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Deficiency of *Trp53* Rescues the Male Fertility Defects of *Kit*$^{W\text{-}v}$ Mice but Has No Effect on the Survival of Melanocytes and Mast Cells

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Mutations of the receptor tyrosine kinase, Kit, or its ligand, mast growth factor (Mgf), affect three unrelated cell populations: melanocytes, germ cells, and mast cells. Kit signaling is required initially to prevent cell death in these lineages both *in vitro* and *in vivo*. Mgf appears to play a role in the survival of some hematopoietic cells *in vitro* by modulating the activity of p53. Signaling by Mgf inhibits p53-induced apoptosis of erythroleukemia cell lines and suppresses p53-dependent radiation-induced apoptosis of bone marrow cells. We tested the hypothesis that cell survival in *Kit* mutant mice would be enhanced by p53 deficiency *in vivo*. Double-mutant mice, which have greatly reduced Kit receptor tyrosine kinase activity and also lack *Trp53*, were generated and the affected cell lineages examined. Mast cell, melanoblast, and melanocyte survival in the double *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ mutants was not increased compared to the single *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ mutants. However, double-mutant males showed an increase in sperm viability and could father litters, in contrast to their homozygous *Kit* mutant, wild-type p53 littermates. This germ cell rescue appears to be male specific, as female ovaries were similar in mice homozygous for the *Kit* mutant allele with or without p53. We conclude that defective Kit signaling *in vivo* results in apoptosis by a p53-independent pathway in melanocyte and mast cell lineages but that in male germ cells apoptosis in the absence of Kit is p53-dependent.

Key Words: **mice; melanocytes; germ cells; mast cells; Kit$^{W\text{-}v}$; Trp53; Mgf.**

INTRODUCTION

Mutations of the mouse receptor tyrosine kinase, *Kit*, or its ligand, mast cell growth factor (*Mgf*) (stem cell factor or steel factor) are also known as the classical dominant white spotting (*Kit*^{W}) and steel (*Mgf*^{Sl}) mutants. Numerous mutant alleles have been identified at both loci that profoundly affect the development of three apparently unrelated stem cell populations, namely melanoblasts, primordial germ cells, and hematopoietic stem cells. Mice homozygous for a loss of function at either the *Kit* or the *Mgf* locus show severe macrocytic anemia, have decreased numbers of mast cells, are infertile, and have almost a complete lack of coat pigmentation. Mgf binds to Kit as a dimer and induces homodimerization of the receptor and subsequent intracellular signaling (Philo *et al.*, 1996).

Point mutations in the Kit receptor, such as *Kit*$^{W\text{-}v}$, which affect tyrosine kinase activity, demonstrate an antimorphic or dominant negative phenotype when heterozygous. Half of the wild-type receptor monomers are inactivated by dimerization with mutant partners producing effectively 25% of normal active Kit receptors on the cell surface (Nocka *et al.*, 1990). The protein encoded by *Kit*$^{W\text{-}v}$ harbors a point mutation which significantly reduces its tyrosine kinase activity compared to the wild-type receptor. Heterozygous *Kit*$^{W\text{-}v}$ animals display a characteristic pigmentation phenotype: lightening of the coat color, a white belly and head spot, and reduced pigmentation of the feet and tail. Although fertile, they are also slightly anemic. Homozygotes have the typical phenotype of severe loss of function of Kit. They have almost no coat pigmentation, although some animals have slightly pigmented skin on the pinna (external ear); they are also anemic, have a deficiency of mast cells, and are infertile.

Melanocytes originate from neural crest cells and migrate, as melanoblasts, to peripheral sites between days 10

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and 16 of mouse embryonic development. We have previously shown that the survival of melanoblasts *in vivo* depends crucially on Kit signaling after embryonic day 11 (E11) (Steel *et al.*, 1992, Cable *et al.*, 1995, MacKenzie *et al.*, 1997). We have also suggested that Kit signaling plays a vital role in melanoblast proliferation (MacKenzie *et al.*, 1997). Others have shown a requirement of this signaling pathway for melanocyte survival, proliferation, and differentiation in culture (Murphy *et al.*, 1992, Morrison-Graham and Weston, 1993, Lahav, 1994); for migration of melanoblasts from the neural crest (Wehler-Haller and Weston, 1995); and for the continued survival of melanocytes after their migration to the skin and hair follicles (Nishikawa *et al.*, 1991).

Germ cells require Kit signaling at several stages of their development. Kit is highly expressed in primordial germ cells (PGCs) during their proliferative phase from E7.5 to E13 (Manova and Bachvarova, 1991; Bachvarova *et al.*, 1993), but is not required for their initial specification. Survival of PGCs *in vitro* requires stimulation of Kit by Mgf expressed on feeder cells (Dolci *et al.*, 1991; Godin *et al.*, 1991) as does proliferation of PGCs in similar systems (Matsui *et al.*, 1991; Buehr *et al.*, 1993). In addition, anti-Kit antibodies (Matsui *et al.*, 1991; Pesce *et al.*, 1997) block the adhesion of PGCs to somatic cells (Sertoli and Leydig cells).

Both male and female germ cells are dependent on active Kit for subsequent postnatal maturation. Kit expression has been detected in Leydig cells, spermatogonia, primary spermatocytes, and round spermatids of the testis (Manova *et al.*, 1990, 1993; Rossi *et al.*, 1993; Sandlow *et al.*, 1997) and Kit signaling is necessary for the proliferation and postnatal maturation of spermatogonia (Yoshinaga *et al.*, 1991). Follicle-stimulating hormone stimulates the expression of Mgf by Sertoli cells in the testes, which in turn activates DNA synthesis in spermatogonia (Rossi *et al.*, 1993).

Kit is highly expressed at all stages of postnatal development in mouse oocytes, starting in the diplotene stage around the time of birth after which primordial follicles are formed (Manova *et al.*, 1990). Normally, female germ cells complete mitotic divisions and begin to enter meiosis before birth. A single layer of granulosa cells surrounds prenatal germ cells to form primordial follicles (Peters, 1969). Upon activation, the follicles follow a maturation process leading to ovulation, although the majority arrest in atresia. Kit and Mgf are expressed in developing ovarian follicles from the early to the fully mature stages in a contiguous manner (Manova and Bachvarova, 1991; Manova *et al.*, 1993). Kit expression is highest on the surface of oocytes arrested in the diplotene stage of meiosis but declines upon ovulation and the resumption of meiotic maturation (Horie *et al.*, 1991). In contrast, Mgf expression is required to maintain the supporting granulosa cells surrounding the developing oocyte but also ceases upon the transition of the follicle to the antral stage (Kuroda *et al.*, 1988; Motro and Bernstein, 1993). Several stages of postnatal oocyte development including the onset of primordial follicle development, primary follicle growth, and follicle maturation are disturbed *in vivo* using antibodies blocking Kit function (Yoshida *et al.*, 1997). Kit is also required for growth of oocytes in culture (Packer *et al.*, 1994). Thus Kit signaling appears to play a role not only in the development of primordial germ cells but also in postnatal ovarian folliculogenesis.

PGCs of mice with severe *Kit* and *Mgf* mutations are present in normal numbers at E8.5, but decline to about 2% of the normal level by E12.5 (Mintz and Russell, 1957; McCoshen and McCallion, 1975). Some germ cells populate the gonads in mice carrying weaker *Kit* or *Mgf* mutations, but defects are seen in later stages of germ cell development, often with more severe effects on either male or female gonads and fertility (Geissler *et al.*, 1981; Copeland *et al.*, 1990). Comparable numbers of β-gal-positive PGCs are detected in *Kit*$^{W\text{-}LacZ/+}$ and *Kit*$^{W\text{-}LacZ/W\text{-}LacZ}$ E9.5 embryos. However, the homozygous mutant cells show aberrant migration to the ventral half of the gut and have completely disappeared by E12.5 (Bernex *et al.*, 1996).

Hematopoietic progenitor cells also express Kit (Ogawa *et al.*, 1991). Mice which lack Mgf (*Mgf*^{Sl} homozygotes) or which have Kit blocked by antibody have a severe reduction in hematopoietic stem cells (Ikuta and Weissman, 1992; Ogawa *et al.*, 1993). The hematopoietic defect in *Kit*^{W} mice is first apparent in the blood islands of the embryonic yolk sac and then persists as the stem cells migrate to the fetal liver and to the sites of neonatal and adult hematopoiesis. In addition to depletion of hematopoietic precursors, Kit- or Mgf-deficient mice show a specific deficit of mast cells. Mast cell precursors are present in these mutant mice in apparently normal numbers and will, through action of IL-3, differentiate into bone marrow-derived mast cells (BMMC). However, absence of Kit signaling results in failure of these precursors to proliferate, adhere, and migrate (Reith *et al.*, 1990).

Thus, stimulation of the Kit receptor pathway is essential for several functions in cells of diverse lineages. The initial requirement for Kit activation, in most if not all lineages, is survival of the progenitor cells. We wished to address the question of how the progenitor cell survival is mediated by examining the mechanism by which the cell is lost in the absence of signaling.

The p53 tumor suppressor protein plays a critical role in regulating apoptosis and G1/S cell cycle arrest by acting as an integral part of the cellular response to DNA damage. Cells lacking p53 fail to arrest in G1 following exposure to ionizing radiation and are resistant to apoptosis induced in response to DNA damage (Lowe *et al.*, 1993; Clarke *et al.*, 1993). Cell death in absence of growth factor stimulation is mediated by p53 for a number of cell lines. Expression of p53 in a myeloid leukemia cell line results in the cells becoming dependent on interleukin 6 (IL-6) for survival. In its absence the cells die by apoptosis, unless the p53 protein is inactivated (Yonish-Rouach *et al.*, 1991, 1993). Furthermore, murine hematopoietic cell lines will become apoptotic after irradiation unless stimulated by IL-3 and functional p53 is required to initiate death (Canman *et al.*, 1995).

Transformed Friend erythroleukemia cells do not normally express p53 and can grow independent of erythropoietin (Epo). Expression of p53 in these cells leads to a rapid loss in viability unless exogenous Epo is added; withdrawal of Epo results in apoptosis (Johnson *et al.*, 1993).

Trp53 mutant mice have a complete absence of p53 protein, yet develop normally. They are, however, predisposed to spontaneous tumor formation and develop predominantly lymphomas. The majority of animals develop tumors or are dead by 10 months of age (Donehower *et al.*, 1992).

Significantly, several studies have shown that defective Kit/Mgf signaling leads to cell death *in vitro* and this death is mediated by a p53-dependent pathway. Addition of Mgf to BMMC suppressed the induction of apoptosis by γ-irradiation or growth factor deprivation (Yee *et al.*, 1994). Interestingly, p53-deficient BMMC continued to undergo apoptosis upon withdrawal of Mgf, but at a lower rate than normal BMMC.

Lotem and Sachs (1993) concluded that bone marrow myeloid progenitor cells and irradiated thymocytes from p53-deficient mice are more resistant to induction of apoptosis in culture when the concentration of any of several viability factors, GM-CSF, IL-1a, IL-3, IL-6, or Mgf, is low. The loss of one allele of wild-type p53 (cells from heterozygous mice) is sufficient for increased resistance. Finally, the addition of exogenous Mgf can attenuate p53-mediated apoptosis and terminal differentiation in Friend erythroleukemia cells (Abrahamson *et al.*, 1995). These are normally p53-deficient and independent of Mgf, but after the introduction of temperature-sensitive p53 the cells become Mgf-dependent at the permissive temperature and respond to its withdrawal by apoptosis.

All of the studies described above used cells in culture as model systems to examine the relationship between Kit/Mgf signaling and p53. We wished to determine whether germ cell, mast cell, and melanoblast cell death due to defective Kit/Mgf signaling was mediated via a p53-dependent pathway *in vivo.* We produced mice that completely lacked p53 protein and which also were defective in Kit signaling and examined whether these animals showed a reduction in apoptosis and thus increased survival of melanocyte, germ cells, and hematopoietic cells. We find that, unlike cultured cells, a genetic lack of p53 does not rescue a deficiency of Kit signaling in mast cells and melanocytes. However, we do see an increased survival of germ cells in newborn and adult male mice. Kit/Mgf signaling appears to be mediated via a p53-dependent pathway in this lineage in a sex-specific manner.

MATERIALS AND METHODS

Mice

Mice with a targeted null allele of the gene encoding p53 (*Trp53*), designated $Trp53^{-/-}$ in this paper (Clarke *et al.*, 1993), were crossed to mice heterozygous for the $Kit^{W\text{-}v}$ mutation, as homozygous $Kit^{W\text{-}v}$ mice are infertile. The resultant $Kit^{W\text{-}v/+}$:$Trp53^{+/-}$ mice were intercrossed and the melanocyte, mast, and germ cell contribution was assessed in the offspring. To specifically analyze the melanoblast population homozygous *Dct-lacZ* transgenic animals (MacKenzie *et al.*, 1997) were also crossed to the heterozygotes. Compound heterozygotes ($Kit^{W\text{-}v/+}$:$Trp53^{+/-}$) with one parent carrying the *Dct-lacZ* transgene were crossed to generate embryos with one of the nine possible genotypic combinations.

Analysis of Embryos

Embryos were generated from intercrosses of compound heterozygotes ($Kit^{W\text{-}v/+}$:$Trp53^{+/-}$), one of which also contributed the *Dct-lacZ* transgene. The time of gestation was calculated by taking noon of the day of detection of a vaginal plug as E0.5 and also noting the external appearance of the embryo (according to Kaufman, 1992). Expression of the transgene was detected by XGal staining as outlined previously (MacKenzie *et al.*, 1997).

Genotyping

DNA isolated from tail biopsy or extraembryonic membranes was used for PCR assays to identify $Kit^{W\text{-}v}$ mutants (Cable *et al.*, 1995), *Trp53* mutants (Malcomson *et al.*, 1997), and mice carrying the *Dct-lacZ* transgene (MacKenzie *et al.*, 1997).

Histology

To ensure that the study was unbiased, all histological analyses were performed blind to genotype.

Analysis of mast cells in skin. Whole skin was peeled from tail biopsies, pinned out flat, fixed overnight in 4% paraformaldehyde at 4°C, and processed for paraffin embedding. Seven-micrometer sections were stained for 1 min in Azur blue solution. The number of mast cells was quantified by counting the number of stained cells in five 0.142-mm^2 fields (the microscope field of an ×20 objective (Zeiss Axioplan 2)). The mean number of mast cells in these fields was calculated for each genotype.

Analysis of germ cells from ovaries and testes. Testes and ovaries from newborn and adult mice were fixed overnight in Bouin's fixative and processed for paraffin embedding. Five-micrometer sections were stained with hematoxylin and eosin. Sperm cell counts were carried out as outlined previously (Searle and Beechey, 1974). Gonocyte counts were recorded from newborn testes sections.

RESULTS

To study interactions between the Kit signaling pathway and p53 *in vivo,* we used mice carrying the spontaneous Kit mutation, $Kit^{W\text{-}v}$, and mice with a targeted null allele of the gene encoding p53 (*Trp53*). We generated mice defective for both genes by intercrossing compound heterozygous ($Kit^{W\text{-}v/+}$:$Trp53^{+/-}$) mice to generate all nine possible genotypes. The offspring from these crosses were used to study melanocytes and their precursor cells mast cells and germ cells.

Melanocytes

Over 30 adult $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{-/-}$ mice were generated from the compound heterozygote intercrosses and they all

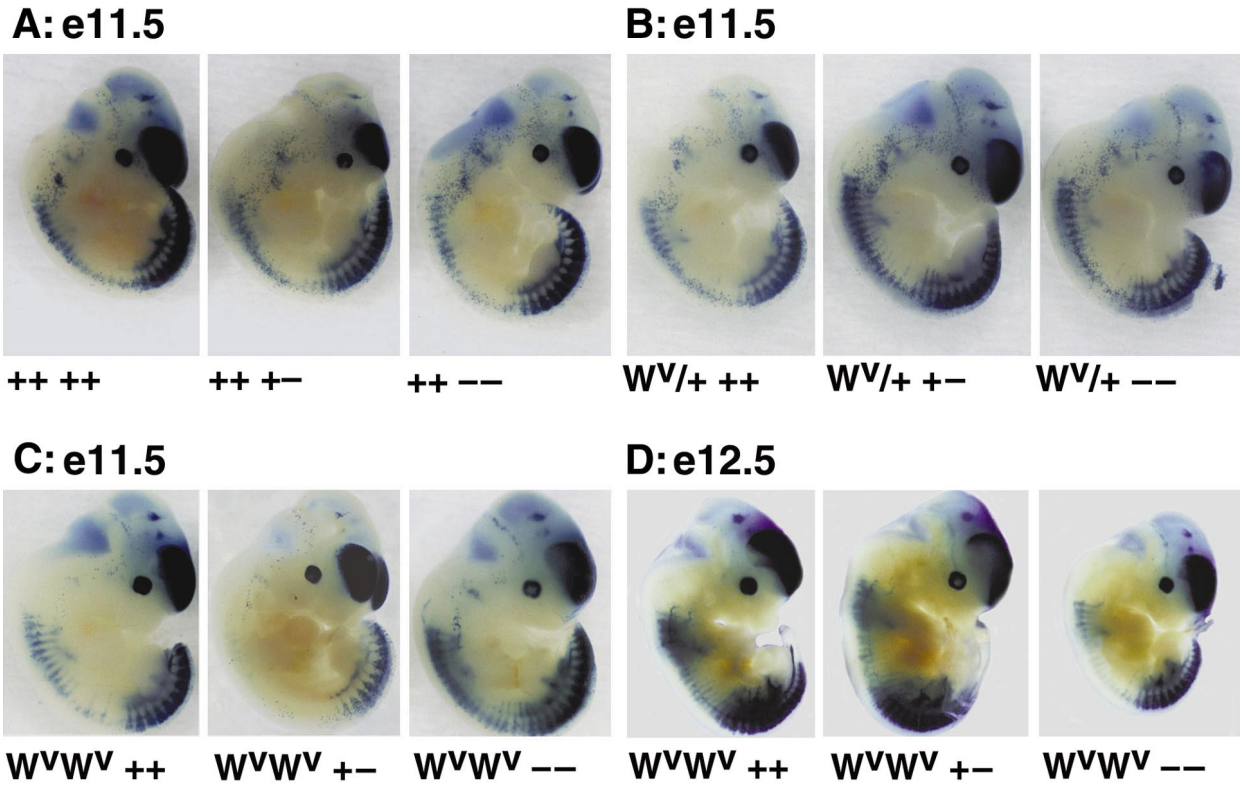

FIG. 1. Melanocyte distribution in the *Kit*$^{W\text{-}v}$:*Trp53* mutant embryos carrying a *Dct-lacZ* reporter gene. (A) E11.5. Left, +/+:*Trp53*$^{+/+}$ embryo, center +/+:*Trp53*$^{+/-}$, and right, +/+:*Trp53*$^{-/-}$. Equal numbers of neural-crest-derived melanoblasts, stained blue by XGal, are observed in all three embryos. (B) E11.5. Left, *Kit*$^{W\text{-}v/+}$:*Trp53*$^{+/+}$ embryo, center *Kit*$^{W\text{-}v/+}$:*Trp53*$^{+/-}$, and right, *Kit*$^{W\text{-}v/+}$:*Trp53*$^{-/-}$. All heterozygous *Kit*$^{W\text{-}v/+}$ embryos regardless of their Trp53 genotype show a reduced melanoblast number compared to wild-type embryos (A). (C) E11.5. Left, *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ embryo, center *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$, and right, *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$. Very few melanoblasts can be detected in all three *Kit*$^{W\text{-}v/W\text{-}v}$ embryos compared to wild-type embryos (A). (D) E12.5. Left, *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ embryo, center *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$, and right, *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$. No melanoblasts can be detected in all three embryos, indicating cell death still occurs in the absence of Trp53.

showed a lack of coat pigmentation identical to their *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ *and Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$ littermates. Some of the double mutants showed pigmentation of their pinna that is also occasionally seen in *Kit*$^{W\text{-}v/W\text{-}v}$ mice. Absence of p53 therefore does not increase melanocyte survival in adult *Kit*$^{W\text{-}v/W\text{-}v}$ mutant mice. Similarly, heterozygous *Kit*$^{W\text{-}v/+}$ mice that lacked p53 showed reduced coat color pigmentation and spotting identical to their *Kit*$^{W\text{-}v/+}$:*Trp53*$^{+/+}$ and *Kit*$^{W\text{-}v/+}$:*Trp53*$^{+/-}$ littermates.

Our previous analyses, using a *Dct-lacZ* reporter transgene, showed that early melanoblasts can be detected emerging from the neural crest from day E10.5, well before they begin to produce pigment. A comparison of wild-type and homozygous *Kit*$^{W\text{-}v}$ embryos shows that at E10.5 mutant embryos contain fewer melanoblasts, although their spatial distribution over the head and rostral trunk is similar to wild type (MacKenzie *et al.*, 1997). By E11.5, the number of melanoblasts in mutant embryos has greatly decreased so that very few remain at E12.5 and none are present at later stages.

To examine whether doubly mutant *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ embryos show prolonged survival of melanoblasts during this critical period (E10.5–13.5), the *Dct-lacZ* transgene was crossed onto the compound heterozygote *Kit*$^{W\text{-}v/+}$:*Trp53*$^{+/-}$ animals. Embryos retrieved from matings between intercrosses of the compound heterozygotes, one of which contributed the *Dct-lacZ* transgene, were stained using XGal to assay the effects of both mutations on melanoblast development. Figures 1A–1C show the nine possible combinations of genotypes for *Kit* and *Trp53* obtained for embryonic stages E11.5 and Fig. 1D shows the transgene staining at E12.5 for *Kit*$^{W\text{-}v/W\text{-}v}$ embryos with all three *Trp53* genotypes. No increase in melanoblast survival was noted for the doubly mutant *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ embryos compared to their *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ and *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$ littermates for the stages examined (E10.5–13.5). By E13.5, no stained melanoblasts were detected in any *Kit*$^{W\text{-}v/W\text{-}v}$ embryo regardless of their p53 status. Thus the signal provided by Kit for melanoblast survival appears to act independent of p53 at both embryonic and adult stages.

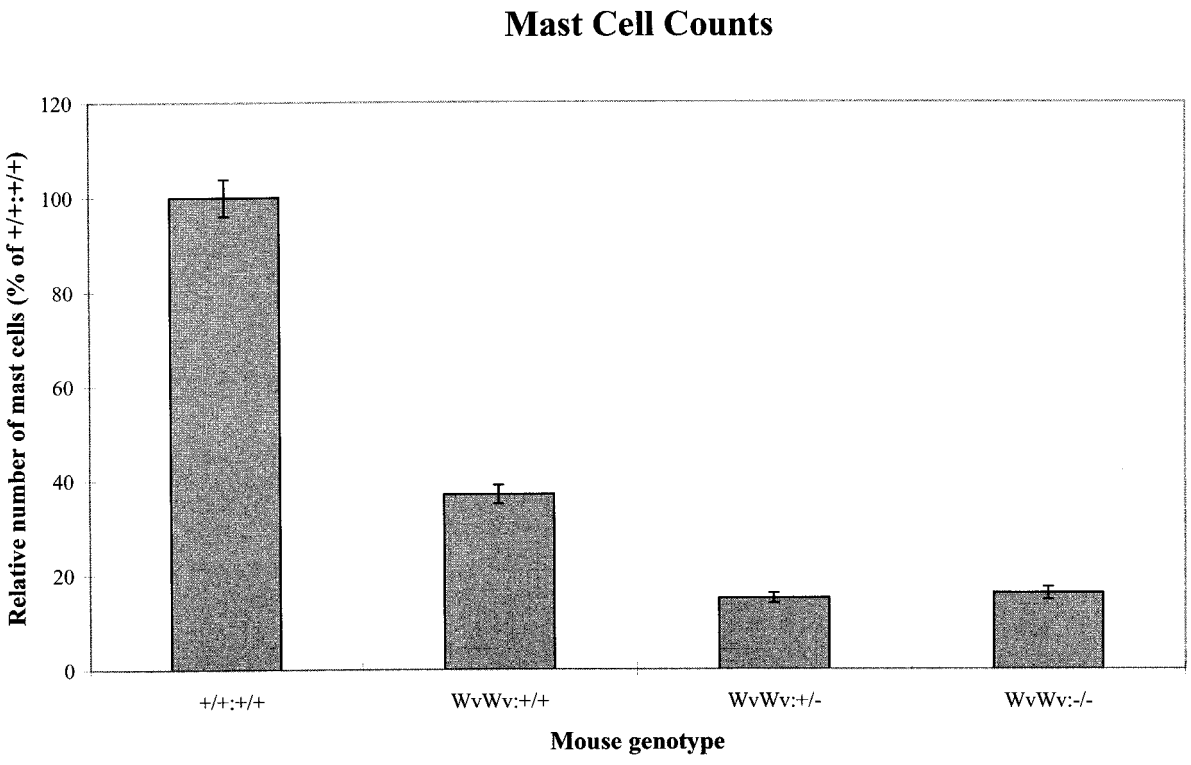

FIG. 2. Analysis of mast cell numbers present in the skin of $Kit^{W\text{-}v}$ and *Trp53* double-mutant mice. Tail skin was fixed and stained with Azur blue (see Materials and Methods). Eight mice from each genotype were analyzed and the mean number of Azur blue-positive cells from five microscope fields (×20 objective) was determined. The relative number of mast cells present in sections of skin from +/+:+/+, $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{+/+}$, $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{+/-}$, and $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{-/-}$ is expressed as a percentage of the number of mast cells present in +/+:+/+ mice.

Mast Cells

Kit homozygous mutant mice exhibit the hematopoietic phenotype of severe macrocytic anemia and mast cell deficiency. We analyzed tail skin from the offspring of compound heterozygotic intercrosses for the presence of mast cells using Azur blue staining. Eight mice were examined for each genotype. The number of mast cells in the skin of homozygous $Kit^{W\text{-}v/W\text{-}v}$ mice is significantly reduced to 37% (±2) relative to wild-type littermates ($P < 0.1$, $\alpha = 0.01$) (Fig. 2). $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{-/-}$ mice had a further decrease in mast cell number to 16% (±1.4) compared to wild-type littermates. Absence of Trp53 function does not lead to increased survival of these cells and may actually enhance mast cell death, although this data set does not provide statistical support for this hypothesis.

Germ Cells

We examined the gonads of the mutant animals for changes in germ cell numbers. Testes and ovaries were removed from adult mice (>6 weeks) generated from the compound heterozygous intercrosses. Histological sections of the gonads from at least three age-matched animals (both male and female) were examined for each of the nine different genotypes. The results of the histological analyses of control and $Kit^{W\text{-}v/W\text{-}v}$ mice (carrying varying numbers of mutant p53 alleles) are outlined in Table 1.

The ovaries and testes from $Kit^{W\text{-}v/W\text{-}v}$ mice regardless of their p53 status were reduced by approximately 85% in size compared to $Kit^{W\text{-}v/+}$ and $Kit^{+/+}$ mice. Only traces of Graafian follicles and no ova were present in $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{+/+}$, $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{+/-}$, and $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{-/-}$ ovaries compared to controls wild type at *Kit* with the three *Trp53* genotypes (Figs. 3A–3C). Similar numbers of follicles and mature ova were detected in $Kit^{W\text{-}v/+}$ ovaries, regardless of *Trp53* genotype. Homozygous $Kit^{W\text{-}v/W\text{-}v}$ ovaries, regardless of p53 status, show a densification of the fibrous stromal structure with ovarian stromal cells frequently arranged in thick swirling cords, consistent with what was previously observed for $Kit^{W\text{-}v/W\text{-}v}$ mutants (Coloumbre and Russell, 1954).

The mean testes masses from $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{+/+}$and $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{-/-}$ males were 15.9% (±1.4) and 26.6% (±3.3) of wild type, respectively. Histological examinations revealed abnormalities in many regions of the seminiferous tubules of $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{+/+}$ and $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{+/-}$ male testes. Vacuolization of the Sertoli cell cytoplasm and degenera-

TABLE 1
Histological Data from Adult Testes and Ovaries

Genotype	Sex	Description of gonads[a]	Sperm count $\times 10^6$ (mean ± SD)[b]
+/+:+/+	Male	All stages normal	354 ± 27
Kit$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$	Male	33% tubules have Sertoli and spermatogonia cells only Some pachytene cell arrest Increase in interstitial cells between tubules No sperm present in epididymis	0
Kit$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$	Male	10–38% tubules have Sertoli and spermatogonia cells only Partial pachytene arrest	3.5 ± 0.4
Kit$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$	Male	Most normal cells present ~1% tubules have Sertoli and spermatogonia cells only Lumen of some tubules packed with cells Majority of tubules show germ cell activity Sperm seen in most segments of epididymis	24 ± 0.2
+/+:+/+	Female	Normal histology: Graafian follicles and developing oocytes	
Kit$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$	Female	Traces of Graafian follicles Densification of the ovary, ovarian stromal cells in thick swirling cords	
Kit$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$	Female	Traces of Graafian follicles Densification of the ovary, ovarian stromal cells in thick swirling cords	
Kit$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$	Female	Traces of Graafian follicles Densification of the ovary, ovarian stromal cells in thick swirling cords	

[a] Histological sections were examined from three mice of each sex.
[b] Sperm counts were taken from three males.

tion of primary spermatocytes along with hyperplasia of the supporting cells were detected (Table 1, Figs. 3E and 3H). Approximately 33% of the tubules within the testes of these animals contained only Sertoli cells and spermatogonia with no mature sperm visible. Moreover, no sperm were detected in cross sections from the epididymis isolated from the mutant *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ and very few sperm were detected in the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$ animals.

In contrast, sections of the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ testes resembled the normal control testes (Table 1, Figs. 3D, 3F, 3G, and 3I). Only 1% of the tubules contained only Sertoli cells and spermatogonia and the majority showed some germ cell activity. Many areas within the tubules contained mature sperm (Fig. 3I) and sperm were also seen in most segments of the epididymis. However, the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ testes also retained some characteristics of the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ mutants with an increase in interstitial cell number between the tubules and an overall disorganized cellular appearance within the tubule lumens. In some cases apoptotic cells were clearly visible within the tubules (Fig. 3F).

The apparent histological differences between the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ testes and those from *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ and *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$ animals prompted us to determine mean epididymal sperm cell counts for each of the genotypes (Table 1). Three male mice were tested for each genotype. Compared to a sperm count of 354×10^6 from control wild-type littermates (+/+:*Trp53*$^{++}$), all the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ males tested had no detectable sperm. Interestingly, *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$ mice had a mean sperm count of 3.5×10^6, which increased to 24×10^6 for the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ mice. Thus in agreement with the histological data there is an increased survival of sperm in the double mutants.

In order to determine whether the rescue to approximately 10% of normal adult sperm counts in the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ mice could bring about a reversal of the sterility seen in *Kit*$^{W\text{-}v/W\text{-}v}$ homozygotes, paired matings were set up between mutant mice of either sex and control proven breeder F1 mice. Three mice each, of both sexes, with the following genotypes were tested: *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$, *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$, and *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$. Copulatory plugs were evident in the females when caged with the males, indicating that mating had occurred. All of the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ and *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$ mice appeared sterile and did not yield any offspring. The three *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ male mice each mated with three females as evidenced by copulatory plugs. Eight of these nine females gave birth to normal-sized litters (6–12 pups), further confirming the survival of mature sperm in the double male mutants. One of the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ males gave rise to three successive generations of offspring from the same female over a period of 3 months prior to being sacrificed for histological analysis. However, double-mutant females were infertile and failed to produce any offspring from matings with wild-type males.

We next examined testes from newborn males to compare numbers of germ cells at this stage between the

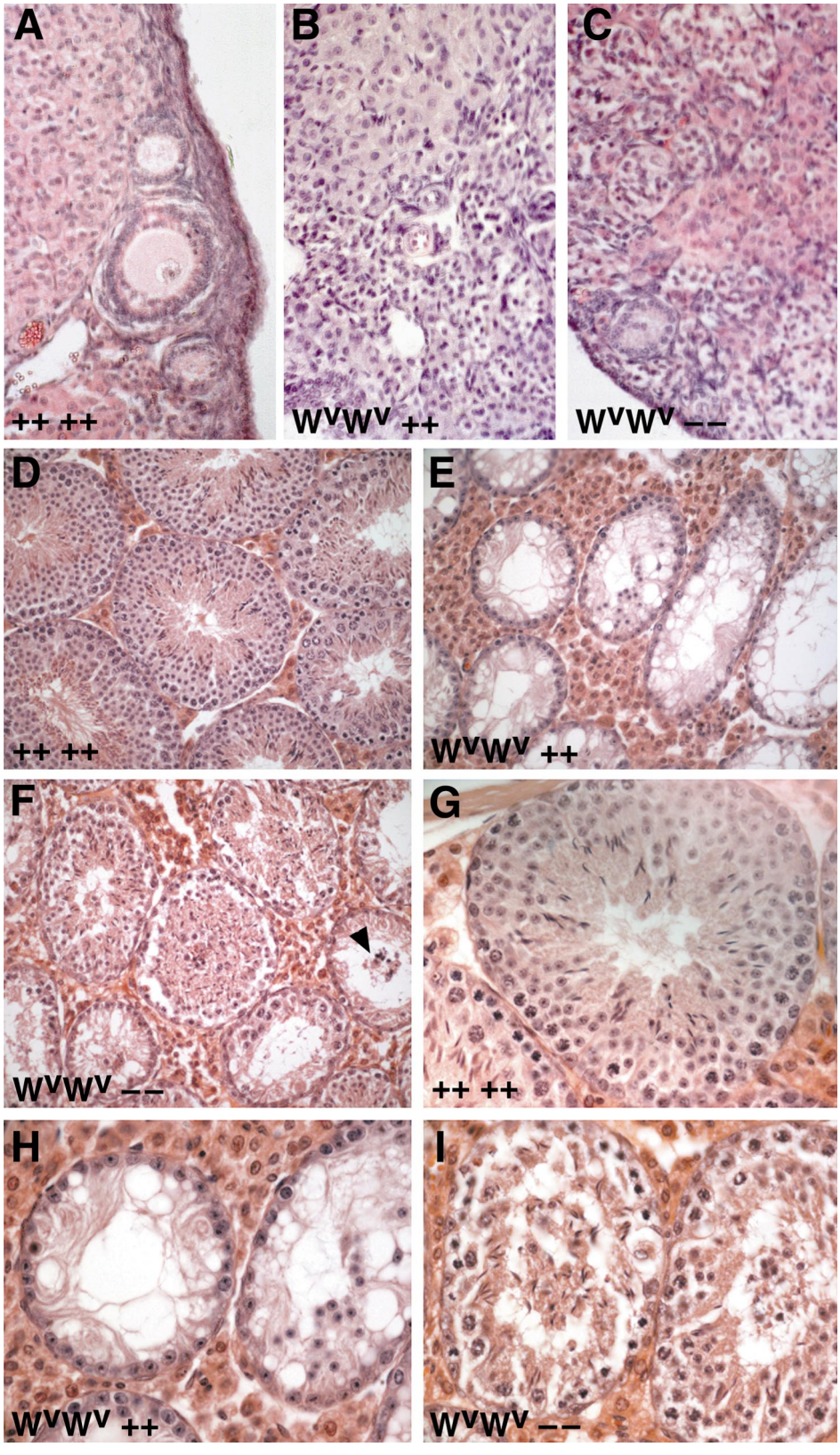

TABLE 2
Mean Genocyte Counts from Newborn Testes

Genotype	Genetic background	No. examined	Gonocyte count[a] (mean ± SD)	% of wild-type count
+/+:+/+	CBA, 129P/Ola	3	2.42 ± 0.39	
Kit$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$	CBA, 129P/Ola	5	0.30 ± 0.13	12.3 ± 5.3
Kit$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$	CBA, 129P/Ola	2	0.21 ± 0.04	8.6 ± 1.5
Kit$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$	CBA, 129P/Ola	3	0.82 ± 0.11	33.8 ± 4.5
+/+:+/+	129S2/SvPas	3	3.59 ± 1.06	
Kit$^{Lac\text{-}Z/LacZ}$:*Trp53*$^{+/+}$	129S2/SvPas	2	0.00	0

[a] Mean gonocyte cell count taken per 50 cross sections.

Kit$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ mice and control, *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$, and *Kit*$^{W\text{-}v/W\text{-}}$:*Trp53*$^{+/-}$ animals. Mean gonocyte counts were taken from 50 testes cross sections for each genotype (Table 2). Interestingly, the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ mice had 33.8% (±4.5) of the wild-type counts compared to 12.3% (±5.3) and 8.6% (±1.5), respectively, for the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ and *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$ animals.

To determine whether prenatal germ cells have an increased ability to survive we have stained mutant gonads from E13.5 embryos using alkaline phosphatase to assay germ cell numbers (data not shown). Female *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ gonads show a substantial reduction in stained cells compared to wild-type. There appears to be somewhat more germ cell staining in the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ gonads compared to those from *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ and *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$ embryos, suggesting some rescue of the germ cell population at this stage. However, this slight rescue is insufficient to support fertility in adult females. Male gonads from wild-type and *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ embryos show similar levels of intense germ cell staining while both *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ and *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$ gonads displayed patchy stained areas.

We have considered the possibility that differences in genetic background result in different phenotypic effects of the *Kit*$^{W\text{-}v}$ mutation on male germ cells. The *Kit*$^{W\text{-}v}$ mutation has been maintained on a CBA background, and the *Trp53* mutation was produced in ES cells, which derive from 129/Ola (129P/Ola) mice, and have been maintained on that background. As the rescue of male germ cells cosegregates with the *Trp53* mutation, if the effect were not caused by the mutation but by genetic background, then this could be explained only by a modifier of *Kit* mutant phenotype deriving from the 129P/Ola strain that maps in the vicinity of the *Trp53* gene. In order to exclude this possibility we examined the effect on germ cells of male mice mutant at *Kit* on a 129 strain background. Male mice homozygous for the *Kit*$^{W\text{-}LacZ}$ mutation, which was made by homologous recombination in ES cells, were used (Bernex *et al.*, 1996). As these mice die at birth or a few days later, usually before they are 3 days of age, newborn testes were examined. No gonocyte cells could be found in the *Kit*$^{W\text{-}LacZ/W\text{-}LacZ}$ testes, while wild-type littermate controls gave numbers similar to those obtained from a CBA background (Table 2). Thus, this 129 strain background (129/SvPas now called 129S2/SvPas) does not rescue the male germ cell defect of *Kit* mutation. Although there are some genetic differences between the Ola and the Sv substrains of 129 mice, simple sequence length polymorphic markers do not show differences between the substrains in the region of chromosome 11 around the *Trp53* locus, in contrast to other regions of the genome (Threadgill *et al.*, 1997; Simpson *et al.*, 1997). It appears that the increased germ cell survival and reversal of sterility observed in the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ males are due solely to the lack of the p53 apoptotic-inducing signal.

FIG. 3. Histological analyses of ovaries and testes from adult *Kit*$^{W\text{-}v}$ and *Trp53* double-mutant mice. (A–C) Ovaries from *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$, *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$, and *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ adult (9 week) mice stained with hematoxylin and eosin. No mature Graafian follicles or oocytes can be detected in the ovaries. Original magnification: ×200. (D and G) Testes sections from +/+:+/+ adult (9 week) male stained with hematoxylin and eosin. Tubules are in different stages of spermatogenesis and show a normal cell distribution. Original magnification: ×200 in D, ×400 in G. (E and H) Testes sections from *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$ adult (9 week) male stained with hematoxylin and eosin. Vacuolization of the Sertoli cell cytoplasm and hyperplasia of the supporting cells are evident. No mature spermatocytes can be detected. Original magnification: ×200 in E, ×400 in H. (F and I) Testes sections from *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ adult (9 week) male stained with hematoxylin and eosin. The majority of seminiferous tubules show some germ cell activity and mature spermatocytes are present. Cells undergoing apoptosis can be detected (arrowhead). Original magnification: ×200 in F, ×400 in I.

DISCUSSION

Activation of the signaling pathway controlled by the Kit receptor promotes cell division and the growth of three distinct lineages, melanocytes, mast cells, and germ cells. Activation of p53 leads to apoptosis in several cell types. Several stimuli, including withdrawal of growth factors, can cause p53-dependent apoptosis. Previous cell studies using bone marrow-derived mast cells (Yee *et al.*, 1994), bone marrow myeloid progenitor cells, irradiated thymocytes (Lotem and Sachs 1993), and erythroleukemia cells (Abrahamson *et al.*, 1995) suggest that the cell death induced by p53 can be inhibited the action of Mgf.

We have taken advantage of mice mutant in Kit and p53 to dissect the role of p53 in regulating cell death in the absence of Kit signaling *in vivo*. In contrast to the cultured cell studies we have found no evidence that deficiency of p53 prevents cell death due to the absence of Kit signaling in melanoblasts, melanocytes, or mast cells. It appears that cell death in these lineages occurs by a mechanism independent of p53. However, homozygosity for a mutation in *Trp53* ameliorated one aspect of the $Kit^{W\text{-}v}$ phenotype, namely male sterility.

No Increased Survival of Melanocytes or Mast Cells

Death of melanoblasts occurs in $Kit^{W\text{-}v/W\text{-}v}$ mutants from E11.5 and at later gestational stages no cells can be detected. Cell death of melanoblasts still occurs in embryonic double, $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{-/-}$, mutants and the adult double mutants are devoid of coat pigmentation. Melanocyte survival in the absence of Kit/Mgf interactions is not ameliorated by the absence of *Trp53*.

Exogenous Mgf acts as a survival factor for hematopoietic cells in culture (Brandt *et al.*, 1994) and mast cells derived from *W* mutant mice exhibit reduced or no survival in the presence of Mgf, depending on the severity of the mutation on Kit kinase activity (Reith *et al.*, 1990; Nocka *et al.*, 1990; Fujita *et al.*, 1988). We have shown that the loss of mast cells *in vivo* is not mediated by p53. The mast cell deficiency associated with the Kit mutation is ameliorated in mice doubly homozygous for mutations in both the Kit receptor ($Kit^{W\text{-}v}$) and the hemopoietic cell phosphatase ($Hcph^{me}$) genes (Paulson *et al.*, 1996). They show that the double $Kit^{W\text{-}v}/Kit^{W\text{-}v}$: $Hcph^{me}/Hcph^{me}$ mutant mice have approximately 75% of wild-type levels of mast cells, indicating that the absence of hemopoietic cell phosphatase function largely compensates for the almost complete loss of Kit receptor tyrosine kinase activity.

Rescue of the Male $Kit^{W\text{-}v/W\text{-}v}$ Sterility Defect in the Absence of p53

A two-step requirement for active Kit signaling in germ cells has been well documented. Initially, Kit is required for the migration of the germ cells from the yolk sac to the genital ridges in the embryo and after birth for the maturation of the spermatogonia (McLaren, 1980; Mintz and Russell, 1957; Coloumbre and Russell, 1954; Sorrentino *et al.*, 1991). From our analyses it would appear that the initial Kit requirement for migration of the germ cells to the germinal ridges and survival at this early stage during development is rescued by the lack of p53 in the double mutants. The $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{-/-}$ mutants show ~30% of wild-type gonocyte cell numbers at birth, but this decreases to ~10% of sperm counts by adulthood. In contrast, the $Kit^{W\text{-}v/W\text{-}v}$ mice have 12% of wild-type germ cells at birth and no sperm by adulthood. Therefore, the increased numbers of cells that survive to birth in the absence of Kit and Trp53 function are sufficient to allow maturation to spermatogonia and to support fertility in mutant males. This sperm maturation process can occur even in the presence of only low levels of Kit signaling. All the tested adult $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{-/-}$ males fathered offspring although their actual sperm levels were only approximately 10% of wild-type levels.

The panda mutation at the *Mgf* locus ($MgF^{Sl\text{-}pan}$), which results in a fivefold reduction in Mgf, allows normal production of sperm (Huang *et al.*, 1993; Bedell *et al.*, 1995). Interestingly, germ cell numbers in panda males at birth were reduced to 30% of controls similar to the levels observed in our $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{-/-}$ neonates. Meiotic spermatocytes were produced on schedule in juvenile panda males and there was no appreciable effect on male fertility. It would appear that levels of 10–30% of wild-type gonocytes and approximately 10% of adult sperm numbers are also sufficient to ensure fertility in these mutant males.

Absence of p53 Sperm Defects

Defects in sperm production or tubule histology have been previously reported for p53-deficient mice on varying genetic backgrounds. An increased level of multinucleate giant cells, spreading from the periphery into the lumen of the seminiferous tubules, was observed in p53-null mice from an inbred 129/Sv background. Homozygous tubules of C57BL/6J × 129/Sv mixed genetic background exhibited apparently normal tubules (Rotter *et al.*, 1993). Our analyses of single $Trp53^{-/-}$ or double-mutant $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{-/-}$ mice do not show any giant cell defects. Neither have we observed any increase in sperm head or tail defects in the $Trp53^{-/-}$ mice, in contrast to the higher numbers of abnormal spermatozoa and reduced fertility of Trp53-null mice on a C57BL/6J background (Yin *et al.*, 1998). The numbers of spermatozoa obtained from the $Trp53^{-/-}$ and control mice were comparable on our mixed CBA × 129/Ola genetic background.

Apoptotic cells were visible in the tubules from the $Kit^{W\text{-}v/W\text{-}v}$:$Trp53^{-/-}$ mice (Fig. 3F), despite the loss of Trp53. Thus cell death still occurs in the mutant tubules by a p53-independent pathway.

Cell Death in Female Germ Cells Is p53 Independent

Histological analysis indicates that lack of p53 in female germ cells cannot rescue death due to a reduction of Kit signaling. The mutant ovaries from the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/+}$, *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{+/-}$, and *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ mice all show similar defects. Oocyte growth and follicle cell growth and proliferation advance only to the earliest stages with no further progression for all three genotypes. In addition the double-mutant females failed to produce any progeny when mated with wild-type males. Germ cell death in the absence of Kit signaling appears to be independent of p53 in females.

Only 20% of normal oocytes survive at birth in *Sl*^{pan} female mutants (Huang *et al.*, 1993). The initiation and maintenance of ovary follicle development in juvenile and adult *Sl*^{pan} mice were affected and subsequent follicle development arrested at the one-layered cuboidal stage (Huang *et al.*, 1993; Bedell *et al.*, 1995). This phenotype is very similar to our observations of the ovarian defects in the *Kit*$^{W\text{-}v}$ mutants regardless of p53 genotype. Hence, a higher threshold level of germ cells is required at birth to enable complete oocyte maturation compared with that required for spermatogonia development.

Are p53-Independent Pathways Involved in Apoptosis Due to a Lack of Kit/Mgf?

If the melanocytes, mast cells, and female germ cells lacking p53 die in the absence of Kit what mechanism is involved? Cell death by p53-independent pathways has been previously documented both *in vivo* and *in vitro*. The remodeling of mammary gland tissue during involution proceeds through p53-independent pathways *in vivo* (Li *et al.*, 1996; Shibata *et al.*, 1996). Furthermore, in the developing mouse nervous system, cell death is p53-dependent in the central nervous system but p53-independent in the peripheral nervous system (Macleod *et al.*, 1996). More recently, it has also been shown that primary mast cell cultures derived from p53-null mice are still dependent on IL-3 for survival (Silva *et al.*, 1997). In contrast, another study (Gottlieb and Oren, 1998) showed that functional p53 is required for efficient apoptosis in response to IL-3 withdrawal in lymphoid cell lines. The pathways determining survival or death appear tissue and sex specific as from our analyses different mechanisms operate in both male and female germ cells.

The intracellular signaling pathways by which growth factors promote survival are poorly understood and much of the work has been carried out in disparate cell types and may not reflect the true situation *in vivo*. In recent years a number of secondary messengers including protein kinase C, mitogen-activated kinases, and phosphatidylinositol (PI)-3 kinases have been shown to be induced by Kit/Mgf signaling (Blume-Jensen *et al.*, 1993; Timokhina *et al.*, 1998; Hemesath *et al.*, 1998; Blume-Jensen *et al.*, 1994). Functional signaling presumably directs downstream signal transducers to block cell death. In the absence of Kit/Mgf signaling and subsequent secondary messenger activation, the apoptotic response can be engaged without a specific requirement for p53. Mgf appears unlikely to interfere directly with the apoptotic machinery, because the expression of the p53-responsive genes *bax* and *bcl-2* is not directly affected by its action (Digiuseppe *et al.*, 1999).

PI-3 kinase is required for Mgf-induced mitogenesis and cell survival and is itself activated by Kit/Mgf signaling (Blume-Jensen *et al.*, 1994; Serve *et al.*, 1995). A cascade of events occurs, mediated by PI-3 kinase, including activation of the serine/threonine protein kinase Akt, which in turn can activate and phosphorylate Bad, the proapoptotic molecule (Blume-Jensen *et al.*, 1998). Upon phosphorylation, Bad in turn is sequestered, preventing an apoptotic response (Zha *et al.*, 1996). Lack of Kit signaling in the *Kit*$^{W\text{-}v/W\text{-}v}$:*Trp53*$^{-/-}$ mutant melanocytes and germ cells may prevent the phosphorylation and subsequent removal of Bad, thereby inducing cell death even in the absence of *Trp53*.

In summary, we have shown that in the absence of Kit and p53 signaling, melanocyte and mast cell death still occurs; however, sufficient male germ cells can survive to support fertility. Different mechanisms appear to operate in different cell types *in vivo* in order to bring about life or death.

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