



Published in final edited form as:

*Dev Cell*. 2008 January ; 14(1): 62–75. doi:10.1016/j.devcel.2007.10.017.

## Synergistic Function of E2F7 and E2F8 is Essential for Cell Survival and Embryonic Development

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### Summary

The novel *E2f7* and *E2f8* family members are thought to function as transcriptional repressors important for the control of cell proliferation. Here we have analyzed the consequences of inactivating *E2f7* and *E2f8* in mice and show that their individual loss had no significant effect on development. Their combined ablation, however, resulted in massive apoptosis and dilation of blood vessels, culminating in lethality by embryonic day E11.5. A deficiency in *E2f7* and *E2f8* led to an increase in *E2f1* and p53, as well as in many stress-related genes. Homo- and hetero-dimers of E2F7 and E2F8 were found on target promoters, including *E2f1*. Importantly, loss of either *E2f1* or p53 suppressed the massive apoptosis in double mutant embryos. These results identify E2F7 and E2F8 as a unique repressive arm of the E2F transcriptional network that is critical for embryonic development and control of the E2F1-p53 apoptotic axis.

### Keywords

E2Fs; embryo development; apoptosis; cell cycle; transcriptional regulation

### Introduction

A large body of work suggests that E2Fs function to activate and repress the transcription of many essential genes involved in cell proliferation, apoptosis and differentiation (Dimova and Dyson, 2005). The effects of deregulated E2F activity are pleiotropic and vary in different experimental settings. A decrease in E2F activity is generally associated with a reduction in the proliferation capacity of cells (Leone et al., 1998; Humbert et al., 2000b; Wu et al.,

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**Competing interests statement:** The authors declare that they have no competing financial interests.

2001), whereas an increase in E2F activity is often associated with inappropriate cell proliferation and/or apoptosis (DeGregori et al., 1997). The regulation of E2F activity during the cell cycle is thought to be critical for cellular homeostasis. Thus, a significant amount of genetic currency has been invested to finely control E2F activity in cells, including by direct binding of the Rb tumor suppressor, by transcription, by post-transcriptional mechanisms involving miRNAs, and by post-translational mechanisms involving protein degradation, phosphorylation and acetylation (Muller et al., 2000; O'Donnell et al., 2005).

Further complexity in the regulation of E2F function has been afforded by the evolution of numerous family members and isoforms. The mammalian E2F proteins are encoded by eight distinct genes (E2F1-8) and specific roles for each family member in controlling cell cycle transitions and apoptosis have been reported (Bracken et al., 2004; DeGregori and Johnson, 2006). Based on structure-function studies and sequence analysis, the E2F family can be conveniently divided into two general subclasses, transcription repressors and activators. Members of the activator subclass, consisting of E2F1, E2F2 and E2F3a, accumulate late in G<sub>1</sub> and are transiently recruited to E2F-binding elements on target promoters and participate in their acute activation. Consistent with an important function for activator E2Fs in regulating gene expression during G<sub>1</sub>-S, their overexpression in quiescent cells can potently transactivate many E2F-responsive genes and drive cells to enter S phase (DeGregori et al., 1997). Conversely, the combined loss of the three activators results in a decrease of E2F-target gene expression and a severe block in cell proliferation (Wu et al., 2001). The repressor subclass consists of E2F3b, E2F4, E2F5, E2F6, E2F7 and E2F8. A subset of this group that includes E2F3b, E2F4 and E2F5, serves to recruit Rb-related pocket proteins and associated co-repressors to target promoters during G<sub>0</sub> and to repress their expression (Attwooll et al., 2004). As cells are stimulated to enter the cell cycle, cyclin dependent kinases (CDKs) phosphorylate pocket proteins, resulting in dissociation of E2F-pocket protein repressor complexes and derepression of E2F-target genes (Muller et al., 2000; Seville et al., 2005). The E2F6 repressor is part of a multi-subunit complex that includes polycomb group proteins as well as Mga and Max, and appears to act on a different subset of target genes than E2F4 (Ogawa et al., 2002; Giangrande et al., 2004). The mechanism of how E2F7 and E2F8 repress gene expression is less clear. While the molecular basis for how E2F repressors and activators orchestrate the acute induction of E2F targets as cells transit through G<sub>0</sub>-G<sub>1</sub>-S is fairly well understood, how E2F-target genes are subsequently downregulated as cells proceed through S-G<sub>2</sub> phases of the cell cycle is not known.

The E2F7 and E2F8 proteins are conserved in mice and humans, and related E2F-like proteins exist in *Arabidopsis* (Kosugi et al., 2002; Mariconti et al., 2002; de Bruin et al., 2003; Di Stefano et al., 2003; Logan et al., 2004; Christensen et al., 2005; Logan et al., 2005; Maiti et al., 2005). These two novel factors have several unique features that distinguish them from other members in the E2F family. They lack the leucine-zipper domain required for dimerization with partner proteins DP1/2 and instead possess two DNA binding domains that are predicted to interact with each other and foster DP-independent DNA-binding activity. The expression of E2F7 and E2F8 during the cell cycle is also different from that of other E2Fs, with peak levels found later in the cell cycle during S-G<sub>2</sub>. Moreover, their overexpression in fibroblasts can, unlike that of other E2Fs, repress E2F-target gene expression and block cell proliferation (de Bruin et al., 2003; Di Stefano et al., 2003; Maiti et al., 2005), suggesting a role for these two E2Fs in facilitating cell cycle transitions. Here we utilized homologous recombination strategies to inactivate *E2f7* and *E2f8* in mice and rigorously investigate their functions *in vivo*. From these studies we conclude that *E2f7* and *E2f8* represent a unique repressive arm of the E2F transcriptional network that is critical for embryonic development and cell survival.

## Results

### E2F7 and E2F8 are essential for embryonic viability

To investigate E2F7 and E2F8 function *in vivo*, we utilized homologous recombination techniques and cre-*loxP* technology to disrupt *E2f7* and *E2f8* function in mice. Targeting the inactivation of *E2f7* and *E2f8* was achieved by flanking exon 4 of *E2f7* and exons 3 and 4 of *E2f8* with *loxP* sites (Figure 1A). Cre-mediated recombination of *loxP*-flanked sequences resulted in the ablation of domains required for DNA binding and in a shift of the open reading frames, leading to premature termination of translation. Pups lacking the *neo* cassette (conditional knockout allele: *E2f7<sup>loxP</sup>* or *E2f8<sup>loxP</sup>*; Figure 1A) or lacking both the *neo* cassette and the *loxP*-flanked regions of *E2f7* or *E2f8* (conventional knockout allele: *E2f7<sup>-</sup>* or *E2f8<sup>-</sup>*; Figure 1A) were identified by Southern blot and PCR genotyping analysis (Figure 1B and data not shown).

To investigate the requirement for these E2Fs in development we interbred *E2f7<sup>+/-</sup>* or *E2f8<sup>+/-</sup>* animals and found that *E2f7<sup>-/-</sup>* or *E2f8<sup>-/-</sup>* pups were born. Mutant pups developed normally through puberty and lived to old age (data not shown). Real-time RT-PCR analysis of gene expression revealed that *E2f7* and *E2f8* mRNA levels were unperturbed in *E2f8<sup>-/-</sup>* and *E2f7<sup>-/-</sup>* embryos, respectively (Figure 1C), indicating that the absence of abnormalities in single knockout animals is not due to simple compensation at the expression level. We then explored functional redundancy between E2F7 and E2F8 by examining the biological consequence of ablating both simultaneously. To this end, we intercrossed *E2f7<sup>+/-</sup>E2f8<sup>+/-</sup>* animals and analyzed the resulting offspring. Whereas *E2f7<sup>-/-</sup>E2f8<sup>+/-</sup>* and *E2f7<sup>+/-</sup>E2f8<sup>-/-</sup>* pups were born at the expected Mendelian ratios, no *E2f7<sup>-/-</sup>E2f8<sup>-/-</sup>* double knockout (DKO) pups were found in newborn litters (P0) (Table 1). This demonstrates that at least one allele of *E2f7* or *E2f8* is required for embryonic development and viability. The contribution of E2F7 and E2F8 towards postnatal development, however, does not appear to be equal. Young and adult *E2f7<sup>+/-</sup>E2f8<sup>-/-</sup>* mice were developmentally normal, whereas most *E2f7<sup>-/-</sup>E2f8<sup>+/-</sup>* animals appeared runted (Figure 2A) and died within the first three months of life (Figure 2B; P90,  $p < 0.001$ ).

### Genetic ablation of *E2f7* and *E2f8* *in vivo* induces massive apoptosis in embryos

Analysis at earlier stages of embryonic development showed that all DKO embryos had a beating heart at embryonic day 9.5 (E9.5). These embryos were noticeably smaller than *wild type* littermates (Table 1 and Figure 2C, top panels) but macroscopic inspection did not reveal other obvious defects. By E10.5, only 46% of DKO embryos were alive ( $p = 0.0015$ ) and by E11.5 all DKO embryos were dead ( $p < 0.0007$ ). Live E10.5 embryos often had vascular defects in the yolk sac and in the embryo proper, which were characterized by large dilated blood vessels associated with multifocal hemorrhages (Figure 2C, bottom panels). Other phenotypes were also manifested in tissues distinct from the vasculature. Specifically, multiple areas within the head mesenchyme, branchial arch, somites and neural tube of DKO fetuses contained cells with pyknotic nuclei surrounded by bright eosinophilic cytoplasm (Figure S1A and data not shown).

These latter observations prompted us to examine proliferation and apoptosis in *E2f7<sup>-/-</sup>E2f8<sup>-/-</sup>* embryos more closely. Since a significant fraction of DKO embryos died by E10.5, proliferation and apoptosis were measured in E9.5 embryos. When assessed by immunohistochemistry using BrdU-specific antibodies, we observed no detectable difference in the percentage of cells incorporating BrdU between *wild type* and DKO embryos (Figure S2). We did observe, however, a marked increase in cells labeled by TdT-mediated dUTP nick end-labelling (TUNEL) in areas of DKO embryos previously noted to contain abundant pyknotic nuclei, confirming widespread apoptosis in these tissues (Figure 2D-E, S1B-C). In

summary, global deletion of *E2f7* and *E2f8* resulted in a spectrum of embryonic defects impacting the vasculature and cell survival.

### **E2F7 and E2F8 form homo-dimers and hetero-dimers**

Previous studies suggested that E2F7 and E2F8 form homo-dimers (Di Stefano et al., 2003; Logan et al., 2004; Maiti et al., 2005). Co-immunoprecipitation (Co-IP) assays using HEK 293 cells co-expressing flag-tagged and HA-tagged versions of E2F7 or E2F8 confirmed their ability to form homo-dimers (Figure 3A and data not shown). We also explored the possibility that E2F7 may physically interact with E2F8. Because immunoprecipitation-quality antibodies for E2F7 and E2F8 are not yet available, we assessed the ability of flag-tagged E2F7 to complex with endogenous E2F8 and *vice versa*. As shown in Figure 3B (and data not shown), these immuno-affinity purification assays revealed an association between E2F7 and E2F8. Hetero-dimerization was confirmed by Co-IP assays using HEK 293 cells co-expressing flag-E2F7 and HA-E2F8 (Figure 3C).

Given the redundant functions of E2F7 and E2F8 in development, we evaluated their preferred dimerization state in cells. To this end, HEK 293 cells were co-transfected with flag-E2F7, HA-E2F7 and myc-E2F8, or alternatively with flag-E2F8, HA-E2F7 and myc-E2F8. We then measured the relative amounts of homo- versus hetero-dimers by immunoprecipitation with flag-antibodies followed by immunoblotting with either HA- or myc-antibodies; the amount of E2F7 and E2F8 on blots was normalized to 1% of the input material. Quantification of three independent experiments showed that E2F7 had a greater binding affinity to itself than to E2F8 (Figure 3D-E). On the other hand, E2F8 had a greater binding affinity for E2F7 than for itself. From this analysis we conclude that, at least in cultured cells, the preferred state of dimerization is E2F7:E2F7 > E2F7:E2F8 > E2F8:E2F8.

### **E2f1 is a direct target of E2F7 and E2F8**

We then utilized chromatin immunoprecipitation (ChIP) assays to assess the ability of E2F7 and E2F8 to bind known E2F target promoters. Quantitative real-time PCR assays showed that flagged-tagged versions of E2F7 and E2F8 were recruited to E2F binding sites present on the *E2f1* and *cdc6* promoters but not to irrelevant sequences in these genes (exons 1) or the *tubulin* promoter (Figure 3F-G, S4A-B and data not shown). This recruitment was specific since IgG failed to immunoprecipitate target promoter sequences. Moreover, anti-flag antibodies failed to immunoprecipitate target promoters from cell lysates expressing mutant forms of E2F7 or E2F8 that are incapable of binding DNA.

We then used sequential ChIP techniques to address whether E2F7 and E2F8 could be recruited to the target promoters as homo- and hetero-dimers. To this end, HEK 293 cells expressing various combinations of HA-tagged and flag-tagged versions of E2F7 and E2F8 were first immunoprecipitated with anti-flag antibodies. Immunoprecipitated DNA-protein complexes were then eluted with excess flag peptide and re-immunoprecipitated with HA-specific antibodies. The final immunoprecipitates were amplified with primers specific for the *E2f1*, *cdc6* or *tubulin* promoters as described above (Figure 3H-J, S4C-E). These sequential ChIP assays showed that both homo- and hetero-dimers of E2F7 and E2F8 could occupy E2F binding sites on E2F-target promoters, including *E2f1*.

### **E2f1 expression is derepressed in *E2f7<sup>-/-</sup>E2f8<sup>-/-</sup>* MEFs**

To determine whether the recruitment of E2F7 and E2F8 to the target promoters had any functional consequence on its expression, we initially examined E2F1 protein and mRNA levels in mouse embryo fibroblasts (MEFs) deficient for both *E2f7* and *E2f8*. Because *E2f7<sup>-/-</sup>E2f8<sup>-/-</sup>* embryos died early during mouse development, we derived MEFs from E13.5 *E2f7<sup>loxP/loxP</sup>E2f8<sup>loxP/loxP</sup>* embryos and then ablated *E2f7* and *E2f8* expression *in vitro* using a

*cre* recombinase-expressing retrovirus. PCR genotyping of genomic DNA and real-time RT-PCR analysis of *E2f7* and *E2f8* expression confirmed their efficient deletion (Figure 4A). These experiments showed that DKO cells have higher E2F1 protein and mRNA levels than control treated MEFs (Figure 4B and 4C, respectively). Interestingly, there was also an increase of p53 protein in DKO MEFs, consistent with the ability of E2F1 to induce the accumulation of p53 (Hsieh et al., 2002; Pomerantz et al., 1998; Rogoff et al., 2002; Russell et al., 2002). Introduction of an *E2f1* luciferase promoter construct into *wild type* and DKO MEFs revealed higher luciferase reporter activity in DKO cells (Figure 4D), indicating that the higher levels of E2F1 mRNA and protein in DKO MEFs is likely due to transcriptional deregulation. Together with the ChIP assays shown in Figure 3F-J, these data suggest that the repression of *E2f1* by E2F7 and E2F8 is direct.

To examine whether E2F7 and E2F8 mediated repression might be cell-cycle dependent, we compared E2F-target expression in synchronized populations of *wild type* and DKO MEFs stimulated to progress through the cell cycle. MEFs were synchronized in G<sub>1</sub>/S by serum deprivation followed by re-stimulation with medium containing 15% serum and 1mM hydroxyurea (HU). Cells were then washed and incubated with medium lacking HU and harvested at various times following HU-release. Cell cycle progression was monitored by flow cytometry (Figure 4E). As expected, the expression of *E2f1* and *cdc6* in *wild type* cells peaked at G<sub>1</sub>/S and subsequently decreased as cells entered S phase and progressed through G<sub>2</sub> (Figure 4F and S5A). Strikingly, expression of *E2f1* and *cdc6* in DKO cells continued to increase during S and G<sub>2</sub>, accumulating up to 12-fold higher levels than in *wild type* cells. These data suggest an important role for E2F7- and E2F8-mediated repression during S and G<sub>2</sub>, coinciding with the time of the cell cycle when these two E2Fs accumulate maximally (de Bruin et al., 2003; Maiti et al., 2005).

### **DKO cells are hypersensitive to DNA damage**

Given the marked increase in the expression of multiple E2F-target genes in cells deficient for *E2f7* and *E2f8*, we analyzed proliferation rates in these double mutant cells. These analyses failed to reveal any significant difference in the proliferation of DKO and *wild type* cells (Figure S5B and data not shown), even though DKO cells appeared to transit through S phase 2-3 hours faster than control cells (Figure 4E). Presumably, faster progression through S phase was compensated by a concomitant delay in other phases of the cell cycle as previously reported for cells lacking *Rb* or overexpressing *dE2f1* (Resnitzky et al., 1994; Reis and Edgar, 2004). Because overexpression of *E2f1* has been shown to elicit apoptosis in cells treated with DNA-damage inducing agents (Stevens and La Thangue, 2004), we tested the sensitivity of DKO cells to camptothecin and cisplatin, two DNA-damage-inducing drugs. To this end, asynchronously proliferating *wild type* and DKO MEFs were treated with camptothecin or cisplatin and cell viability was determined by trypan blue exclusion. These drugs induced a significant acceleration of cell death in DKO MEFs when compared to *wild type* MEFs (Figure 4G and data not shown). Drug-treated DKO cells detached from tissue culture plates and exhibited the characteristic blebbing morphology of apoptotic cells (Figure S5C). Consistent with this observation, drug treatment preferentially triggered cleavage of caspase-3 in DKO MEFs (Figure 4H). We also evaluated the levels of E2F1 and p53 in drug-treated DKO MEFs. As expected, E2F1 and p53 protein levels accumulated to higher levels in camptothecin-treated DKO cells than in similarly treated *wild type* cells (Figure S5D). The increase in p53 protein corresponded with a marked increase in the expression of p53-responsive genes, including *gadd45*, *noxa*, and *pidd* (Figure 5I). These results suggest that E2F7 and E2F8 may attenuate DNA-damage induced apoptosis by preventing the aberrant accumulation of E2F1 and p53.

## Induction of apoptosis in *E2f7<sup>-/-</sup>E2f8<sup>-/-</sup>* embryos is dependent on E2F1 and p53

Given the above observations, we hypothesized that the apoptosis in *E2f7<sup>-/-</sup>E2f8<sup>-/-</sup>* embryos might be mediated through the induction of E2F1 and/or p53. To test this possibility, *E2f7<sup>+/-</sup>E2f8<sup>-/-</sup>* animals were bred with either *E2f1<sup>-/-</sup>* or *p53<sup>-/-</sup>* animals in order to generate cohorts of triple knockout embryos (TKO). TUNEL assays performed on E9.5 TKO embryos showed that the loss of either *E2f1* or *p53* suppressed the massive apoptosis caused by a deficiency in *E2f7* and *E2f8* (Figure 5A and 5B). From these results we conclude that E2F7 and E2F8 represent a critical regulatory arm of the E2F network that controls apoptosis through the E2F1-p53 axis.

Interestingly, both cohorts of TKO embryos harvested at E10.5 had dilated vessels and extensive hemorrhaging similar to DKO embryos (data not shown). Importantly, no live TKO embryos could be observed at E12.5 (Figure 5C-D). These results suggest that the widespread apoptosis observed in DKO embryos, which was suppressed in TKO embryos, is independent of vascular defects and embryonic lethality. These results also indicate that misregulated E2F7- and E2F8-target genes beyond *E2f1* are likely to be involved in the mortality of DKO embryos.

Consistent with this view, global analysis of gene expression showed that among the ~ 39,000 transcripts analyzed, 88 were upregulated and 33 were downregulated at least 3-fold in *E2f7<sup>-/-</sup>E2f8<sup>-/-</sup>* embryos relative to *wild type* embryos (Figure 6A). Real-time RT-PCR assays confirmed the altered expression of many of these genes (Figure 6B). Functional annotation of misregulated genes revealed a bias for gene products known to be activated in response to stresses, including hypoxia, nutrient deprivation and apoptosis (Figure 6C and S6). These analyses also confirmed that *E2f1*, *cdc6* and additional E2F targets were increased in DKO versus *wild type* embryos, albeit the increase in their expression was only ~2-fold (data not shown). Interestingly, a subset of the 122 transcripts (88 up- and 33 down-regulated) were partially misregulated in *E2f7<sup>-/-</sup>*, *E2f8<sup>-/-</sup>*, and/or *E2f7<sup>+/-</sup>E2f8<sup>+/-</sup>* embryos, underscoring the synergistic role of these two E2F factors in the control of gene expression during embryonic development.

## Discussion

The E2F7 and E2F8 transcription factors likely represent the final members of the E2F family to be identified in mammals. We show here that these two novel factors are strictly required for embryonic development and are critical direct regulators of the E2F1-p53 apoptotic axis.

While disruption of *E2f7* or *E2f8* had little impact on mouse development, their combined ablation resulted in widespread apoptosis, vascular defects and hemorrhaging, leading to embryonic death by E11.5. Provision of even one functional allele from either locus was sufficient to carry fetuses through development all the way to birth. The contribution of E2F7 and E2F8 in postnatal development, however, does not appear to be equal. Young and adult *E2f7<sup>+/-</sup>E2f8<sup>-/-</sup>* mice were developmentally normal, whereas most *E2f7<sup>-/-</sup>E2f8<sup>+/-</sup>* animals appeared runted and died within the first three months of life. A bias in homo- versus hetero-dimerization may explain the differential requirement for *E2f7* and *E2f8* in postnatal development. We found that in tissue culture experiments, under conditions where expression levels can be compared (by epitope tagging) and experimentally equalized, the formation of E2F7:E2F7 homo-dimers was preferred over E2F7:E2F8 hetero-dimers, and E2F8:E2F8 homo-dimers appeared to be the least preferred state (E2F7:E2F7 > E2F7:E2F8 > E2F8:E2F8). While the molecular basis for homo- versus hetero-dimerization is not yet clear, these data suggest that inefficient homo-dimerization may compromise the ability of E2F8 to compensate for the loss of E2F7, an effect that might be aggravated in circumstances of limiting amounts of E2F8 (i.e. *E2f7<sup>-/-</sup>E2f8<sup>+/-</sup>*). Thus, the observed bias for E2F7 homo-dimerization may explain the stricter postnatal requirement for this subunit. While this interpretation is likely an

oversimplification, these results do provide a molecular explanation for their functional redundancy in development. It will be interesting to investigate how dimerization might be impacted by the levels of E2F7 and E2F8 proteins *in vivo* or by tissue-specific signals.

Because of the intense interest in E2Fs as major regulators of the cell cycle and apoptosis, individual E2F family members, including *E2f1* through *E2f6*, have been extensively studied *in vivo* by gene targeting approaches in mice. With the exception of *E2f3* knockout mice, defects in embryos deficient for each of the known E2Fs are rather subtle (Field et al., 1996; Yamasaki et al., 1996; Lindeman et al., 1998; Humbert et al., 2000a; Murga et al., 2001; Storre et al., 2002; Rempel et al., 2004). Even the disruption of *E2f3* when in a mixed genetic background yields viable mice (Humbert et al., 2000b; Wu et al., 2001). The virtual absence of cell proliferation and apoptotic defects in E2F deficient embryos has raised questions about the physiological importance of these factors, leaving the impression that E2Fs must either not be critical for the control of these processes *in vivo* or that there is sufficient functional redundancy among family members to accommodate for a deficiency in any single member. Previous tissue culture experiments have provided evidence for functional redundancy among E2F1-3 activators and E2F4-6 repressors (Gaubatz et al., 2000; Wu et al., 2001; Giangrande et al., 2004) in the control of proliferation. The current work underscores functional redundancy among E2F7 and E2F8 in the control of apoptosis *in vivo*.

Too much or unrestrained E2F activity can also compromise cell homeostasis. The importance of restraining E2F activity is highlighted by the fact that at least four independent mechanisms have evolved to achieve this. One such repressive mechanism involves the binding and inhibition of E2F proteins by the Rb tumor suppressor. Indeed, disruption of Rb leads to unregulated proliferation and ectopic apoptosis that is partly suppressed by the concomitant loss of E2F activators (Ziebold et al., 2001; Saavedra et al., 2002). A second mechanism involves binding of cyclinA/cdk2 to E2F1, E2F2 and E2F3a proteins during S phase, leading to the phosphorylation of their dimerization partners DP1/2 and the inhibition of E2F/DP DNA binding activity (Dymlacht et al., 1994). A third mechanism involves the p45SKP2/F-box-dependent degradation of the three E2F activators during S phase (Leone et al., 1998; Marti et al., 1999). Here we described a fourth repressive mechanism to keep E2F activity in control that involves direct transcriptional repression of *E2f1* by E2F7 and E2F8. In contrast to other repressor E2Fs (E2F3b, E2F4-6), which play a predominant role in silencing gene expression in G<sub>0</sub>-G<sub>1</sub>, these two novel E2Fs may have a particularly important role in repressing E2F targets as cells transit through S phase and into G<sub>2</sub>. Gene expression analysis in synchronized *E2f7<sup>-/-</sup>E2f8<sup>-/-</sup>* MEFs revealed that *E2f1* mRNA levels, as well as that of many other E2F-target genes, increased acutely during S and G<sub>2</sub>. Thus, by participating in the repression of E2F-target genes as cells transit through S-G<sub>2</sub>, E2F7 and E2F8 represent the first transcriptional mechanism described in mammals that contributes directly to the downswing in the oscillating pattern of cell cycle-specific gene expression.

*In vitro* and *in vivo* experiments described here provide clear-cut evidence for a role of E2F7 and E2F8 in the control of apoptosis that involves, at least in part, the regulation of *E2f1* expression. Genetic inactivation of *E2f7* and *E2f8* resulted in the accumulation of *E2f1* mRNA and a corresponding increase in its protein product. Consistent with a direct role in repression, ChIP experiments showed that E2F7 and E2F8 are recruited to the *E2f1* promoter and that this requires intact DNA binding activity. The increase of E2F1 protein levels in *E2f7<sup>-/-</sup>E2f8<sup>-/-</sup>* cells coincided with the accumulation of p53 protein. Several mechanisms of how E2F1 may lead to the accumulation and activation of p53 have been described (Hsieh et al., 2002; Pomerantz et al., 1998; Rogoff et al., 2002; Russell et al., 2002). The increase of E2F1 and p53 in *E2f7<sup>-/-</sup>E2f8<sup>-/-</sup>* cells is of physiological significance since ablation of either *E2f1* or *p53* suppressed the widespread apoptosis observed in DKO embryos. The fact that these TKO embryos still died suggests that apoptosis in *E2f7<sup>-/-</sup>E2f8<sup>-/-</sup>* embryos is not simply due to an

indirect consequence associated with embryonic lethality but rather due to a specific signal emanating from deregulated E2F1. These results also indicate that additional targets repressed by E2F7 and E2F8 must be involved in the many pathologies that likely underlie the lethality of DKO embryos. Indeed, profiling of global gene expression in DKO embryos confirmed the deregulation of many additional genes. Interestingly, a substantial portion of these included gene products known to be involved in various responses to stress such as hypoxia, nutrient deprivation and apoptosis. We view these results to mean that physiological stress, whether induced by vascular or other defects in DKO embryos or by the addition of DNA damaging agents in DKO MEFs, exacerbates the activation of the E2F1-p53 axis and unleashes a massive apoptotic response.

In summary, we show that E2F7 and E2F8 are essential for embryonic development. Their synergistic function may be viewed as a distinct arm of the E2F network involved in repression of transcription during S-G<sub>2</sub>, where *E2f1* represents a particularly critical target that if not appropriately repressed can illicit widespread apoptosis in developing embryos.

## Experimental Procedures

### Generation of *E2f7* and *E2f8* knockout mice

*E2f7* and *E2f8* specific probes were used to screen a 129Sv/Ev bacterial artificial chromosome library. A 7.0 kb *Xba*I fragment of *E2f7* spanning exon 4 and 5 was isolated from *RPC1 22 431 H17* and a 6.6 kb fragment of *E2f8* containing exon 3 and 4 was isolated from *RPC1 22 539 P23*. Standard cloning techniques were used to generate *E2f7* and *E2f8* targeting vectors, which were confirmed by direct sequencing. Targeting vectors were linearized with *Not*I and electroporated into TC1 129Sv/Ev embryonic stem (ES) cells. ES cells were selected in medium containing G418 plus ganciclovir and correct recombination was confirmed by Southern blots using the indicated probes in Figure 1A. Selected ES clones were injected into C57BL/6 blastocysts to generate chimeric mice, which were bred with Black Swiss females to obtain germline transmission. Appropriate offspring were then bred to *EIIa-Cre* mice (Lakso et al., 1996) to produce mice with conventional and conditional null alleles as illustrated in Figure 1A. Genotypic analysis of offspring was performed by Southern blot analysis and multiplex PCR using allele-specific primers described in Figure S7.

### Real-time RT-PCR

Total RNA was isolated using Qiagen RNA miniprep columns as described by the manufacturer, which included the optional DNase treatment before elution from columns. Reverse transcription of total RNA was performed using Superscript III reverse transcriptase (Invitrogen) and RNase Inhibitor (Roche) according to the manufacturer's protocol. Real-time PCR was performed using a BioRad iCycler and reactions were performed in triplicate and relative amounts of cDNA were normalized to GAPDH. The *E2f7* and *E2f8* real-time primers are located within the deleted regions of *E2f7* and *E2f8* respectively. Primer sequences for the indicated genes are included in Figure S7.

### Whole mount *in situ* hybridization

*Wild type* E9.5 day-old embryos were harvested and fixed in 4% paraformaldehyde. Whole mounts were performed as described (Riddle et al., 1993). Probes for *E2f7* and *E2f8* were generated by PCR amplification (see Figure S7). The resulting PCR products were subcloned into pBluescript KS (Stratagene) and pGEM-TEasy vector (Promega), respectively. Sense and antisense RNA probes were transcribed from the appropriate promoters using T3 and T7 RNA polymerase (Roche).



### Affymetrix microarray analysis

Total RNA was prepared from E10.5 embryos using the RNasy Kit (Qiagen) and included the optional DNase treatment before elution from purification columns. RNA was processed as described in [www.osucc.osu.edu/microarray/](http://www.osucc.osu.edu/microarray/) and hybridized to oligonucleotide microarrays (Mouse Genome 430 2.0 array, Affymetrix, Santa Clara, CA). Scanned image files were quantified with GENECHIP 3.2 (Affymetrix). The Raw gene expression data is presented in Supplemental Figure S8. Genes that increased or decreased at least 3-fold ( $p < 0.008$ ) in DKO samples relative to *wild type* samples were used to generate the heatmaps illustrated in Figure 6A.

### Co-immunoprecipitation assays (Co-IP)

Human embryonic kidney (HEK) 293 cells were cultured in DMEM medium supplemented with 15% FBS and used for co-immunoprecipitation assays. Transient transfections with the indicated constructs were performed by standard calcium chloride techniques. Cells were harvested and washed in PBS at 4°C and cell pellets were lysed in 10 volumes of lysis buffer (0.05M sodium phosphate pH7.3, 0.3M NaCl, 0.1% NP40, 10% glycerol with protease inhibitor cocktail, Roche). Co-IP was performed as described before (Maiti et al., 2005).

### Immuno-affinity purification (IAP)

HeLa cells were transduced with retroviruses expressing a bicistronic mRNA encoding human E2F7 or E2F8 (flag-HA-7 and flag-HA-8) and interleukin-2 receptor (IL-2R)- $\alpha$  (Ogawa et al., 2002). The transduced population was incubated with magnetic beads linked to IL-2R specific antibodies. E2F7/8-IL-2R expressing cells were then enriched by separating magnetic beads-bound cells from non-bound cells using a magnetic plate. Nuclear extracts from enriched E2F7/8 expressing cells were prepared by a modified Dignam preparation protocol using Buffer C-450 (20mM HEPES, pH 7.9, 450 mM KCl, 1.5 mM MgCl<sub>2</sub>, 25% glycerol, 0.2mM EDTA) (Dignam et al., 1983). Resulting extracts were incubated with pre-equilibrated flag M2 affinity resin (A2220, Sigma) for 3h. Resin/extract mixtures were decanted onto Bio-Spin columns (BioRad) and allowed to empty by gravity. After extensive washing with Wash Buffer (20mM HEPES, pH 7.9, 150mM KCl; 10% glycerol, 0.2mM EDTA), complexes were eluted by incubation with 200 $\mu$ g/mL of flag peptide (F3290, Sigma). The flag eluents were subsequently subjected to Western blot analysis using anti-E2F8 antibody (M01, Abnova). All nuclear extraction and immuno-affinity purification steps were performed at 4°C and fresh protease inhibitors were added to all working solutions.

### Chromatin immunoprecipitation (ChIP) and sequential ChIP assays

For ChIP assays, the EZ CHIP™ assay kit (Upstate) was used as described by the manufacturer. Briefly, harvested HEK 293 cells overexpressing flag-E2F7 and/or flag-E2F8 were crosslinked and chromatin was sonicated to an average size of 200-1000 bp. Lysates were subsequently pre-cleared with Salmon Sperm DNA/Protein G agarose slurry. Antibodies specific to flag, HA, or normal mouse IgG (Oncogene) were then added to each sample and incubated overnight at 4°C. Antibody-protein-DNA complexes were recovered by addition of 30  $\mu$ l of Salmon Sperm DNA/Protein G agarose slurry and incubation for 1h at 4°C. Following extensive washing, the complexes were eluted and decrosslinked at 65°C for 4h. Finally, samples were treated with Proteinase K (Roche) and Rnase A (Roche) and purified through Qiaquick columns (Qiagen). Real-time PCR quantification of immunoprecipitated DNA was performed using the Biorad iCycler machine with primers specific for the indicated promoter regions. All primer sequences are listed in Figure S7. Reactions were performed in triplicate and normalized to 1% of the total input.

Sequential ChIP-PCR was carried out similarly using the same EZ CHIP™ protocol, except two rounds of chromatin immunoprecipitation steps were performed. The first round of immunoprecipitation was performed by incubation of extracts with M2 anti-flag antibody and Salmon Sperm DNA/Protein G agarose slurry. Precipitated DNA-protein complexes were eluted with excess flag peptide (200µg/mL) for 1h at 4°C and then subjected to a second round of precipitation with primary antibodies as indicated in Figure 3H-J. After extensive washing, sequentially precipitated complexes were recovered by addition of 30µl of Salmon Sperm DNA/Protein G agarose slurry and incubation for another 1h at 4°C. Protein-DNA complexes collected from sequential ChIP were treated and subjected to real-time PCR analysis as described above for ChIP experiments.

### Western blot and antibodies

Immunoblot analyses were performed by standard procedures using ECL reagents as described by the manufacturer (Amersham Biosciences). The following commercial antibodies were used as indicated in the figures: flag (M2, Sigma), HA (12C5A, Roche), Myc (9E10, Santa Cruz), E2F1 (C-20, Santa Cruz), tubulin (T6199, Sigma), caspase-3 (9662, Cell Signaling), p53 (Ab-1, Oncogene).

### Cell culture and viability assay

*E2f7<sup>loxP/loxP</sup>E2f8<sup>loxP/loxP</sup>* mouse embryo fibroblasts (MEFs) were isolated from E13.5 embryos and maintained in DMEM medium containing 15% fetal bovine serum (FBS). Immortalized cell lines were generated from primary MEFs using the standard 3T9 protocol. Immortalized MEFs were treated with retrovirus expressing *cre* recombinase using standard methods (Wu et al., 2001).

Apoptosis was measured in MEFs at the indicated times following treatment with 10µm cisplatin (Sigma) or 20µm camptothecin (Sigma) for 18h. Cell viability was determined by trypan blue exclusion.

### Reporter assay

Luciferase reporter assays were performed in triplicate as described previously (de Bruin et al., 2003). Briefly, *cre*-treated *wild type* and *E2f7<sup>loxP/loxP</sup>E2f8<sup>loxP/loxP</sup>* MEFs were transfected with *E2f1-Luc* reporter plasmid (Araki et al., 2003), along with a thymidine kinase renilla luciferase construct as an internal control. Cells were harvested and luciferase activity was detected using dual luciferase system (Promega) following the manufacturer's protocol. *E2f1-Luc* reporter plasmid has been described previously.

### FACS analysis

*Cre*-treated *wild type* and *E2f7<sup>loxP/loxP</sup>E2f8<sup>loxP/loxP</sup>* MEFs were synchronized by starvation in DMEM containing 0.2% FBS for 48h and blocked at the G<sub>1</sub>/S transition by the addition of DMEM containing 15% FBS with 1mM hydroxyurea (HU) for 18h. Cells were then washed 3 times with PBS and incubated with fresh medium containing 15% FBS. Cells were harvested at the indicated time points and fixed in 70% ethanol, followed by incubation in propidium iodide and analyzed by flow cytometry using standard methods.

### BrdU and TUNEL assays

Pregnant females at 9.5 days postcoitum were injected intraperitoneally with BrdU (100µg/grams of body weight) 30min prior to harvesting. Embryos were fixed in formalin and 5µm paraffin embedded-sections were used for immunohistochemistry. After deparaffinization, anti-BrdU antibody (MO-0744, DAKO) and Vectastain Elite ABC reagents (Vector labs) were used to detect BrdU incorporation according to the manufacturer's instructions. Apoptotic cells

were detected using TUNEL (S7101, Chemicon) assays, performed according to the manufacturer's protocol. All slides were counterstained with hematoxylin.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

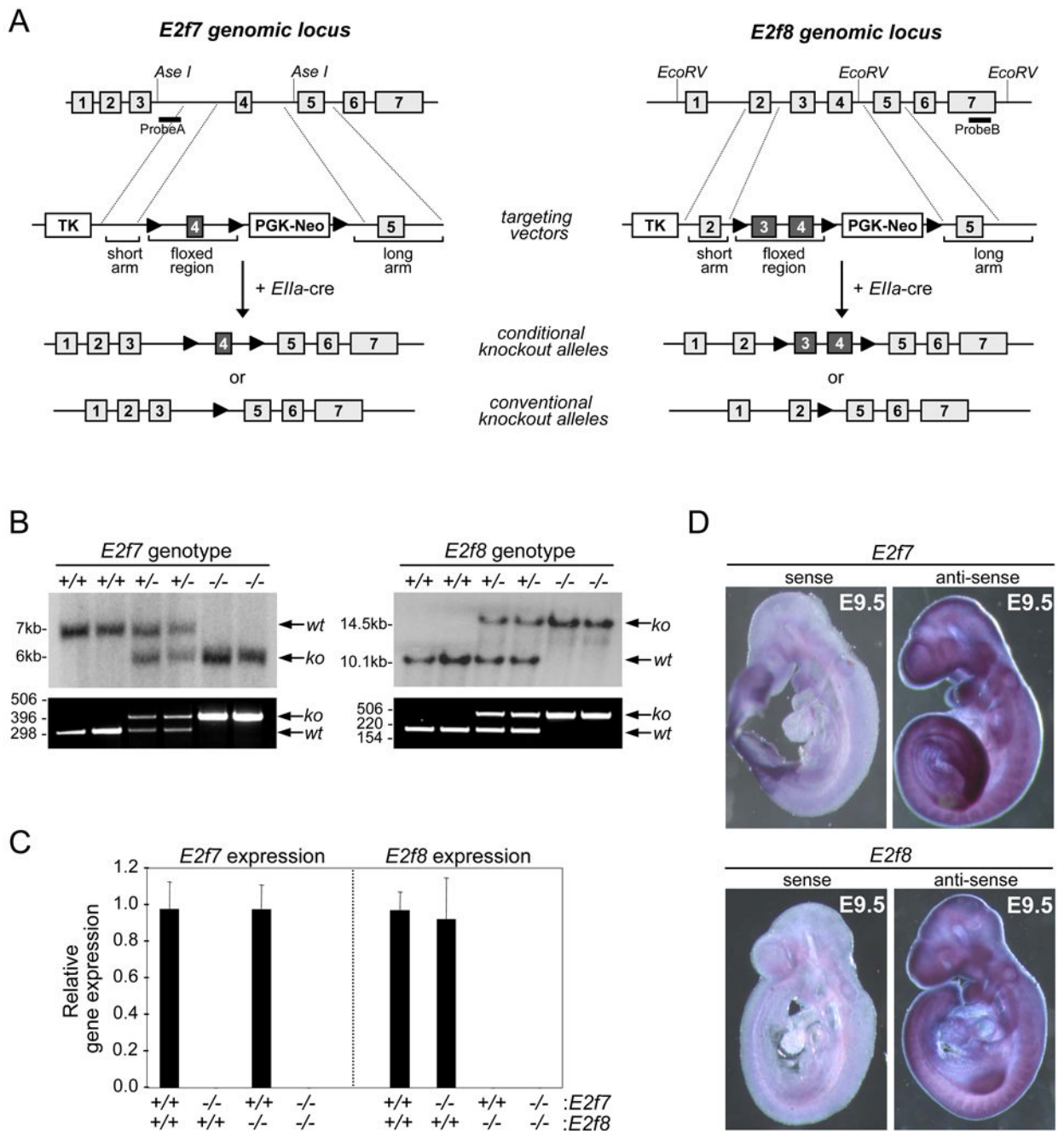
We are grateful for technical assistance provided by J. Moffitt and J. Opavska. We also thank members of the Leone lab for helpful suggestions and Pam Wenzel for critical reading of the manuscript. This work was funded by NIH grants to G.L. (R01CA85619, R01CA82259, R01HD047470, P01CA097189), NIH grant to M.W. (R01HD42619) and DoD award to A.d.B. (BC030892). G.L. is the recipient of The Pew Charitable Trust Scholar Award and the Leukemia & Lymphoma Society Scholar Award.

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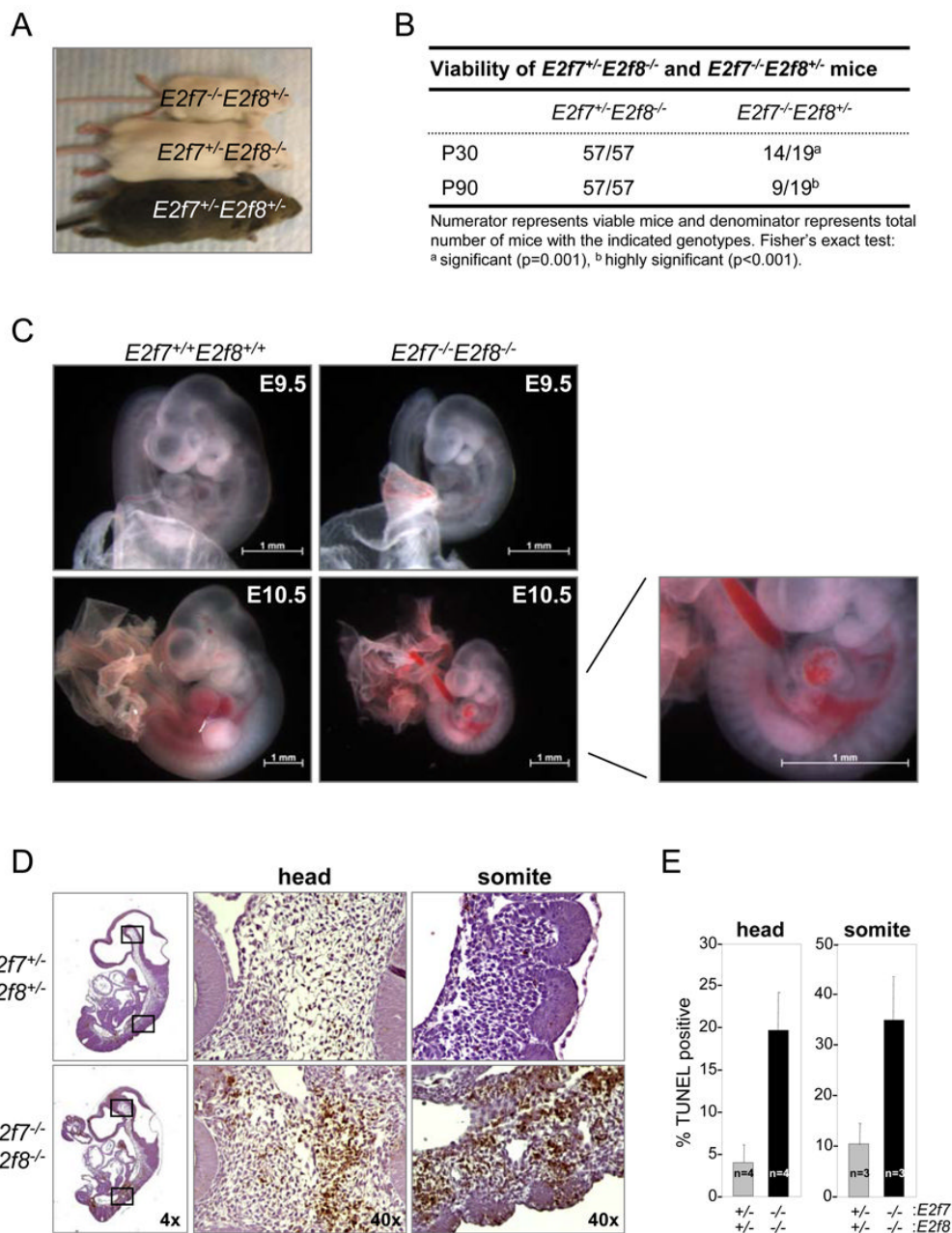
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**Figure 1.**

Generation of *E2f7* and *E2f8* knockout mice. **(A)** Targeting strategies used to inactivate *E2f7* (left) and *E2f8* (right). Top panels: partial exon/intron structures of the *E2f7* and *E2f8* genes. The black bars indicate the position of probes used for Southern analysis. Middle panels (targeting vectors): position of the TK and Neo cassettes, as well as loxP sites (filled triangles) are indicated. Bottom two panels (conditional knockout alleles and conventional knockout alleles): illustrate the two desired alleles resulting from possible recombination events. **(B)** Top panels: Southern analysis of genomic DNA isolated from conventional knockout mice using *AseI* for *E2f7* (left) and *EcoRV* for *E2f8* (right), and hybridized using probes A and B, respectively. Bottom panels: genotyping of tail DNA was performed using allele-specific PCR

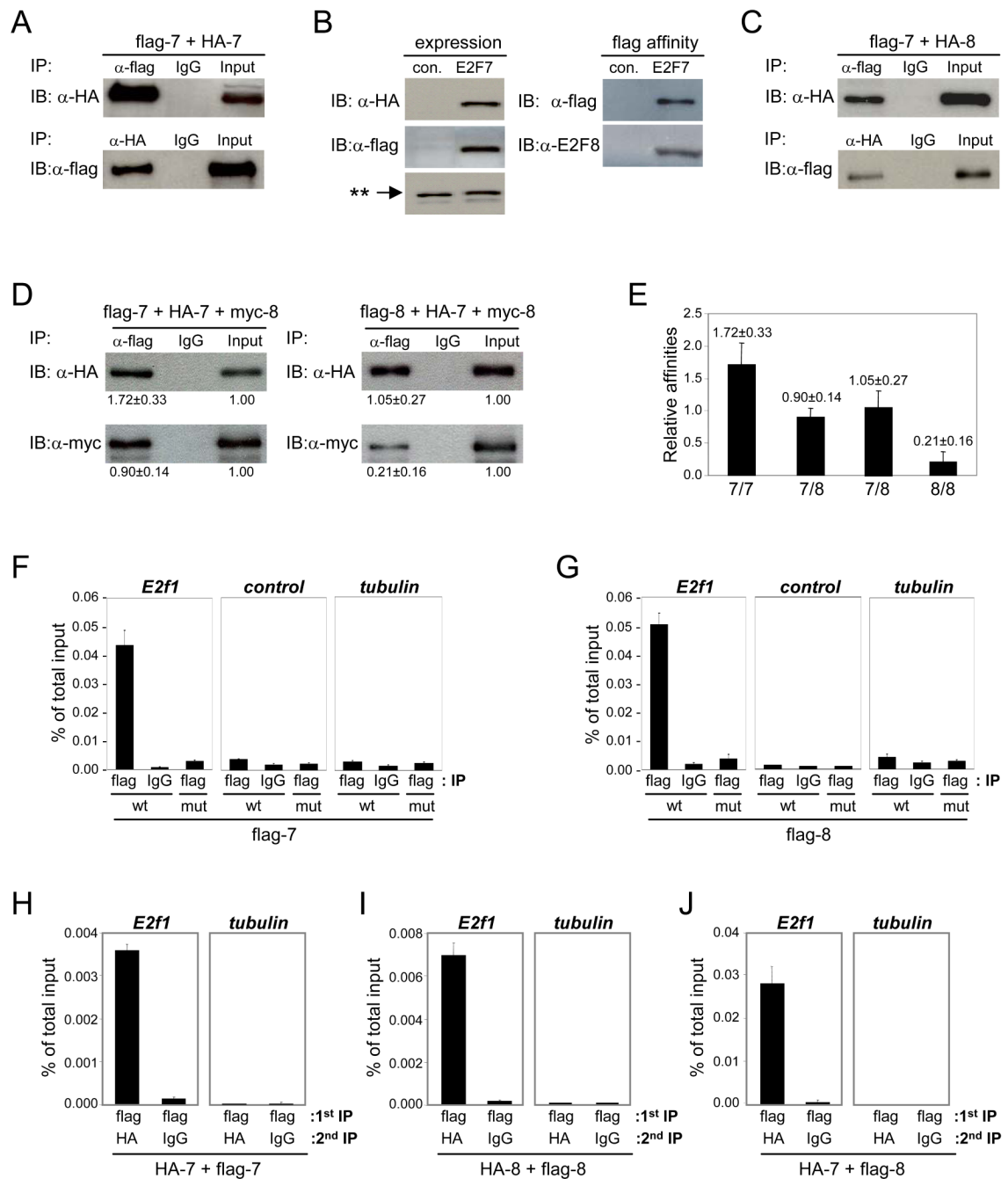
primers. **(C)** Real-time RT-PCR analysis of *E2f7* or *E2f8* expression in embryos with the indicated genotypes using primers described in Figure S7. **(D)** *Wild type* E9.5 embryos were subjected to whole mount *in situ* hybridization using sense and antisense probes specific to *E2f7* (top panels) and *E2f8* (bottom panels).



**Figure 2.** Global deletion of  $E2f7$  and  $E2f8$  results in developmental delay, vascular defects and widespread apoptosis *in vivo*. (A) Micrograph of  $E2f7^{-/-}E2f8^{+/-}$ ,  $E2f7^{+/-}E2f8^{-/-}$  and  $E2f7^{-/-}E2f8^{+/-}$  mice at 21 days of age. (B) Tabulated number of  $E2f7^{+/-}E2f8^{-/-}$  and  $E2f7^{-/-}E2f8^{+/-}$  mice that live until 30 (P30) and 90 (P90) days of age. (C) Gross pictures of  $E2f7$  and  $E2f8$  double knockout embryos at E9.5 (top panels) and E10.5 (bottom panels). The right panel is a higher magnification view of the vascular defects in E10.5  $E2f7^{-/-}E2f8^{-/-}$  embryos. (D) E9.5 embryos with the indicated genotypes were analyzed by TUNEL assays. Far left panels: low magnification pictures of whole embryos. Right two panels: high magnification images of representative areas demarcated by the box in the low magnification images. (E) Quantification of TUNEL-positive cells in the indicated tissue areas are presented

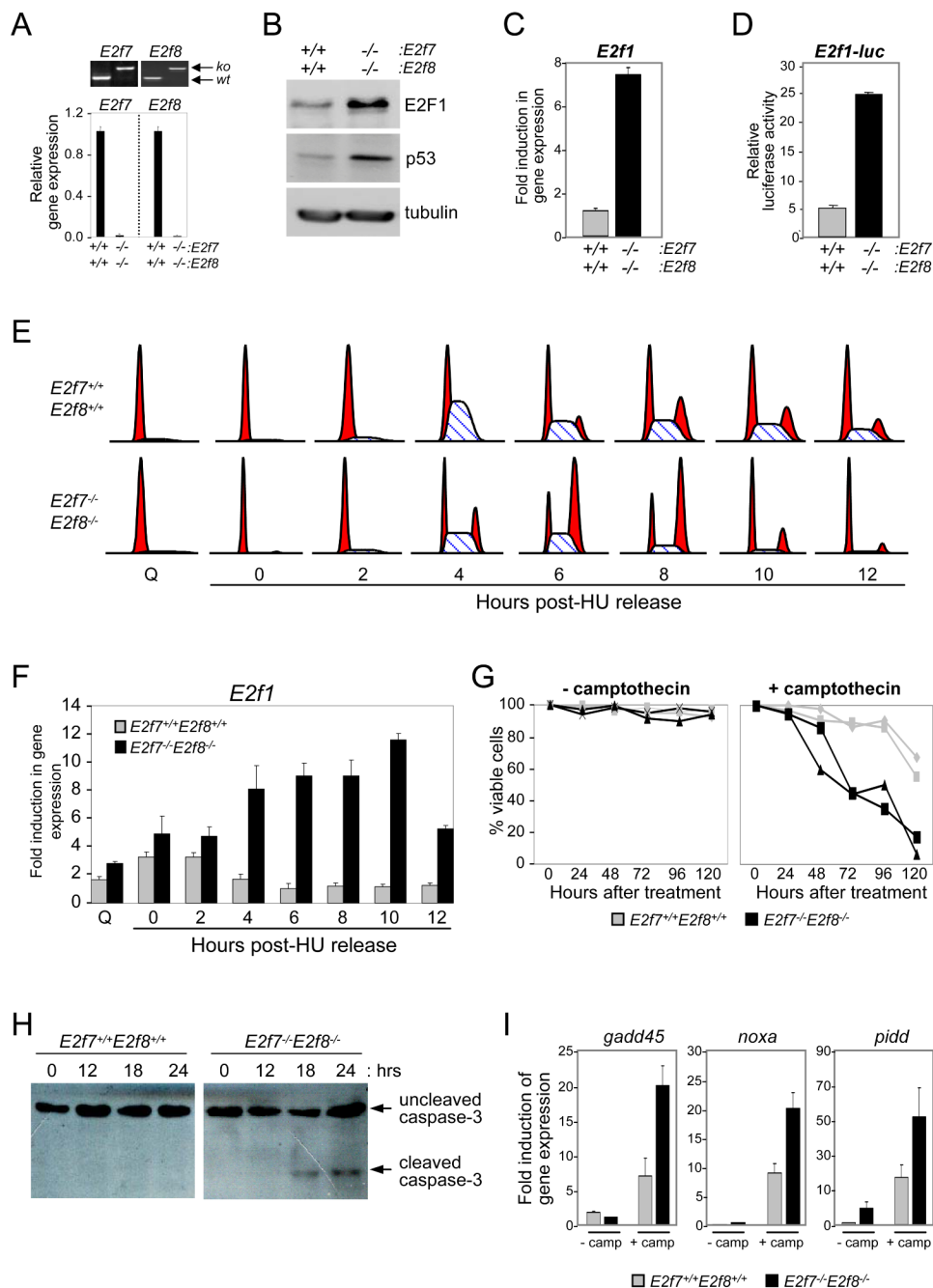


as the average  $\pm$  SD percentage of cells that are TUNEL-positive. Three sections per embryo and at least three different embryos for each genotype were analyzed.

**Figure 3.**

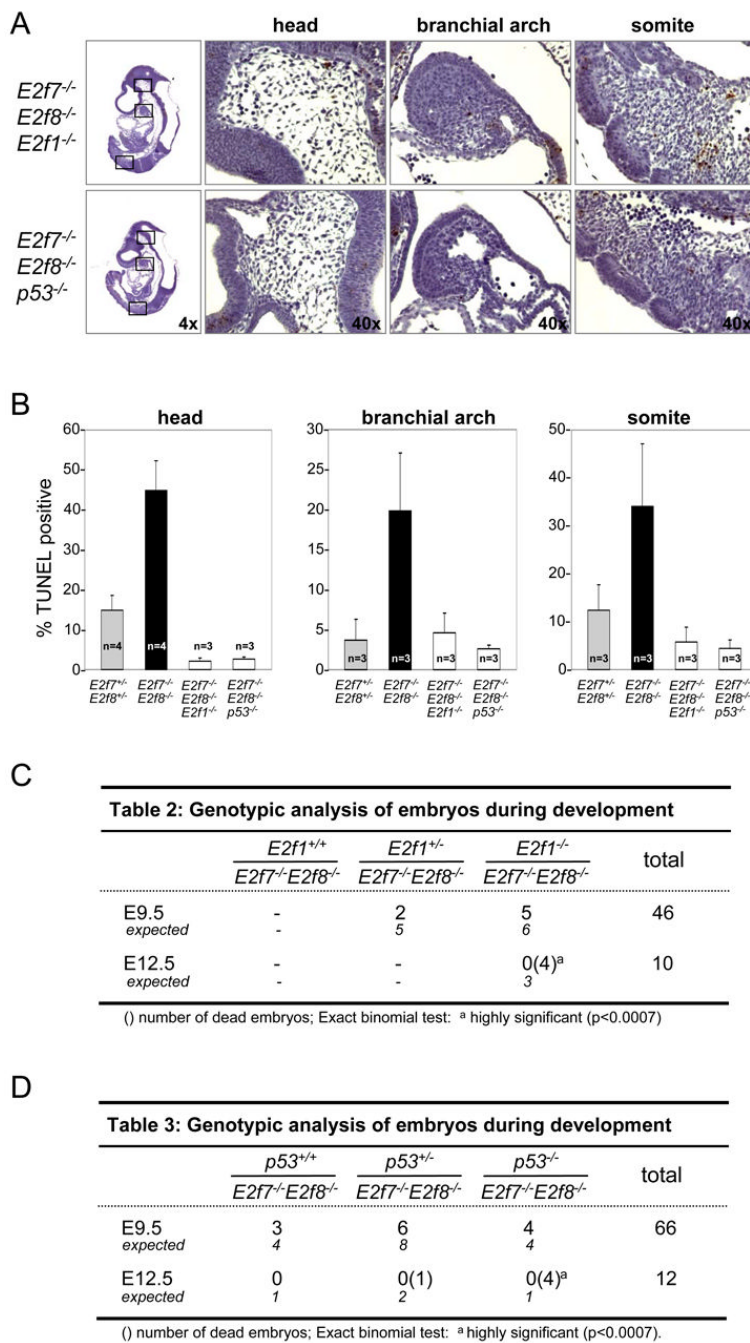
E2F7 and E2F8 homo- and hetero-dimerize. **(A)** Homo-dimerization of E2F7. Lysates from cells transfected with both flag-tagged E2F7 (flag-7) and HA-tagged E2F7 (HA-7) were immunoprecipitated (IP) and immunoblotted (IB) with anti-flag and anti-HA antibodies as indicated. Immunoprecipitation with normal mouse IgG was used as a negative control. **(B)** Left panels: stable expression of flag-HA-E2F7 in HeLa cells. Flag-HA-E2F7 transduced HeLa cell lysates were subjected to Western blotting. The presence of a non-specific protein indicated by (\*\*\*) was used as a loading control. Right panels: Endogenous E2F8 associates with flag-HA-E2F7. Nuclear extracts from flag-HA-E2F7 expressing cells were immunoprecipitated with flag antibody affinity resins as described in Experimental Procedures and eluents were

immunoblotted with E2F8-specific antibodies. Mock-transduced HeLa cells (con.) were used as a negative control. **(C)** Hetero-dimerization of E2F7 and E2F8. Lysates derived from cells overexpressing both flag-E2F7 and HA-E2F8 were immunoprecipitated (IP) with anti-flag antibodies and immunoblotted (IB) with anti-HA antibodies (top panel) or *vice versa* (bottom panel). Immunoprecipitation with normal mouse IgG was used as a negative control. **(D)** Kinetic analysis of dimerization. HEK 293 cells overexpressing the indicated constructs were subjected to anti-flag immunoprecipitation (IP), followed by anti-HA or anti-myc immunoblotting (IB) as indicated. The amount of detected E2F7 and E2F8 was measured by densitometry and quantified relative to 1% of the input material. The relative levels indicated at the bottom of each lane are presented as the average  $\pm$ SD of 3 independent experiments where Input is always equal to 1.00. **(E)** Bar graph presentation of the kinetic analysis of dimerization shown in (D). **(F)** E2F7 binds to the *E2f1* promoter. Chromatin from cells overexpressing *wild type* flag-E2F7 (wt) or flag-E2F7DBD1,2 (mut) was immunoprecipitated (IP) with anti-flag or IgG control antibodies. Immunoprecipitated DNA was amplified using primers specific for the *E2f1* promoter (*E2f1*), irrelevant sequences in exon 1 of *E2f1* and the *tubulin* promoter (*control* and *tubulin*, respectively). **(G)** E2F8 binds to the *E2f1* promoter. Cells overexpressing *wild type* flag-E2F8 (wt) or flag-E2F8DBD1,2 (mut) was immunoprecipitated (IP) with anti-flag or normal mouse IgG antibodies. Immunoprecipitated DNA was amplified using primers specific for the *E2f1* promoter (*E2f1*), exon 1 of *E2f1* (*control*) and the *tubulin* promoter (*tubulin*). **(H-J)** Homo-dimers and hetero-dimers of E2F7 and E2F8 occupy the *E2f1* promoter. Cell extracts expressing ectopic HA-E2F7 and flag-E2F7 **(H)**, HA-E2F8 and flag-E2F8 **(I)**, HA-E2F7 and flag-E2F8 **(K)** were used for sequential ChIP assays as described in Experimental Procedures. Antibodies used for the first and second round of immunoprecipitation are indicated (1<sup>st</sup> IP and 2<sup>nd</sup> IP, respectively). Immunoprecipitated DNA collected after two rounds of ChIP was amplified using primers specific for the *E2f1* promoter (*E2f1*) or for the *tubulin* promoter (*tubulin*). For all the ChIP experiments, real-time PCR was performed in triplicate and cycle numbers were normalized to 1% of the input DNA.

**Figure 4.**

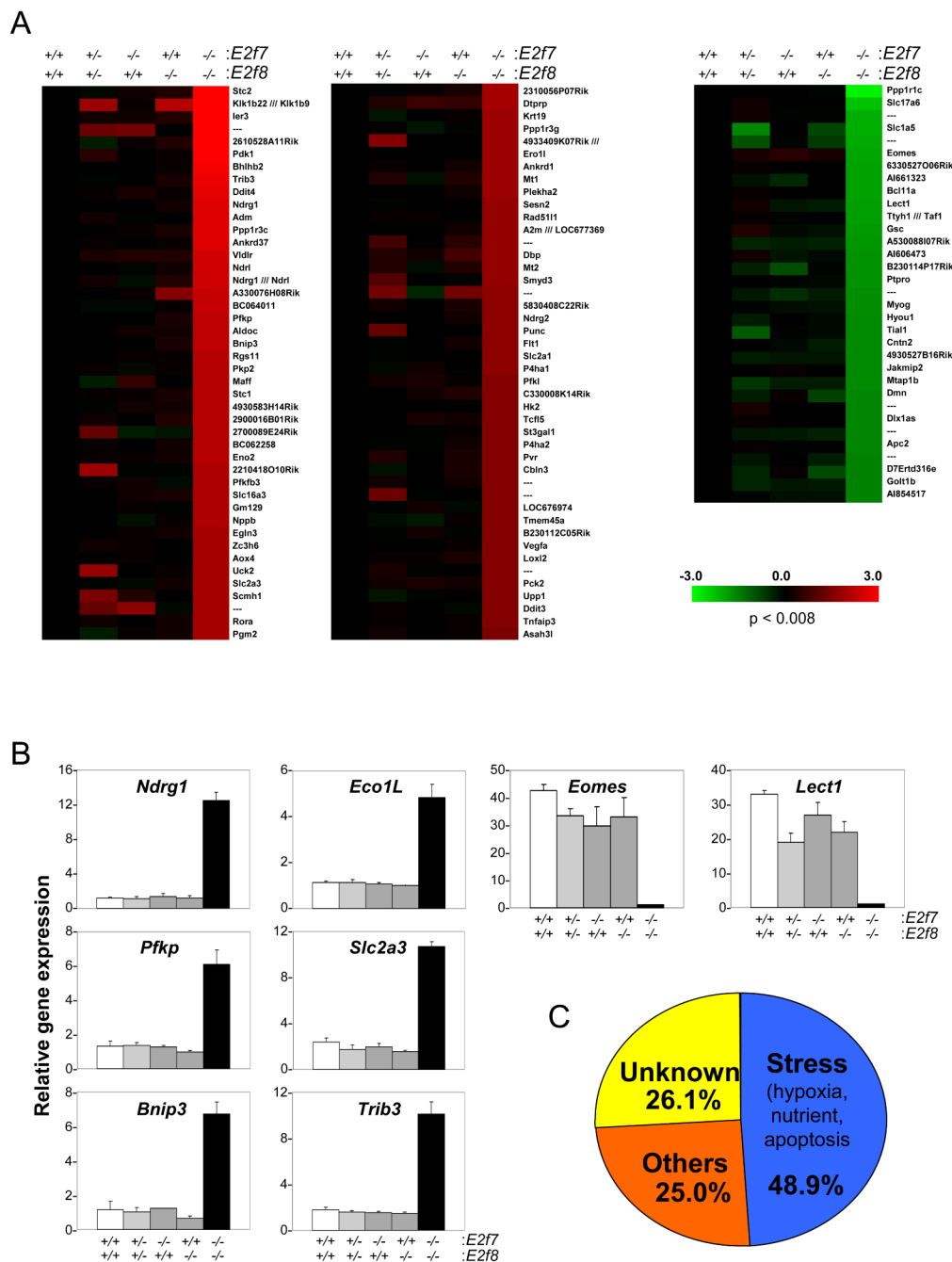
(A-F) Deregulation of *E2f1* and *p53* expression in MEFs deficient in *E2f7* and *E2f8*. (A) Top panels: PCR genotyping analysis of DNA isolated from *E2f7*<sup>+/+</sup>*E2f8*<sup>+/+</sup> and *E2f7*<sup>loxP/loxP</sup>*E2f8*<sup>loxP/loxP</sup> MEFs treated with *cre*-expressing retroviruses. The knockout (*ko*) and wild type (*wt*) alleles are indicated. Bottom graphs: Real-time RT-PCR analysis of *E2f7* and *E2f8* expression in cells. For convenience, *cre*-deleted *loxP* alleles were labeled as (-/-) at bottom of graphs. (B) Western blot analysis of lysates described in (A) using antibodies specific for E2F1 and p53 as indicated. Tubulin-specific antibodies were used as an internal loading control. (C) Expression of *E2f1* in MEFs treated as in (A) was measured by real-time RT-PCR. (D) Cells treated as in (A) were co-transfected with the *E2f1* firefly luciferase and thymidine

kinase renilla luciferase reporter plasmids (internal control). Relative *E2f1-luc* reporter activity was normalized to renilla luciferase activity. A representative of at least three independent experiments is shown. **(E)** FACS analysis of synchronized cre-treated *E2f7<sup>+/+</sup>E2f8<sup>+/+</sup>* and *E2f7<sup>loxP/loxP</sup>E2f8<sup>loxP/loxP</sup>* MEFs. For convenience, cre-deleted *loxP* alleles were labeled as (-/-). MEFs were synchronized by serum deprivation and hydroxyurea (HU) block as described in Experimental Procedures. At the indicated time points, cells were harvested and stained by propidium iodide for flow cytometry. Q: quiescent cells kept in serum-deprived medium. **(F)** Total RNA isolated from same synchronized MEFs samples as in (E) was analyzed by real-time RT-PCR assays specific for *E2f1* expression. **(G-I)** MEFs deficient in *E2f7* and *E2f8* are hypersensitive to DNA damage induced apoptosis. **(G)** Cre-treated *E2f7<sup>+/+</sup>E2f8<sup>+/+</sup>* and *E2f7<sup>loxP/loxP</sup>E2f8<sup>loxP/loxP</sup>* MEFs were mock treated with DMSO (-camptothecin) or with camptothecin (+camptothecin). At the indicated intervals cells were harvested and counted in triplicate as described in Experimental Procedures. The percentages of viable cells at the indicated time points are presented. **(H)** Lysates derived from MEFs treated as in (G) were analyzed at indicated time points by Western blotting using caspase-3 specific antibodies. **(I)** Total RNA was extracted from cells treated for 18h as in (G) and expression of the indicated p53 target genes was measured by real-time RT-PCR.

**Figure 5.**

Loss of  $E2f1$  or  $p53$  suppresses apoptosis in  $E2f7^{-/-} E2f8^{-/-}$  embryos. **(A)** Micrographs of TUNEL stained E9.5  $E2f7^{-/-} E2f8^{-/-} E2f1^{-/-}$  (top panels) and  $E2f7^{-/-} E2f8^{-/-} p53^{-/-}$  embryos (bottom panels). Far left panels: low magnification pictures of whole embryos. Right three panels: high magnification images of representative areas demarcated by the box in the low magnification images. **(B)** Quantification of TUNEL-positive cells in the indicated tissue areas are presented as the average  $\pm$  SD percentage of cells that are TUNEL-positive. Three sections per embryo and at least three different embryos for each genotype were analyzed. For comparison purposes, data derived from  $E2f7^{+/+} E2f8^{+/+}$  and  $E2f7^{-/-} E2f8^{-/-}$  in Figure 2E, S1C was included. **(C)** Genotypic analysis of embryos derived from  $E2f1^{+/+} E2f7^{+/+} E2f8^{-/-}$

intercrosses at the indicated stages of development. **(D)** Genotypic analysis of embryos derived from  $p53^{+/-}E2f7^{+/-}E2f8^{-/-}$  intercrosses at the indicated stages of development.



**Figure 6.** Microarray analysis of E10.5 embryos. **(A)** Heat maps of probe sets in Affymetrix microarrays that showed at least a 3-fold induction (left and middle) or a 3-fold reduction (right) of expression in  $E2f7^{-/-}E2f8^{-/-}$  relative to *wild type* embryos. Data are presented as the medium of 4 embryos from *wild type*,  $E2f7^{-/-}E2f8^{+/+}$  and  $E2f7^{+/+}E2f8^{-/-}$  genotype groups, and the medium of 2 embryos from  $E2f7^{+/+}E2f8^{+/+}$  and  $E2f7^{-/-}E2f8^{-/-}$  genotype groups. Red and green indicate induction and reduction respectively. Probe sets representing genes of interest are indicated. **(B)** The expression of 8 target genes (6 up-regulated and 2 down-regulated) was evaluated by real-time RT-PCR assays. **(C)** Pie chart diagrams illustrate the non-random distribution of the stress-related genes among the total 88 up-regulated genes in DKO embryos.



Table 1

**Genotypic analysis of embryos during development**Genotypic analysis of embryos derived from  $E2f7^{+/-}E2f8^{+/-}$  or  $E2f7^{+/-}E2f8^{-/-}$  intercrosses at the indicated stages of development.

	$E2f7^{+/+}$				$E2f7^{+/-}$				total	
	$E2f8^{+/+}$	$E2f8^{+/-}$	$E2f8^{+/-}$	$E2f8^{+/+}$	$E2f8^{+/+}$	$E2f8^{+/-}$	$E2f8^{+/-}$	$E2f8^{-/-}$		
E9.5 <i>expected</i>	6 5	16 28	29 22	16 19	72(2) 75	53(1) 56	12 13	58(1) 47	33 34	299
E10.5 <i>expected</i>	9 7	21 20	13 13	7(1) 16	45 43	28(1) 28	4(1) 8	28(1) 23	6(7) <sup>a</sup> 14	172
E11.5 <i>expected</i>	- -	6 5	4 5	2 3	15(2) 16	14(2) 13	2(1) 3	8(1) 10	0(5) <sup>b</sup> 8	62
E12.5 <i>expected</i>	- -	- -	3 5	- -	4 4	17(2) 15	- -	3 4	0(9) <sup>b</sup> 10	38
P0 <i>expected</i>	7 8	22 18	16 10	24 18	45 39	17 21	5 10	18 21	0 <sup>b</sup> 11	154

() number of dead embryos recovered; Exact binomial test:

<sup>a</sup> significant (p=0.0015)<sup>b</sup> highly significant (p<0.0007).