HHS Public Access

Fas-activated mitochondrial apoptosis culls stalled embryonic stem cells to promote differentiation

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SUMMARY

The intrinsic (mitochondrial) apoptotic pathway is a conserved cell death program crucial for eliminating superfluous, damaged, or incorrectly specified cells [1], and the multi-domain pro-death BCL-2 family proteins BAX and BAK are required for its activation [2, 3]. In response to internal damage or developmental signals, BAX and/or BAK permeabilize the mitochondrial outer

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membrane [4, 5], resulting in cytochrome *c* release and activation of effector caspases such as Caspase-3 (Casp3) [6]. While the mitochondrial apoptotic pathway plays a critical role during late embryonic development in mammals [3], its role during early development remains controversial [7, 8]. Here, we show that $Bax^{-/-}Bak^{-/-}$ murine embryonic stem cells (ESCs) display defects during the exit from pluripotency, both in culture and during teratoma formation. Specifically, we find that when ESCs are stimulated to differentiate, a subpopulation fails to do so and instead upregulates FAS in a p53-dependent manner to trigger Bax/Bak-dependent apoptosis. Blocking this apoptotic pathway prevents the removal of these poorly-differentiated cells, resulting in the retention of cells that have not exited pluripotency. Taken together, our results provide further evidence for heterogeneity in the potential of ESCs to successfully differentiate, and reveal a novel role for apoptosis in promoting efficient ESC differentiation by culling cells that are slow to exit pluripotency.

Graphical Abstract
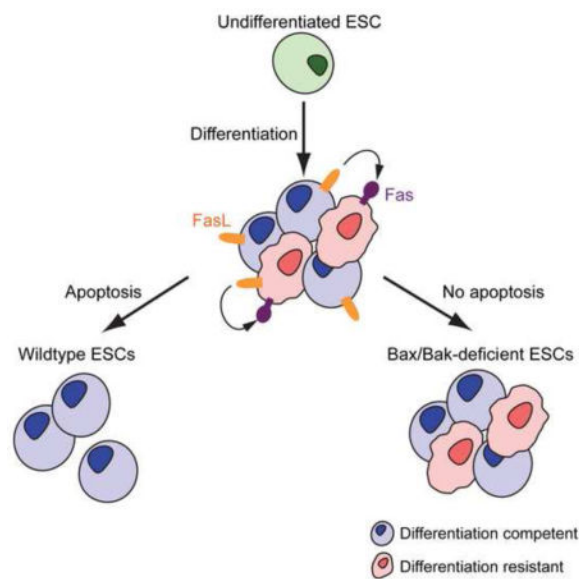
RESULTS

Bax/Bak-dependent mitochondrial apoptosis is critical for proper development during late embryogenesis, as most $Bax^{-/-}Bak^{-/-}$ mice die around embryonic day 17 due to defects in neural tube closure. The rare viable $Bax^{-/-}Bak^{-/-}$ mice only survive a few weeks and possess many physical abnormalities [3]. Mice genetically deficient for other key players of this apoptotic pathway, such as Apaf-1 and Caspase-9, exhibit phenotypic defects similar to, but less dramatic than, those of $Bax^{-/-}Bak^{-/-}$ mice [9–12]. However, the role of mitochondrial apoptosis during early embryogenesis remains poorly defined. A previous study reported that Casp3 cleaves the pluripotency factor Nanog to promote murine embryonic stem cell (mESC) differentiation without inducing apoptosis [13]. Conversely, others have reported that ESCs undergo apoptosis after withdrawal of the pluripotency-promoting cytokine leukemia inhibitory factor (LIF) [7] or during differentiation into

cardiomyocytes [8]. Therefore, it remains unclear whether apoptosis has critical functions during ESC differentiation.

To study the role of the intrinsic apoptotic pathway during ESC differentiation, we generated ESCs deficient for *Bax* and *Bak*, which together are required for mitochondrial apoptosis upstream of Casp3. As germline-deficient $Bax^{-/-}Bak^{-/-}$ mice generally die *in utero* [2], we isolated three different ESC lines (Figure S1A) from C57BL/6 $Bax^{f/f}Bak^{-/-}$ mice [14]; these were transiently transfected with *Cre* recombinase to obtain three *Bax/Bak*-deficient (double knockout, DKO) ESCs lines (Figures S1B, C). Subsequently, two lines (DKO#17 and DKO#18) were found to lack stable integration of *Cre* and were chosen for further analysis (Figure S1D); unless otherwise indicated, data shown are from line DKO#17. Deletion of *Bax/Bak* had no observable effects on size, shape, growth rate, or expression of the pluripotency factors Oct4 or Nanog [15–17] in undifferentiated ESCs (Figure S1E, F). Furthermore, DKO#17 and DKO#18 had normal karyotypes (40, XY) (Figure S1G).

Next, we tested whether loss of *Bax* and *Bak* affected ESC differentiation. DKO ESCs were differentiated with retinoic acid (RA) and compared to wildtype C57BL/6 ESCs ($Bax^{+/+}Bak^{+/+}$, WT), parental cells ($Bax^{f/f}Bak^{-/-}$), and $Casp3^{-/-}$ ESCs. We first performed colony formation assays with RA-treated ESCs and found that both DKO and $Casp3^{-/-}$ ESCs inappropriately formed colonies expressing the pluripotency marker alkaline phosphatase (AP) [18] (Figures 1A, B), consistent with the previous Casp3 study [13]. Interestingly, DKO ESCs were more delayed in losing AP expression than $Casp3^{-/-}$ ESCs. To further assess this differentiation defect, we measured mRNA levels of *Nanog* and *Oct4*, and found significantly higher expression in DKO ESCs relative to WT cells after 1d and 2d of RA treatment (Figure 1C). Similarly, loss of Oct4 and Nanog proteins was delayed in both DKO ESC lines during differentiation (Figure 1D, S2A, B). We next examined DKO ESC differentiation during embryoid body (EB) formation and found elevated levels of Nanog and Oct4 protein in DKO relative to WT ESCs 4d after initiating EB formation (Figure 1E), indicating that the observed defect is not restricted to RA-induced differentiation. Thus, DKO ESCs exhibit a marked delay in exiting pluripotency *in vitro*.

To account for potential artifacts from Cre expression, we transiently expressed Cre in WT ESCs and selected individual clones without stable expression (Figure S1H), as was done to generate DKO cells. In two different clones, transient expression did not impair ESC differentiation (Figure S1I, J). Separately, to test whether differentiation-resistant DKO colonies could be stably propagated, we re-grew these cells in ESC media. Even after 4d of previous RA treatment, Oct4 and Nanog expression in these cells was similar to that of undifferentiated ESCs (Figure S1K), indicating that these cells were not stuck in a partially differentiated state. Furthermore, with similar efficiency to unmanipulated DKO ESCs, these ESCs re-differentiated upon treatment with RA (Figure S1L). Thus, the differentiation delay of some DKO ESCs may be a stochastic event and not an inherent defect of these cells.

Next, we tested WT, $Bax^{f/f}Bak^{-/-}$, DKO, and $Casp3^{-/-}$ ESCs for their ability to differentiate *in vivo* during teratoma formation. Hematoxylin and eosin (H&E) stains of teratomas revealed that WT, $Bax^{f/f}Bak^{-/-}$, and to a lesser extent $Casp3^{-/-}$ lines were

mainly comprised of differentiated cells representing all three germ layers (Figure 1F). In contrast, DKO teratomas largely consisted of sheets of undifferentiated cells. Moreover, Oct4 immunohistochemistry showed significantly more undifferentiated, Oct4-positive nuclei in DKO teratomas than those from the other lines. Taken together, our results demonstrate that the differentiation defect is more pronounced in $Bax^{-/-}Bak^{-/-}$ than in $Casp3^{-/-}$ ESCs. Interestingly, this mirrors the greater resistance to apoptosis in $Bax^{-/-}Bak^{-/-}$ cells compared to $Casp3^{-/-}$ cells [19].

Previously, Casp3 was reported to have a nonapoptotic role in promoting ESC differentiation [13]. To test this, we quantified apoptosis in ESCs during differentiation using AnnexinV and propidium iodide (PI). We found that levels of apoptotic cells were extremely low (~3%) in untreated ESCs, while ~12% were apoptotic after 2d of RA-induced differentiation (Figure 2A, S3A). In contrast, $Casp3^{-/-}$ and both DKO ESC lines lacked evidence of apoptosis under untreated and RA-treated conditions (Figure 2A, S2C). For confirmation, we performed TUNEL staining and found that while WT ESCs exhibited an increase in the proportion of TUNEL+ cells after 2d RA treatment, DKO cells were resistant (Figure 2B). Furthermore, Casp3 was only cleaved and activated after RA treatment in WT and $Bax^{f/f}Bak^{-/-}$ ESCs (Figure 2C, S2D). Therefore, Bax and Bak are required for Casp3 activation and differentiation-induced apoptosis.

We next searched for upstream factors that activate Bax/Bak during ESC differentiation. Based on a small microarray of approximately 100 cell death and survival genes, we found >30 fold upregulation of *Fas* mRNA after 4d of RA treatment; its cognate ligand Fas ligand (*Fas-L*) was upregulated ~3 fold as well (Table S1). We confirmed that during differentiation, *Fas* and *Fas-L* mRNA and protein were markedly upregulated (Figure 2D, F). A prototypical death receptor, Fas can trigger apoptosis through assembly of the death-inducing signaling complex (DISC) and subsequent activation of Caspase-8 (Casp8) [20]. In Type I cells, Fas-mediated activation of Casp8 is sufficient to activate the executioner Casp3; however, in Type II cells, Fas-mediated apoptosis requires Casp8-dependent activation of the BH3-only protein Bid and subsequent engagement of Bax and Bak [20] (Figure 2E).

As Bax and Bak are required for apoptosis during ESC differentiation, we asked whether ESCs are Type II cells. Indeed, we observed cleavage of Casp8 (to the active p18 fragment) and Bid during ESC differentiation (Figure 2F). Importantly, these events occurred upstream of Bax/Bak and in the absence of Casp3, suggesting that Casp8 is an initiator caspase rather than a substrate of downstream caspases. We then costained differentiating ESCs for Fas and AnnexinV and found significant enrichment of apoptotic cells in the Fas-positive subpopulation (Figure 2G, S3B). Furthermore, after sorting differentiating WT ESCs for Fas, we found elevated Casp8 and Casp3 activation in the Fas positive population (Figure 2H).

To determine the generality of Fas upregulation and Casp3 activation during ESC differentiation, we first analyzed EB formation and observed Fas upregulation in both WT and DKO ESCs, while cleaved Casp3 was only detected in WT ESCs (Figure 2I). Although Casp3 activation during EB formation could be critical for other developmental steps, such

as cavitation, these data are consistent with our hypothesis that Fas signaling triggers apoptosis during ESC differentiation. We then analyzed RA-treated human ESCs (hESCs) and found transient FAS upregulation and CASP3 cleavage (Figure 2J). This is consistent with the mESCs phenotype and suggests that this apoptotic process is conserved across species.

To determine whether differentiated or undifferentiated ESCs are variably susceptible to apoptosis, we utilized Oct4-GFP reporter ESCs [21] to measure levels of AnnexinV staining after RA treatment. Strikingly, levels of apoptosis were significantly higher in the undifferentiated (GFP^{high}) subpopulation (Figure 3A, B). We then sorted these cells for GFP and found that the GFP^{high} subpopulation contained significantly more cleaved Casp3 than the GFP^{low} subpopulation (Figure 3C). Thus, a subgroup of poorly-differentiating ESCs preferentially initiates apoptosis through the Bax/Bak pathway.

We next determined the distribution of *Fas* and *Fas-L* among differentiating ESCs. Using Oct4-GFP ESCs, we found that Fas-expressing cells were predominantly undifferentiated (GFP^{high}) (Figure 3D). Likewise, after sorting differentiating WT ESCs for Fas, we found that Fas^{high} cells had markedly higher levels of Oct4 than Fas^{low} cells (Figure 3E). To determine the source of Fas-L, we isolated mRNA from GFP-sorted Oct4-GFP ESCs and found equivalent upregulation of *Fas-L* mRNA in undifferentiated and differentiated cells (Figure 3F), suggesting that poorly-differentiated cells acquire this signal through both autocrine and paracrine sources.

To test the roles of Fas and Casp8 in ESC differentiation, we performed siRNA knockdowns (Figure 3G). In comparison to cells transfected with nontargeting control siRNA, knockdown of Fas or Casp8 significantly reduced the levels of apoptotic cells (Figure 3H). Furthermore, knockdown of Fas or Casp8 increased the number of AP-positive colonies and delayed the loss of Nanog after RA treatment (Figures 3I, J). Taken together, our data strongly suggest that engagement of Fas is a critical upstream activator of the intrinsic apoptotic pathway during ESC differentiation, and that deletion of *Bax* and *Bak* prevents the elimination of poorly differentiating cells.

Next, we tested known transcriptional regulators of *Fas* and discovered that its expression was largely dependent on p53 (Figure 4A–C). $p53^{-/-}$ ESCs could not efficiently upregulate Fas, and these cells were protected against apoptosis and Casp3 cleavage (Figure 4D, E).

Interestingly, we found that p53 mRNA and protein levels decline during WT ESC differentiation (Figure 4F, G), a counterintuitive result consistent with previous reports [22–24]. We reasoned that p53 activity could increase despite a decrease in its levels because its activity is restrained in undifferentiated ESCs, and differentiation could lift this signaling brake. In fact, a previous study demonstrated that Aurora Kinase A (AurA), an important mitotic kinase, phosphorylates p53 in undifferentiated ESCs to inhibit its transcriptional activity [22]. Immunoblotting for AurA revealed significant reductions in its levels during differentiation, irrespective of *Bax/Bak* or *p53* status (Figure 4H, I). However, the pluripotency factors Oct4, Nanog, and Klf4 are not responsible for regulating AurA levels in ESCs (Figure S4). Furthermore, either treatment of undifferentiated ESCs with the AurA-

specific kinase inhibitor MLN8237 or siRNA knockdown of AurA was sufficient to induce p53-dependent upregulation of Fas and apoptosis (Figure 4J–M). Thus, AurA restrains p53 function in undifferentiated ESCs, and its downregulation during differentiation allows p53-mediated upregulation of *Fas* and initiation of Bax/Bak-dependent apoptosis.

As p53 guards the integrity of the genome, we analyzed levels of phosphorylated histone protein H2AX (γ-H2AX), a classical DNA damage marker, and saw a transient increase in WT ESCs but a steady accumulation in DKO ESCs during differentiation (Figure 4N). Staining for γ-H2AX foci confirmed that DKO ESCs retain significantly more γ-H2AX-positive cells than WT ESCs after 4d of RA treatment (Figure 4P). Interestingly, p53 protein levels did not significantly decline in DKO ESCs during differentiation (Figure 4F), suggesting that an inability to remove damaged ESCs precluded downregulation of p53 protein at the population level.

We next used Oct4-GFP ESCs to investigate why Fas upregulation and apoptosis primarily occur in poorly differentiating ESCs. Although we observed no significant difference in AurA or total p53 levels between GFP^{high} and GFP^{low} cells, we found increased levels of γ-H2AX and phospho-Ser15-p53, an activating p53 modification, in undifferentiated ESCs (Figure 4O). Thus, our data suggest that loss of AurA-mediated inhibition licenses p53 activity in all differentiating cells, while activation of p53 primarily occurs in the differentiation-resistant ESC subpopulation (Figure 4Q).

DISCUSSION

Here, we show that upregulation and engagement of Fas on poorly differentiating ESCs selectively triggers their demise. The most parsimonious explanation for the differentiation delay in *Bax$^{-/-}$Bak$^{-/-}$* ESCs is a failure to remove differentiation-resistant cells. This defect is consistent with a previous report studying *Casp3* [13]. However, in contrast to their conclusion that Casp3 does not induce apoptosis, our results support a pro-apoptotic role for Casp3. This discrepancy may be because they used DAPI staining rather than the more sensitive, yet specific, markers employed here. Although our data argue that Casp3 promotes apoptosis in undifferentiated cells during differentiation, Casp3 may have non-apoptotic functions in cells that survive and differentiate. In fact, Fas itself may have additional non-apoptotic functions during differentiation, as it can activate NF-κB signaling [25–27], which may be important for ESC differentiation [28]. Nevertheless, our results clearly identify a pro-apoptotic role for Fas during ESC differentiation.

In contrast to our findings, a previous study reported downregulation of *FAS* during hESC differentiation [29]. This study compared pluripotent hESCs to fetal and adult human tissues, and hESCs differentiated for 20d, while our study focused on early ESC differentiation. Thus, the difference may be partly due to the timing of analysis. However, in closer agreement with our findings, conversion of human fibroblasts or hepatocytes into induced pluripotent stem cells is associated with a reduction in FAS mRNA levels [30, 31], while differentiation of hESCs into pancreatic islet-like cells or mesenchymal stem cells is associated with increased *FAS* mRNA levels [32, 33]. Further studies will be required to resolve these differences.

The accumulation of γ-H2AX in DKO ESCs coupled with its efficient loss in WT ESCs during differentiation suggests that DNA damage may be crucial to activate p53. However, future studies are necessary to determine the potential form of damage and its sensor(s). Furthermore, questions such as whether some undifferentiated ESCs have already accumulated DNA damage, or whether an inability to differentiate itself causes a DNA damage response, remain to be addressed.

Based on the dramatic differentiation defects in apoptosis-deficient (*p53*$^{-/-}$, *Bax*$^{-/-}$*Bak*$^{-/-}$, and *Casp3*$^{-/-}$) ESCs observed here and previously [13, 23], one might expect obvious problems in early embryogenesis in mice genetically deficient for these genes. However, *p53*-deficient mice are born at normal Mendelian ratios without gross developmental defects [34]. *Bax*$^{-/-}$*Bak*$^{-/-}$ and *Casp3*$^{-/-}$ mice also progress through early embryogenesis without obvious problems [3, 12]. We speculate that this may be due to compensation for the loss of these genes, as has been seen in other circumstances [3, 35–38]. Nevertheless, these mutant mice, and *p53* haploinsufficent humans (Li-Fraumeni syndrome), have a predisposition to cancer [34, 39, 40]. Future studies will be required to test whether this elevated risk of cancer is partly due to an inability to delete differentiation-defective cells during early embryogenesis.

In summary, our findings show that the mitochondrial apoptotic pathway is critical for removing poorly-differentiated ESCs during their exit from pluripotency. These results highlight the heterogeneity of isogenic ESCs and reveal the importance of apoptosis in controlling cell fate decisions at the earliest stages of ESC differentiation.

EXPERIMENTAL PROCEDURES

ESC Cultures

Mouse ESC lines were maintained in ESC Media: DMEM containing high glucose (Sigma) with HEPES hemisodium salt (MP Biomedical), 1% L-glutamine and 1% non-essential amino acids (UCSF Cell Culture Facility), 15% ESC-qualified fetal bovine serum and 100 mM β-mercaptoethanol (Gibco), and LIF (made in-house). Cells were maintained on irradiated mouse embryonic fibroblasts (MEFs). For differentiation experiments, ESCs were plated away from MEFs onto gelatinized dishes (0.2% gelatin from porcine skin, Sigma) and treated with 1 μM retinoic acid (Sigma) in ESC media without LIF as indicated. *Casp3*$^{-/-}$ ESCs were a generous gift from T. Zwaka (Mt. Sinai). Embryoid bodies were formed using the hanging drop method, with 400 cells plated in 20 μl drops of ESC media without LIF.

For additional methods, see Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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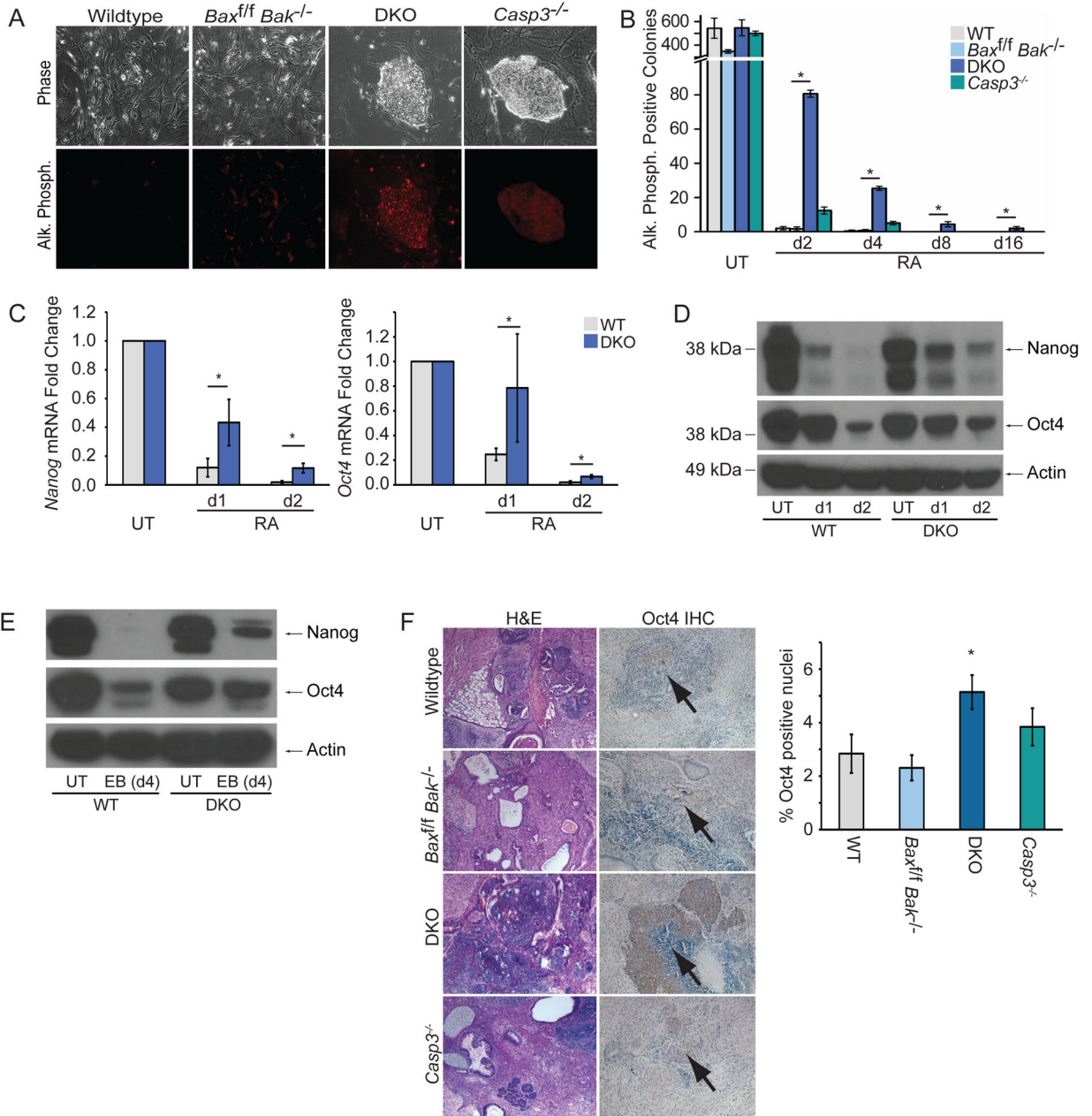
Figure 1. Genetic Deletion of Bax and Bak Results in a Delay in ESC Differentiation
(A) Representative phase and fluorescent images of each ESC line stained for AP after 2d of RA treatment. (B) Quantification of AP positive colonies during RA treatment; data plotted as mean ± SD. (C) *Nanog* and *Oct4* mRNA levels from ESCs analyzed via qPCR; data plotted as mean ± SD. (D) Immunoblot of ESC lysates for Nanog and Oct4. (E) Immunoblot of ESC lysates for Nanog and Oct4 during EB formation. (F) Sections of teratomas stained with hematoxylin and eosin (H&E) (left panel) or for Oct4 expression (right panel), and quantification of Oct4+ nuclei. At least three independent biological samples were used for the AP colony-reforming assay, and *Nanog* and *Oct4* qPCR. * significant at $p < 0.05$ (Student t test). See also Figures S1–S2.
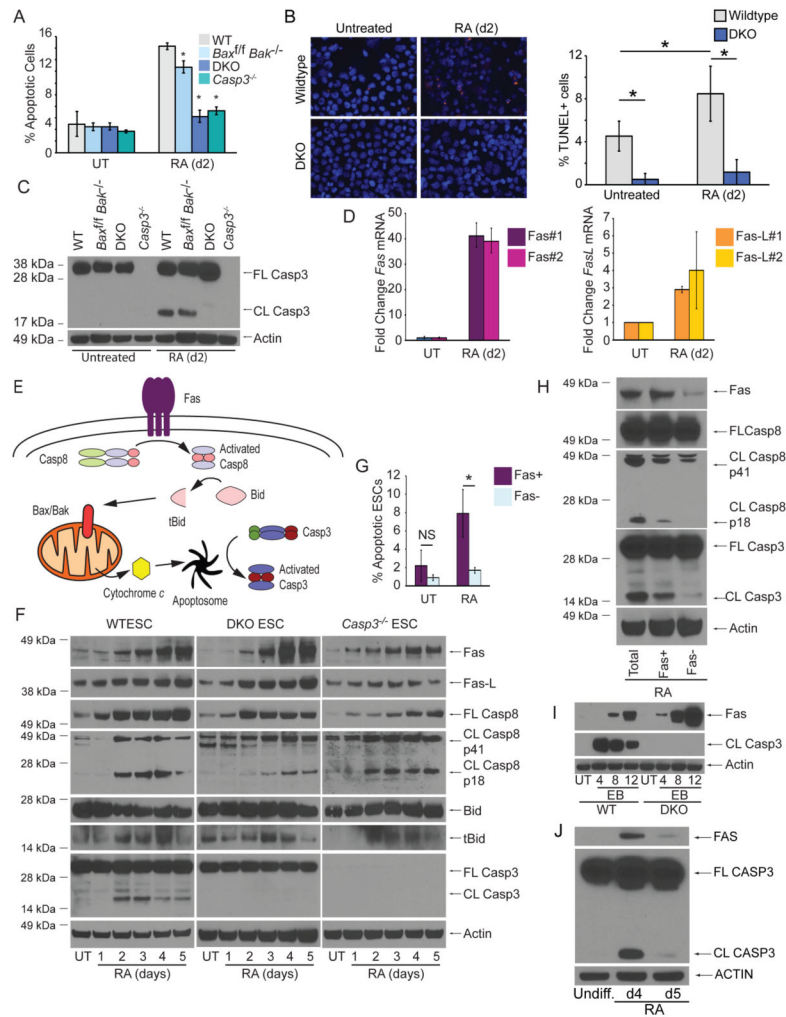
Figure 2. ESCs Undergo Fas-mediated, Bax/Bak-dependent Apoptosis During Differentiation
(A) Quantification of percent apoptotic cells (AnnexinV positive/PI negative) during RA-mediated differentiation. (B) Representative images and quantification of TUNEL staining from untreated and 2d RA-treated WT and DKO ESCs. (C) Immunoblot for full-length (FL) and cleaved (CL) Casp3 from ESCs untreated or treated with RA for 2d. (D) Representative Q-PCR of *Fas* and *Fas-L* mRNA levels during ESC differentiation using two independent sets of primers for each target. (E) Schematic of Fas-mediated activation of Bax/Bak-dependent apoptosis. (F) Immunoblot for components of the Type II Fas death receptor pathway during ESC differentiation. (G) Fas and AnnexinV co-staining in ESCs after 2d of differentiation. (H) Immunoblot for Casp8 and Casp3 in ESCs sorted for Fas after 2d of differentiation. (I) Immunoblots for Fas and Casp3 from WT or DKO cells induced to form embryoid bodies by the hanging drop method. (J) Immunoblot of FAS and CASP3 from UCSF4 human ESCs differentiated with RA as indicated. Three independent biological samples were used for the AnnexinV assays. Data plotted as mean ± SD. * significant at $p < 0.05$ (Student t test). See also Figure S3.
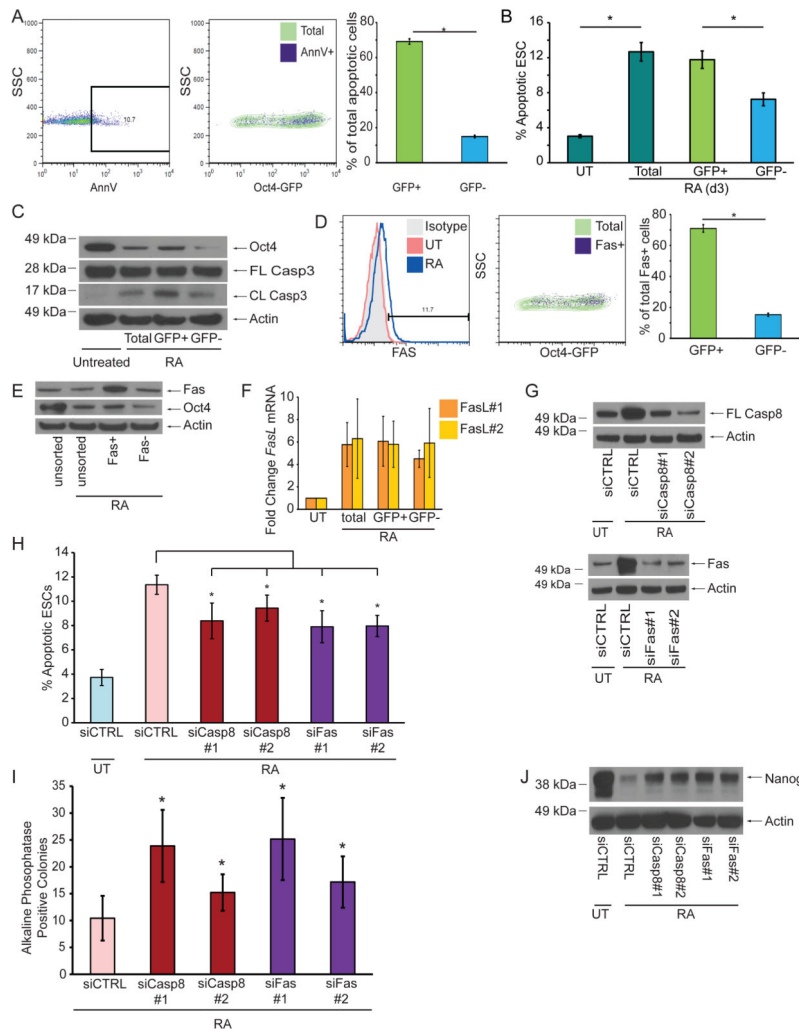
Figure 3. Fas and Casp8 Promote the Removal of Poorly Differentiating cells during ESC Differentiation

(A) Representative FACS plots and quantification of Oct4-GFP ESCs stained for AnnexinV after 2d of RA treatment. (B) Quantification of apoptotic Oct4-GFP ESCs after 3d of RA treatment. (C) Immunoblots of lysates from 2d-treated Oct4-GFP ESCs after sorting for GFP expression. (D) Representative FACS plots and quantification of Oct4-GFP ESCs stained for Fas after 2d of RA treatment. RA-treated ESCs were used for isotype control staining. (E) Immunoblot for Oct4 from 2d-treated WT ESCs after sorting for Fas expression. (F) Q-PCR using two different primer sets for *Fas-L* mRNA from Oct4-GFP ESCs treated or untreated with RA for 2d. (G) Immunoblot of ESC lysates for Casp8 and Fas after siRNA knockdown and 2d RA treatment. (H) AnnexinV staining of ESCs quantified by FACS after indicated siRNA knockdown and 2d RA treatment. (I) Number AP+ colonies after indicated siRNA knockdown and 2d RA treatment. (J) Immunoblot of ESC lysates for Nanog after siRNA knockdown and 2d RA treatment. Three independent biological samples were used for the AnnexinV, qPCR, and AP assays. Data plotted as mean ± SD. * significant at $p < 0.05$ (Student t test).
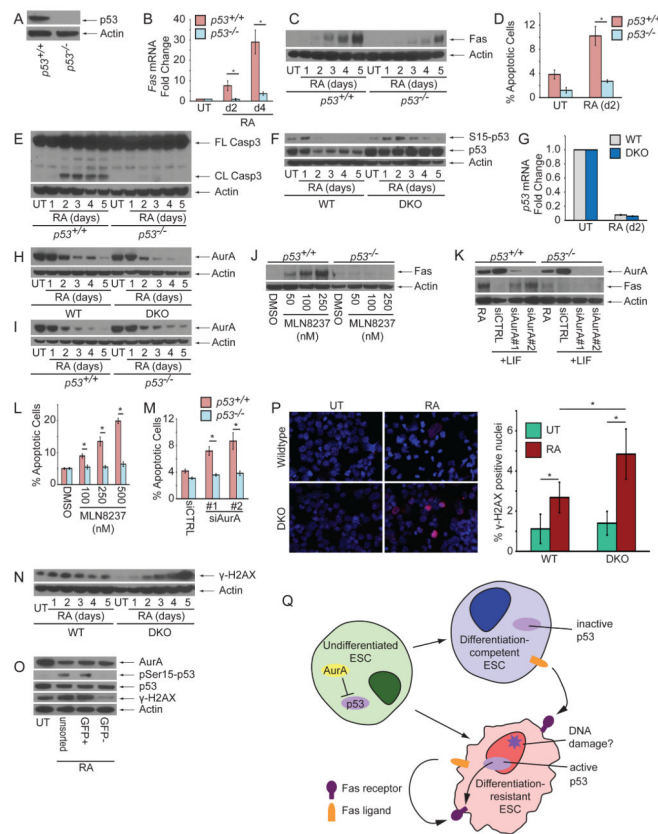
Figure 4. p53 is De-repressed During ESC Differentiation to Initiate Apoptosis in Poorly Differentiating Cells

(A) Immunoblot of p53 from $p53^{+/+}$ and $p53^{-/-}$ ESCs. (B) mRNA and (C) protein levels of *Fas* from RA-treated $p53^{+/+}$ and $p53^{-/-}$ ESCs. (D) AnnexinV staining of $p53^{+/+}$ and $p53^{-/-}$ ESCs after 2d RA treatment. (E) Immunoblot of CASP3 from $p53^{+/+}$ and $p53^{-/-}$ ESCs treated with RA. (F) Immunoblot for p53 from WT and DKO ESCs during RA treatment. (G) mRNA levels of *p53* from WT and DKO ESCs during RA treatment. Immunoblot for AurA from (H) WT and DKO or (I) $p53^{+/+}$ and $p53^{-/-}$ ESCs during RA treatment. (J) Immunoblot for Fas from $p53^{+/+}$ and $p53^{-/-}$ ESCs treated with MLN8237 for 24 hr. (K) Immunoblot for Fas from $p53^{+/+}$ and $p53^{-/-}$ ESCs transfected with control siRNA or siRNA against AurA. AnnexinV staining of p53+/+ and p53−/− ESCs after treatment with (L) MLN8237 for 24 hr or (M) knockdown with control or AurA-specific siRNA. (N) Immunoblot for γ-H2AX from WT and DKO ESCs treated with RA. (O) Immunoblot for AurA, phospho-serine15-p53, total p53, and γ-H2AX from Oct4-GFP ESCs sorted for GFP expression after 2d RA treatment. (P) Representative immunofluorescence images and quantification of γ-H2AX+ nuclei from WT and DKO ESCs untreated or treated with RA for 4d. (Q) Schematic of Fas-L-mediated activation of Fas to induce apoptosis in undifferentiated ESCs. Three independent biological samples were used for the AnnexinV and qPCR assays. Data plotted as mean ± SD. * significant at $p < 0.05$ (Student t test). See also Figure S4.