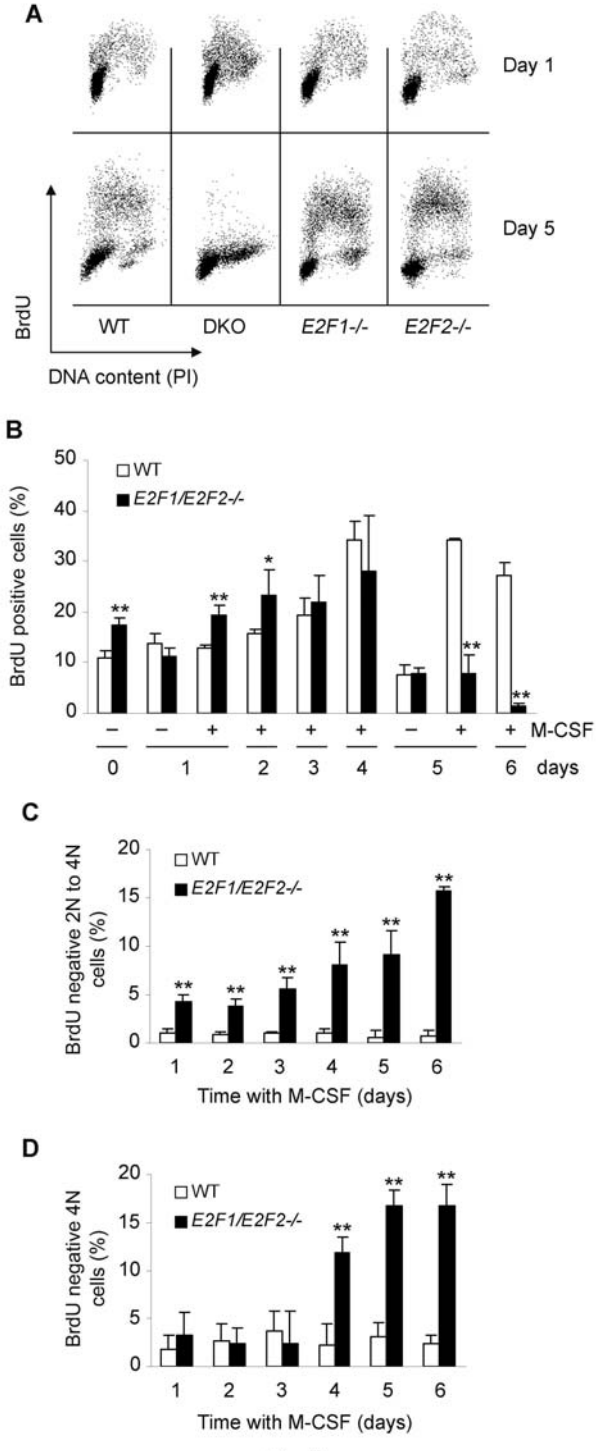
A
Day 1
Day 5
WT
DKO
E2F1-/-
E2F2-/-
BrdU
DNA content (PI)
B
WT
E2F1/E2F2-/-
BrdU positive cells (%)
M-CSF
days
C
BrdU negative 2N to 4N cells (%)
Time with M-CSF (days)
D
BrdU negative 4N cells (%)
Time with M-CSF (days)

Fig.3

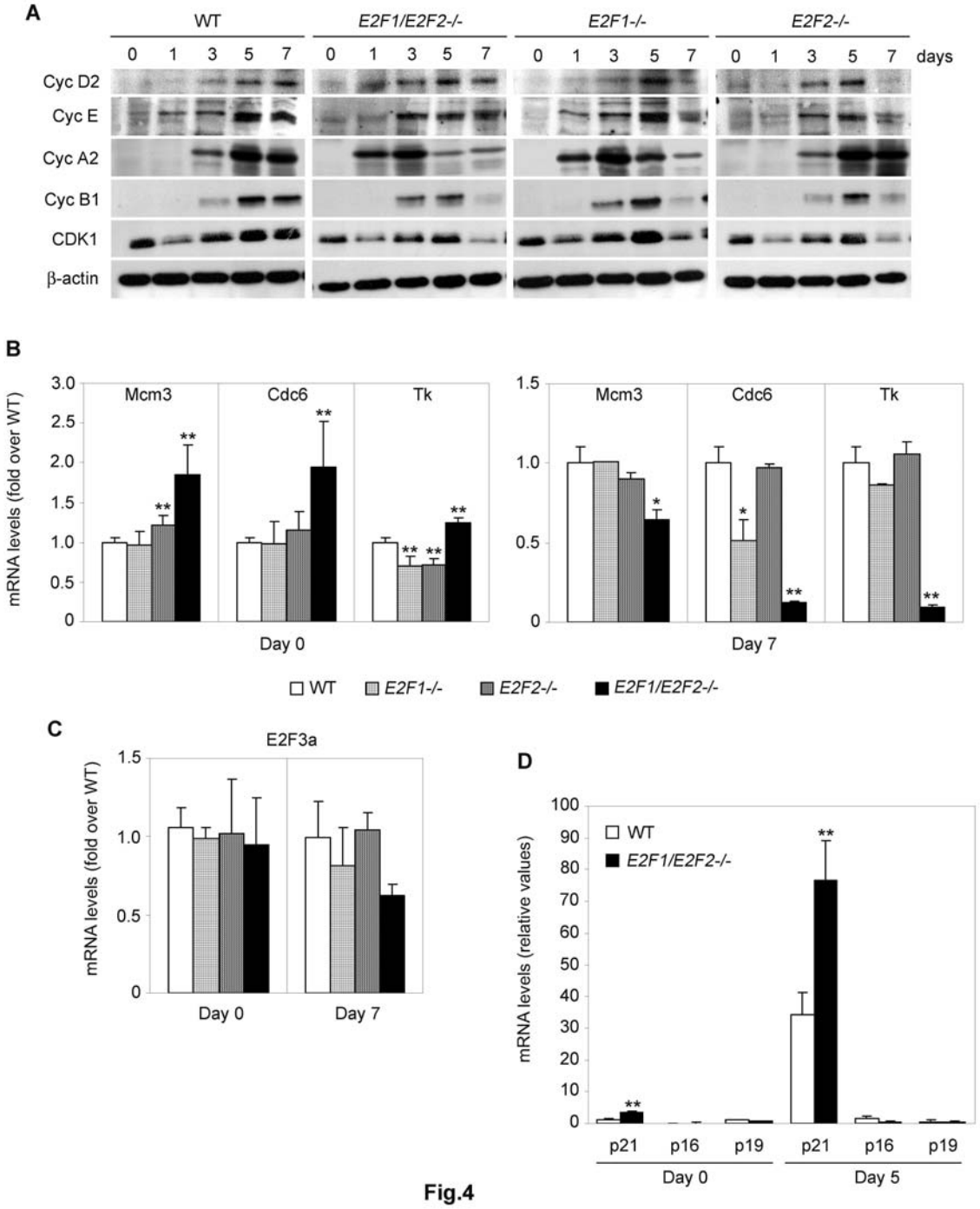
A
WT
E2F1/E2F2-/-
E2F1-/-
E2F2-/-
0 1 3 5 7 days
Cyc D2
Cyc E
Cyc A2
Cyc B1
CDK1
β-actin
B
mRNA levels (fold over WT)
Mcm3
Cdc6
Tk
Day 0
Day 7
WT
E2F1-/-
E2F2-/-
E2F1/E2F2-/-
C
E2F3a
mRNA levels (fold over WT)
Day 0
Day 7
D
mRNA levels (relative values)
WT
E2F1/E2F2-/-
p21
p16
p19
Day 0
Day 5

Fig.4

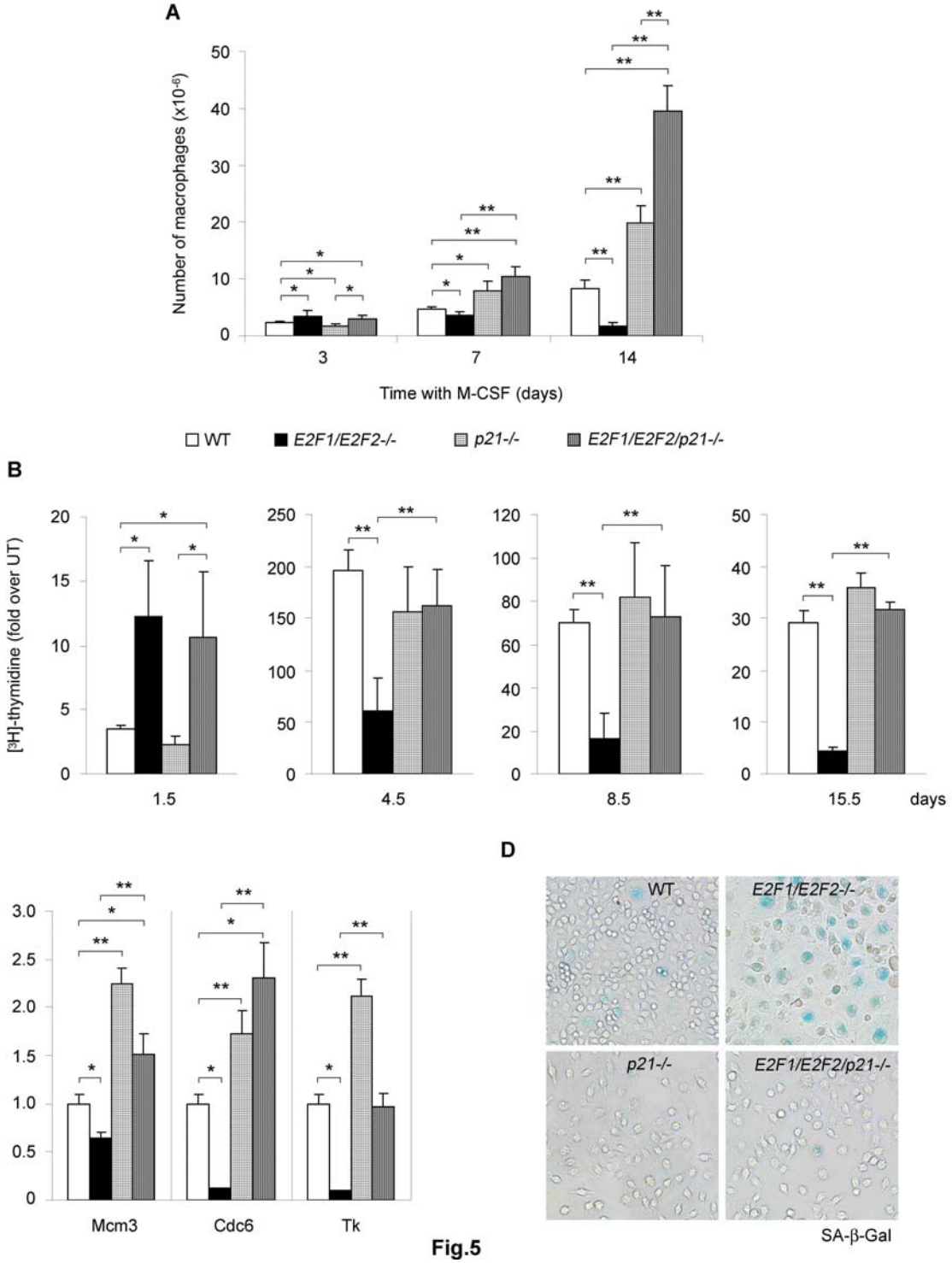
A
Number of macrophages (x10-6)
Time with M-CSF (days)
WT
E2F1/E2F2-/-
p21-/-
E2F1/E2F2/p21-/-
B
[3H]-thymidine (fold over UT)
days
C
mRNA levels (fold over WT)
Mcm3
Cdc6
Tk
D
WT
E2F1/E2F2-/-
p21-/-
E2F1/E2F2/p21-/-
SA-β-Gal

Fig.5

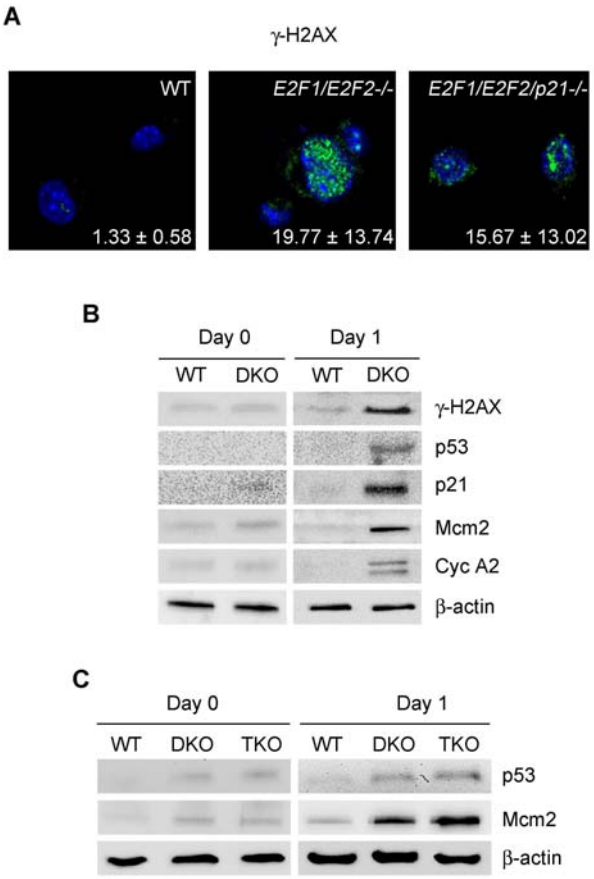
A
γ-H2AX
WT
E2F1/E2F2-/-
E2F1/E2F2/p21-/-
1.33 ± 0.58
19.77 ± 13.74
15.67 ± 13.02
B
Day 0
Day 1
WT
DKO
WT
DKO
γ-H2AX
p53
p21
Mcm2
Cyc A2
β-actin
C
Day 0
Day 1
WT
DKO
TKO
WT
DKO
TKO
p53
Mcm2
β-actin

Fig.6

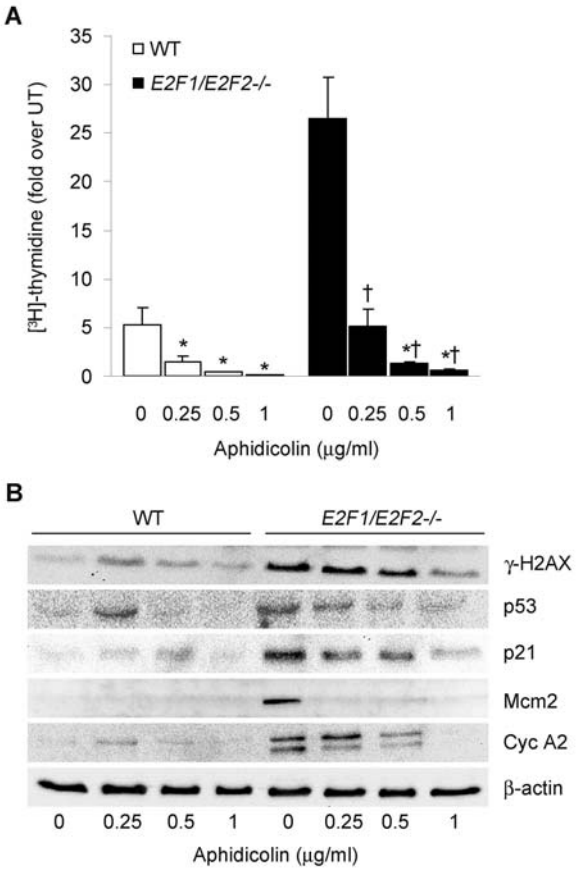
A
35
30
25
20
15
10
5
0
WT
E2F1/E2F2-/-
[3H]-thymidine (fold over UT)
*
*
*
†
*†
*†
0 0.25 0.5 1 0 0.25 0.5 1
Aphidicolin (μg/ml)
B
WT
E2F1/E2F2-/-
γ-H2AX
p53
p21
Mcm2
Cyc A2
β-actin
0 0.25 0.5 1 0 0.25 0.5 1
Aphidicolin (μg/ml)

Fig.7