Expression of Arf Tumor Suppressor in Spermatogonia Facilitates Meiotic Progression in Male Germ Cells

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Abstract

The mammalian Cdkn2a (Ink4a-Arf) locus encodes two tumor suppressor proteins (p16Ink4a and p19Arf) that respectively enforce the anti-proliferative functions of the retinoblastoma protein (Rb) and the p53 transcription factor in response to oncogenic stress. Although p19Arf is not normally detected in tissues of young adult mice, a notable exception occurs in the male germ line, where Arf is expressed in spermatogonia, but not in meiotic spermatocytes arising from them. Unlike other contexts in which the induction of Arf potently inhibits cell proliferation, expression of p19Arf in spermatogonia does not interfere with mitotic cell division. Instead, inactivation of Arf triggers germ cell-autonomous, p53-dependent apoptosis of primary spermatocytes in late meiotic prophase, resulting in reduced sperm production. Arf deficiency also causes premature, elevated, and persistent accumulation of the phosphorylated histone variant H2AX, reduces numbers of chromosome-associated complexes of Rad51 and Dmc1 recombinases during meiotic prophase, and yields incompletely synapsed autosomes during pachynema. Inactivation of Ink4a increases the fraction of spermatogonia in S-phase and restores sperm numbers in Ink4a-Arf doubly deficient mice but does not abrogate γ-H2AX accumulation in spermatocytes or p53-dependent apoptosis resulting from Arf inactivation. Thus, as opposed to its canonical role as a tumor suppressor in inducing p53-dependent senescence or apoptosis, Arf expression in spermatogonia instead initiates a salutary feed-forward program that prevents p53-dependent apoptosis, contributing to the survival of meiotic male germ cells.

Introduction

The Cdkn2a-Cdkn2b gene cluster (also designated Ink4-Arf) encodes two polypeptide inhibitors (p16Ink4a and p19Arf) of cyclin D-dependent kinases (Cdk4 and Cdk6), as well as a third protein (p15Ink5) that antagonizes the Mdm2 ubiquitin E3 ligase to activate p53 [1]. Although the Ink4a and Ink4b genes likely arose through gene duplication, the structure of the Ink4-Arf gene cluster is highly unusual, as major portions of the p16Ink4a and p19Arf proteins are encoded by alternative reading frames of a shared exon [2]. Induction of p16Ink4a and p15Ink5 prevents the phosphorylation of the retinoblastoma protein (Rb), thereby maintaining Rb in its growth suppressive state and preventing entry into the DNA synthetic (S) phase of the cell division cycle. In contrast, p19Arf expression elicits a p53-dependent transcription program that either enforces cell cycle arrest or triggers apoptosis, depending on cell type, physiologic setting, and collateral modulating signals [1]. The Ink4-Arf genes prevent cell proliferation by implementing Rb- and p53-dependent programs that enforce cellular senescence and inhibit tissue regeneration as animals age, but their intimate genetic linkage facilitates their coordinate repression in embryonic and adult tissue stem cells, thereby allowing self-renewal [3,4]. Deleterious growth-promoting stimuli conveyed by activated oncogenes induce Ink4-Arf gene expression and engage both p53 and Rb to counter untoward cellular proliferation. Not surprisingly, bi-allelic deletion of the Ink4-Arf gene cluster abrogates this form of tumor suppression and is one of the more frequent events in human cancer.

Despite its canonical role as an inducer of p53 in response to oncogene signaling, Arf also has p53-independent tumor suppressive activity. Deletion of Arf together with Mdm2 and p53 expands the spectrum and decreases the latency of cancers that spontaneously arise in mice lacking p33, p53 and Mdm2, or Arf alone [5]. Although highly basic p19Arf (~20% arginine) has been reported to physically interact with more than 25 different proteins other than Mdm2, the role of p19Arf, if any, in regulating the functions of these putative “target” proteins remains controversial [6]. Indeed, numerous reports that p19Arf regulates such diverse processes as ribosomal biosynthesis, transcription, DNA repair, apoptosis and autophagy in a p53-independent manner have generally relied on experiments performed with cultured cells but have not been buttressed by more extensive in vivo analyses.

Although the Ink4-Arf locus is not detectably expressed under most normal physiologic conditions, eye and male germ cell development provide notable exceptions [7]. Arf is required for early postnatal regression of the hyaloid vasculature in the
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**Author Summary**

The intimately linked Arf and Ink4a genes, encoded in part by overlapping reading frames within the Cdkn2a locus, are induced by oncogenic stress, activating the p53 and Rb tumor suppressors, respectively, to inhibit proliferation of incipient cancer cells. As such, expression of the p19Arf and p16^Ink4a proteins is undetected in most normal mouse tissues. However, p19Arf is physiologically expressed in mitotically dividing spermatogonia, the progenitor cells that differentiate to form meiotic spermatocytes in which Arf expression is extinguished. We show that, instead of provoking cell cycle arrest or death, Arf expression in spermatogonia facilitates survival of their meiotic progeny, ensuring production of normal numbers of mature sperm. When Arf is ablated, meiotic defects ensue, along with p53-dependent cell death of spermatocytes, indicating an unexpected role of p53 in monitoring meiotic progression. Surprisingly, it is the absence of p19Arf rather than its induction that enforces p53 expression in this setting. Co-inactivation of Ink4a compensates for Arf loss by fueling proliferation of spermatogonial progenitors, but does not correct meiotic defects triggered by Arf loss. Although the Arf and Ink4a tumor suppressors are expected to restrain cellular self-renewal, Arf plays an unexpected role in male germ cells by facilitating their proper meiotic progression.

Arf Deficiency Compromises Sperm Production, But Is Compensated by Ink4a Inactivation

Total body weights of age-matched wild-type, Arf-null, Ink4a-null, and Ink4a-Arf double-null mice are equivalent, but testis weights of Arf-null animals were reduced relative to those of wild-type controls (Figure 2A), and this was associated with a significant reduction in numbers of mature sperm by the time Arf-null mice were two months old (Figure 2B). Nonetheless, young Arf-null males remain fertile, and despite the widespread use of independently derived Arf-null strains by us and others, there is no suggestion that young fertile males produce reduced litter sizes. Hence, defects in spermatogenesis were not previously appreciated.

Knock-in of a C DNA encoding Cre recombinase in place of the first Arf exon creates a functionally null Arf allele that expresses Cre in lieu of p19Arf under the control of the Arf promoter. Crossing Arf^Cre/+ females to homozygous males containing Arf alleles flanked by LoxP sites (“floxed” alleles) specifically results in the inactivation of Arf function in the testis of compound heterozygous Arf^Cre/+Arf^floxed male offspring. Although penetrance of Cre expression is not complete, more than 90% of spermatogonia in the seminiferous tubules of P21 mice had no detectable anti-p19Arf fluorescence signals [9]. Overall, while p19Arf was detected in the testes of haplo-insufficient Arf^Cre/+ mice, any residual levels of the protein in Arf^Cre/+Arf^floxed testes were too low to be detected by immunoblotting analysis (representative data illustrated in Figure 3), confirming significant Cre-mediated Arf deletion in this setting. We therefore used this “targeted” deletion approach to compare the Arf loss-of-function phenotypes of Arf^Cre/+Arf^floxed males with those of Arf^-/- males. Analysis of testis weights revealed no differences between those of Arf^Cre/+Arf^floxed and wild-type controls (Figure 2C). However, the sperm counts of Arf^-/- mice were reduced to levels approaching those of Arf^Cre/+Arf^floxed animals (Figure 2D). Notably, the Arf^Cre or Arf/Flox alleles alone had no significant effects in limiting sperm production unless coexpressed in compound heterozygotes. Therefore, tissue-restricted effects of Arf inactivation independently recapitulated those seen in mice that completely lack Arf function.

**Results**

**Arf Is Expressed in Mitotically Dividing Spermatogonia**

Lineage tracking experiments in the mouse previously revealed that all viable male germ cells are derived from spermatogonial progenitors in which transient Arf expression neither inhibits proliferation nor subsequent meiotic commitment [9]. Underscoring these findings, expression of p19Arf in young adult mice is observed in all types of spermatogonia, but not in Sox3-expressing Sertoli cells on the tubular basement membrane or in DAPI-stained intratubular spermatocytes, spermatids, or sperm (Figure 1A). The fact that p19Arf is not detected in cells that have detached from the basement membrane implies that Arf expression is extinguished at or near the primary spermatocyte stage of germ cell differentiation. Consistent with this interpretation, the Arf protein does not co-localize with Dmc1 [9], a meiotic recombinase expressed in leptotene spermatocytes. In the mature tests, spermatogenesis occurs in waves along the length of the seminiferous tubules, so that cross sections capture tubules in which dividing spermatogonia are in synchronous phases of the cell cycle. When five month-old mice injected intraperitoneally with BrdU were sacrificed two hours later, dual immunofluorescence analysis revealed that many cells on the tubular basement membrane that had synthesized DNA also expressed p19Arf (Figure 1B). Similarly, at P12 when the number of mitotically cycling progenitors exceeds those of more differentiated germ cells, p19Arf was co-expressed with cyclin D1, a G1 phase marker of proliferating spermatogonia [14] (Figure 1C), and strikingly, was detected during all stages of mitosis (Figure 1D, 1E). Therefore, in spermatogonia, p19Arf is expressed throughout the cell division cycle without interfering with proliferation.

**Arf-null mice form a retrolenticular mass predominantly composed of pericytes; the abnormal accumulation of these cells disrupts the retina and lens and leads to blindness [8]. Arf inactivation also results in a significant reduction of sperm production through as yet poorly defined mechanisms, although young male mice remain fertile [9]. In contrast, Arf-null females have no discernable reproductive defects.**

Spermatogenesis involves a stereotyped sequence of mitotic and meiotic divisions followed by sperm differentiation [10]. In mice, male germ cell progenitors (gonocytes) renew in the testis between days 1–7 postpartum (P1–P7) and generate spermatogonia that line the germ cell progenitors (gonocytes) renew in the testis between days 1–7. Meiotic divisions followed by sperm differentiation [10]. In mice, male fertility is restored when mature spermatozoa enter the epididymis. As spermatogenesis rapidly by meiosis-II, and by spermiogenesis (P19–P35), after which the recombination [13]. Meiosis-I is completed by P18, and is followed complexes and exchange genetic information through homologous During the extended prophase of meiosis-I, homologous pairs of membrane, whereas spermatocytes, spermatids, and mature sperm tubules remain localized on the peripheral tubular basement.
Figure 1. **Arf protein expression in mitotically dividing spermatogonia.** Protein expression in sections of seminiferous tubules were determined by immunofluorescence analysis. (A) p19\textsuperscript{Arf} (green, left panel) is expressed in spermatogonia that intervene between Sox9-expressing Sertoli cells (red, middle panel) in the seminiferous tubules of adult 4 month-old mice. The right panel shows merged images documenting no overlap in expression of the two proteins. Unlabeled cells within the lumina of the tubules are visualized with DAPI. (B) After a 2 hour *in vivo* pulse of bromodeoxyuridine (BrdU) in adult 5 month old mice, p19\textsuperscript{Arf} expression (green, left panel) was revealed in spermatogonia that had incorporated BrdU (red, middle panel). The right panel shows merged images documenting co-expression of both markers in many spermatogonia at the tubular periphery (yellow). (C) In seminiferous tubules from P12 mice, cells expressing p19\textsuperscript{Arf} (green, left panel) co-express cyclin D1 (red, middle panel), a protein expressed in actively cycling spermatogonia; a merged image is shown at the right. (D, E) Spermatogonia within the seminiferous tubules of P15 mice express p19\textsuperscript{Arf} (green) during mitosis. Examples of p19\textsuperscript{Arf} expression during metaphase (D) and telophase (E) are shown. DAPI (blue) highlights the nuclei of intratubular germ cells and somatic Leydig cells that occupy the intertubular space. Scale bars: (A, D, E) 50 \textmu m; (B, C) 100 \textmu m. 

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Hormone signaling networks are involved in the proper control of spermatogenesis. Key regulatory gonadotrophins include luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secreted by the anterior pituitary gland, and testosterone produced by testicular interstitial Leydig cells. The considerable day-to-day and even hour-to-hour variation over a 30-fold range in plasma testosterone levels in age-matched mice of a single strain precluded accurate measurements of strain-specific differences, even in a relatively large sample size (Figure 4) [15]. Importantly, however, no discernable defects in pituitary or Leydig cell development have been observed in Arf-null, Ink4a-null, or doubly-deficient mice, and no significant differences were observed in the ranges of serum FSH and LH among all genotypes examined (Figure 4). These findings suggest that spermatogenesis defects in Arf-deficient mice are not a secondary consequence of hormonal imbalances.

Unlike Arf-null males, those lacking functional Ink4a instead exhibit increased testis weights and produce higher numbers of sperm than wild type mice (Figure 2A and 2B). Cdk4, the major target of p16Ink4a protein inhibition in the adult testis, is expressed at maximal levels at the earlier stages of spermatogenesis, where spermatogonia undergoing mitotic cell divisions predominate [16,17], and Cdk4 inactivation compromises male fertility [18,19]. We therefore quantified the in vivo incorporation of BrdU in spermatogonia of young adult wild-type, Arf-null, Ink4a-null, and Ink4a-Arf-null mice by counting stained cells that had entered S phase during a two-hour pulse. The S phase fractions of wild-type and Arf-null spermatogonia did not differ from each other (Figure 5), implying that the failure of Arf-null mice to produce normal numbers of sperm reflects a loss of meiotic cells or their

Figure 2. Decreased sperm production in Arf-null mice is compensated by loss of Ink4a or p53. (A, C) Testes were dissected from adult (2–6 month old) mice of the indicated genotypes and weighed as pairs. (B, D) Caudal epididymides were collected from corresponding mice, and recovered sperm were enumerated using a hemocytometer. Relative testes weights (A) and sperm counts (B) are reduced in Arf-null males but increased in Ink4a-null mice. Ink4a-Arf double-null and p53−/−; Arf−/− double null mice exhibit increased testes weights (A) and sperm counts (B). While testes weights (C) are not significantly reduced in ArfCre/Flox males, reduced sperm counts (D) mimic the Arf loss-of-function phenotype. N = 20–32 mice (A, B) and 5 mice (C, D). Bars represent standard deviations from the mean. P values were determined using a Student’s t-test (*p<0.001, **p<0.0001) and designate significant differences from the wild type genotype.

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immediate progeny rather than spermatogonia. In contrast, we observed a significant two-fold increase (p \textless 0.0001, Student’s t-test) in the relative number of S phase spermatogonia from both strains that lack Ink4a (Figure 5). Looking only at testis weights and sperm counts in Ink4a-Arf double-null animals, Ink4a inactivation appears to compensate for loss of Arf function (Figure 2A and 2B), presumably by fueling the production of a greater number of mitotic progenitors. Together, the consequences of these two independent loss-of-function effects rebalance testis size and sperm output in the doubly null strain. In this sense, these two “tumor suppressor” genes play opposing physiologic roles in male germ cell development.

Arf Tumor Suppressor Regulates Spermatogenesis

Tests from two month-old Arf-null mice exhibited a significant increase in the numbers of apoptotic (TUNEL-positive) cells when compared to age-matched wild-type controls (Figure 6A and 6E). The vast majority of apoptotic cells are spermatocytes as judged by the topological relation of TUNEL-positive cells to the expression of the meiosis-specific strand-exchange protein Dmc1, which is expressed during early prophase-I (Figure 6A). Notably, however, intratubular TUNEL-positive cells were not stained with antibodies to Dmc1, implying that Arf-null cells die during a later stage of germ cell development after Dmc1 expression is greatly diminished. To examine this issue further, we conducted TUNEL staining of meiotic chromosome spreads. Characteristic stages of prophase during meiosis-I can be marked by staining chromosomes with antibodies to synaptonemal complex proteins, such as the axial element component SYCP3 [20], and by several ancillary criteria (see Materials and Methods). Unlike pachytene cells from wild-type mice, those from the Arf-null strain exhibited considerable TUNEL staining (Figure 6C) with a concomitant reduction in the fraction of Arf-null diplotene spermatocytes (Figure 6F) that correlated with decreased sperm production (Figure 2B). Inactivation of Ink4a alone did not trigger spermatocyte apoptosis nor limit apoptosis in the Arf-null background (Figure 6E) reinforcing the conclusion that the two closely linked genes play fundamentally different roles within the male germ line.

Arf Inactivation Leads to p53-Dependent Apoptosis in Primary Spermatocytes

Figure 5. Increased frequency of BrdU-incorporating spermatogonia in Ink4a-null mice. Quantification of BrdU-positive spermatogonia in five month-old wild-type, Arf-null, Ink4a-null, and doubly Ink4a and Arf-null mice was determined two hours after intraperitoneal BrdU administration. BrdU-labeled cells were scored in 100 tubules in testis sections from seven different mice. Error bars indicate standard deviations from the mean. ** p \textless 0.0001 vs wild-type by Student’s t-test.

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Arf”/-; p53”/- doubly-deficient males are even more susceptible to spontaneous tumor development than mice lacking either Arf or p53 alone [5]. However, the young tumor-free males produce sufficient viable sperm to remain fertile. Inactivation of p53 restored testis weights and sperm production in Arf-null males (Figure 2A and 2B), prevented the apoptotic elimination of Arf-null germ cells (Figure 6D and 6E), and restored the number of spermatogonia.
diplotene spermatocytes (Figure 6F). Accordingly, higher levels of p53 were detected in whole testis lysates from Arf-null mice as compared to those in wild-type mice (Figure 7). Thus, in direct contrast to the role of p19Arf in triggering a p53 response following abnormal hyperproliferative stress in somatic cells, it is instead the absence of Arf expression in spermatogonial progenitors that impairs the fidelity of meiotic progression and ultimately leads to p53-dependent elimination of Arf-null primary spermatocytes.

Inappropriate γ-H2AX Accumulation in Arf-Null Germ Cells

Histone H2AX is phosphorylated at serine-139 in response to DNA strand breaks caused by ionizing radiation [21], UV irradiation [22], replication stress [23,24], failure of nucleotide excision repair [25], and at the leptotene stage of meiosis prior to synaptonemal complex formation [26]. H2AX phosphorylation also occurs in spermatocytes in a DNA damage-independent manner during formation of the sex body, a heterochromatic subnuclear domain encompassing the nonhomologous parts of the X and Y chromosomes [27]. Remarkably, staining of testis sections and immunoblotting of whole testis lysates revealed a profound increase in global γ-H2AX levels when Arf was inactivated (Figure 6A and 6B). Again, inactivation of Ink4a neither recapitulated nor ameliorated this Arf-null defect (Figure 6A and 6B). Germ cells at the periphery of Arf-null seminiferous tubules exhibited the greatest increase in γ-H2AX staining, suggesting that more immature cells were the ones most affected (Figure 8A). Microscopic quantification revealed that the number of γ-H2AX-positive spermatogonia, as well as the number of γ-H2AX foci per cell, were increased ~2-fold when Arf was inactivated (Figure 9), but the greatest increase in γ-H2AX staining was observed in primary Arf-null spermatocytes (Figure 8A and 8B; see below). The increased γ-H2AX in meiotic cells is especially striking because these cells do not normally express p19Arf when the gene is present (Figure 1).
Meiotic double-strand DNA breaks are induced by the Spo11 transerase and its accessory factors, which are loaded onto chromatin during the final pre-meiotic S-phase [13]. Autosomal γ-H2AX staining is normally observed during the leptotene and zygote phases of meiosis-I, which are the early stages at which chromatids undergo DNA scission as a prelude to homologous recombination. In contrast, γ-H2AX foci are not normally detected by early pachytene (except in the sex body) once homologous synapsis is complete (Figure 8C, top panels) [28]. Chromosome spreads from meiotic primary spermatocytes from Arf-null males revealed that 150 of 382 individually enumerated pachytene cells (39%) exhibited persistent autosomal γ-H2AX foci in addition to normal sex body staining, whereas very few such cells (6.7%) were detected at the diplotene stage (Figure 8C, bottom panels). It could be that disappearance of γ-H2AX is delayed until diplonema, or that cells with aberrantly elevated γ-H2AX are preferentially eliminated. The latter interpretation is supported by the prophase I apoptosis and depletion of diplotene cells observed in Arf-null mice (Figure 6). Therefore, in the absence of Arf, γ-H2AX accumulates to higher levels than normal starting in the least mature spermatogonia, continuing into meiotic prophase I, and persisting past the time when it would normally disappear from autosomes. Although inactivation of p53 suppresses the increased apoptosis of Arf-null spermatocytes, γ-H2AX persists in cells lacking both of these genes (Figure 8A, lower right panel). In meiotic chromosome spreads from p53; Arf double-null
mice, 20.5% (34 of 166) of diplotene spermatocytes display persistent autosomal $\gamma$-H2AX immunostaining as compared to 6.7% (5 of 74) of singly $Arf^{-/-}$null and less than 1% (1 of 149) of wild-type diplotene spermatocytes. These data underscore the fact that the accumulation of $\gamma$-H2AX in $Arf^{-/-}$ spermatocytes is $p33$-independent, whereas the elimination of defective spermatocytes that retain $\gamma$-H2AX inappropriately is $p33$-dependent.

Additional Meiotic Prophase Defects in $Arf^{-/-}$ Primary Spermatocytes

DNA double-strand breaks induced early in prophase I by Spo11 serve as substrates for the strand exchange proteins Rad51 and meiosis-specific Dmc1, which are required for double strand break repair during homologous recombination. Foci of staining using antibodies to Dmc1 (Figure 10A) and Rad51 (Figure 10B) were readily observed in zygotene spermatocytes from wild-type mice (left panels) but were fewer and less prominent in their $Arf^{-/-}$null counterparts (right panels). The number and average fluorescence intensities of foci in 100 zygotene cells of each genotype were determined using commercial imaging software. In wild-type zygotene spermatocytes, the frequency of Dmc1 and Rad51 foci peaked at 100–125 per cell (Figure 10E and 10F, blue bars) and exhibited a broad distribution of relative intensities over a ~10-fold range (Figure 10E and 10F, blue bars). In contrast, both the number and intensities of Dmc1/Rad51 foci were significantly reduced in $Arf^{-/-}$null cells (average number of foci $\pm$ S.D.: 103 $\pm$ 46 Dmc1 foci in wild-type vs. 44 $\pm$ 34 in $Arf^{-/-}$; 108 $\pm$ 45 Rad51 foci in wild-type vs. 45 $\pm$ 33 in $Arf^{-/-}$; average relative intensities $\pm$ S.D.: 10239 $\pm$ 4959 Dmc1 foci in wild-type vs. 6611 $\pm$ 3005 in $Arf^{-/-}$; 10980 $\pm$ 5783 Rad51 foci in wild-type vs. 5637 $\pm$ 3340 in $Arf^{-/-}$; N = 100, $p < 0.0001$, Student’s t-test; Figure 10C–10F). An accumulation of $Arf^{-/-}$null spermatocytes in zygonema (Figure 6F) suggests that there may be a delay at this stage before progression to pachytene.

Pachytene cells are normally characterized by well developed synaptonemal complexes that stretch the length of autosomal axes and by knob-like accumulation of SYCP3 at teloceres (Figure 11A). However, 34% of $Arf^{-/-}$null cells exhibited defects in synopsis (quantified in Figure 11D), including forked terminal structures and interstitial bubbles on autosomes (Figure 11B) and complete asynapsis of sex chromosomes (arrow, Figure 11C). In addition, interrupted regions of SYCP3 staining (denoted by arrowheads in Figure 11C) were more frequently observed in meiotic chromosome spreads from $Arf^{-/-}$null cells versus those in wild-type cells (191 versus 53 such segments, respectively, in 300 pachytene cells of each genotype). Because synopsis was complete in the majority of $Arf^{-/-}$null pachytene cells, we could not distinguish whether the observed defects arose from regions in which synaptonemal complexes did not form at all, or where complexes had formed but subsequently disassembled. Taken together, $Arf^{-/-}$ deficiency results in a series of abnormalities during prophase I that include reduced loading of the Rad51 and Dmc1 recombinases, defects in synopsis, elevated and persistent $\gamma$-H2AX expression, and p53-dependent apoptosis, ultimately associated with diminished production of mature sperm.

Discussion

With few exceptions, the $Arf^{-/-}$ tumor suppressor is not expressed in normal tissues of healthy mice but is induced by abnormally sustained and elevated thresholds of proliferative signals, activating a p53 response that opposes the deleterious effects of oncogene activation. Notably, p53 responds to a much wider range of $Arf^{-/-}$dependent signal transduction cascades triggered by many other forms of cellular stress, including acute DNA damage, to which the $Arf^{-/-}$ promoter does not respond [6]. By converging on p53, these different signaling pathways inhibit cell cycle progression or trigger apoptosis, acting to suppress tumor formation.

We now document a physiological role of $Arf^{-/-}$ in mouse male germ cell development that is distinct from its tumor suppressive functions in key respects. First, $Arf^{-/-}$ is expressed in spermatogonia, but not in the primary spermatocytes that arise from them. Expression of p19$Arf$ neither arrests spermatogonial mitotic progression nor triggers their p53-dependent apoptosis. However, the absence of $Arf^{-/-}$ expression in spermatogonia leads to p53-dependent apoptosis of spermatocytes before they exit meiosis-I. The defect in spermatogenesis is germ cell autonomous and results in a significant reduction in sperm counts by the time $Arf^{-/-}$null mice are two months old, although residual sperm production maintains fertility in young males. Thus, expression of $Arf^{-/-}$ in mitotic progenitor cells enhances the survival of their meiotic progeny in which $Arf^{-/-}$ expression is normally extinguished. These features indicate that $Arf^{-/-}$ expression initiates a salutary, feed-forward program that facilitates meiotic progression. Indeed, although $Arf^{-/-}$ and Ink4a are widely viewed to convey tumor suppressive functions that coordinate the activities of the p53 and Rb signaling “pathways,” inactivation of $Arf^{-/-}$ and Ink4a in the testes leads to opposing outcomes. Disruption of Ink4a increases the mitotic activity of spermatogonial progenitors to enhance sperm output and, in this respect, compensates for $Arf^{-/-}$ loss of function without eliminating the cellular defects that arise in the $Arf^{-/-}$null setting. In short, loss of Ink4a increases the spermatogonial pool size, but without $Arf^{-/-}$ expression, spermatocytes undergo increased apopto- sis, returning the number of mature sperm to normal levels.

Homologous recombination during meiosis exchanges genetic information between maternally and paternally derived chromosomes and also guides proper segregation of chromosome pairs to maintain correct chromosome numbers in gametes [13]. During meiosis, in contrast to mitotically diving cells, homologous chromosomes are favored over sister chromatids as templates for recombinational DNA repair. Double-strand DNA breaks are formed by the topoisoamerase-II-related transesterase Spo11. This process activates the Atm kinase and leads to phosphorylation of...
the H2AX histone variant near sites of strand breakage during early prophase I. Binding of the RecA family strand exchange proteins, Rad51 and meiosis-specific Dmc1, to Spo11-induced DNA ends generates filaments that search for and invade homologous duplex DNA molecules, leading to pairing of homologous chromosomes. Loading of Rad51 and Dmc1 is normally reversed by early pachytene when chromosomes are fully synapsed, after which γ-H2AX foci are no longer detected.

In the Arf-null setting, a modest but significant increase in γ-H2AX staining was first detected in the least mature spermato- gonia, and primary spermatocytes displayed accentuated signals that persisted inappropriately into the pachytene stage. Arf-null cells also formed fewer Dmc1/Rad51 foci at zygotene and exhibited focal regions of asynapsis at pachytene. Aberrant Arf-null spermatocytes underwent apoptosis at pachytene, resulting in the emergence of fewer diplotene cells and a significant reduction in sperm output. Importantly, Arf<sup>−/−</sup>; p53<sup>−/−</sup> double-null pachytene cells also exhibited persistent γ-H2AX staining, but these cells escaped elimination. Thus, apoptosis was p53-dependent, but aberrant γ-H2AX accumulation was not.

Although the underlying mechanisms remain unknown, we consider here two plausible interpretations of this apoptotic arrest.
First, it may be that reduced Rad51/Dmc1 focus formation and persistent γ-H2AX staining in Arf-null male germ cells connotes a defect in DNA repair that then activates p53 through Atm/Atr-dependent signaling pathways. In this scenario, Spo11-induced DSBs would form at normal levels but Rad51/Dmc1 loading would be impaired such that some DNA damage would persist into pachytene. This might conceivably involve the p53-independent ability of p19Arf to promote the sumoylation of numerous target proteins by inhibiting the SUMO2/3 protease Senp3 [29–31]. SUMO2/3 accumulates at sites of DNA damage in mammalian cells [32,33], and various aspects of DNA repair are regulated by the SUMO conjugation pathway [34]. There is fragmentary evidence that absence of p19Arf compromises nucleotide excision repair in cultured cells [35,36] raising the possibility that Arf may play an as yet undefined role in promoting homologous recombination. All meiotic mutants that cannot properly synapse homologous chromosomes arrest during pachytene [37], and accompanying defects in sex body formation and failure to properly silence transcription of the sex chromosomes during prophase is itself sufficient to eliminate pachytene cells [27,38]. However, spermatocytes can also undergo apoptosis in direct response to un repaired Spo11-induced breaks even if sex body formation is normal [27,39]. Where tested, spermatocyte apoptosis in meiotic mutants with chromosome synopsis errors has been found to be p53-independent [40–42]. Moreover, Spo11-dependent activated phospho-p53 can be transiently detected from leptonema and zygonema in wild-type male mice, and in Drosophila, p53 activity is prolonged in cells defective for meiotic repair [43]. Thus, it remains a formal possibility that meiotic recombination defects can trigger p53-dependent apoptosis.

A second, alternative interpretation rests on the idea that the earlier and less profound accumulation of γ-H2AX in Arf-null spermatogonia might be a symptom of an underlying defect affecting chromatin structure or Atm/Atr signaling. The appearance of γ-H2AX reflects chromatin modifications that flank sites of DNA damage rather than strand breaks themselves, so the kinetics of γ-H2AX formation and dissolution do not necessarily coincide with the appearance and repair of DNA damage [44,45]. Moreover, aberrant Atm/Atr signaling is itself sufficient to activate p53, whether triggered by DNA breaks or not [46]. Thus, it may be that Arf deficiency causes inappropriate Atm/Atr signaling that provokes p53-dependent apoptosis in a DNA damage-independent manner. In this view, the observed meiotic prophase defects in Arf-null spermatocytes may possibly be a separate downstream consequence of this earlier anomaly, and may not be the cause of apoptosis. Regardless of which interpretation is correct, it is important to note that our findings provide strong evidence that p53-dependent monitoring promotes proper meiotic maturation, in addition to the previously documented p53-independent pathway(s). Whatever the underlying mechanisms, the role of Arf in male germ cell development contrasts with the general paradigm of p19Arf acting as an activator of p53. Instead, it is the absence of Arf in spermatogonia that consequently leads to p53-dependent apoptosis of spermatocytes.

Materials and Methods

Ethics Statement

No human or non-human primates were studied. All animal work with mice was performed under established guidelines and supervision by the St. Jude Children’s Research Hospital’s Institutional Animal Care and Use Committee (IACUC), as required by the United States Animal Welfare Act and NIH policy to ensure proper care and use of laboratory animals for research. Experiments were undertaken in an accredited facility of the Association for Assessment of Laboratory Animal Care under the supervision of trained veterinary personnel and in strict compliance with Howard Hughes Medical Institute, St. Jude Children’s Research Hospital, and NIH institutional guidelines. The latter include detailed protocol submission and review of all animal care, monitoring, and experimental procedures prior to initiation of any experiments. Ongoing protocols for animal research not necessitating interim amendments are minimally subjected to annual review by the IACUC. All persons involved in the use of animals have read and understand all implications of pertinent protocols, have received training in the execution of relevant animal-related procedures prior to participation in the protocol, and have participated in educational or training programs deemed necessary by the IACUC or the Animal Resources Center personnel. Studies reported herein did not unnecessarily duplicate previous research, and were undertaken only because suitable non-animal models were unavailable. The number of animals used was consistent with good statistical design. Anesthesia, analgesia and tranquillization were used to relieve pain and distress in accordance with the IACUC recommendations.
Mouse Strains

Arf-null [47], Arf-GFP [7], Arf-Flox and Arf-Cre mice [9] were generated in the Sherr laboratory. Mouse strains deficient for Ink4a [48] and Ink4a-Arf [49] were generously provided by R.A. DePinho (Dana Farber Cancer Center). All genetically engineered mice were backcrossed nine or more times onto a C57Bl/6 background to create isogenic strains. C57Bl/6 mice deficient for p53 were purchased from Jackson Laboratories (Stock Number 2101). Arf-GFP/GFP mice were crossed to p53<sup>-/-</sup> mice, and compound heterozygotes were interbred to generate Arf<sub>GFP/GFP</sub>, p53<sup>-/-</sup> mice functionally null for both genes. Arf<sub>C57Bl/6</sub> females were interbred with Arf<sub>GFP/Flox</sub> males to generate Arf<sub>C57Bl/6</sub>/Arf<sub>GFP/Flox</sub> mice.

Phenotypic Characterization of Mouse Testes and Sperm Count Analysis

Caudal epididymides were harvested before dissection of the testes. For each male mouse, two cauda were minced into 1 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM HEPES buffer (pH 7.5) and 4 mg/ml bovine serum albumin and incubated at 37°C for 20 minutes. Suspensions of sperm were fixed at a 1:25 dilution in 10% formalin and counted on a hemocytometer. All sperm counts were performed between 1:00–3:00 PM. Dissected testes were weighed in pairs.

Immunofluorescence of Testes Sections

Mice were euthanized by CO<sub>2</sub> asphyxiation, and testes were removed and fixed overnight at 4°C in 4% paraformaldehyde followed by saturation in 30% sucrose at 4°C overnight. Tissues were embedded in TBS Tissue Freezing Medium (Fisher Scientific, Pittsburg PA), and then sliced with a HM500M Cryostat (Microm International, Walldorf, Germany) into 10 μm sections. Fixed and frozen samples were sectioned and subjected to antigen retrieval in 0.1 M Na citrate buffer, pH 6.0, followed by one hour incubation at room temperature in a blocking solution of 10% normal goat serum (NGS), 0.1% Triton-X 100 in phosphate-buffered saline (PBS), and then by overnight incubation at 4°C in primary antibodies diluted in 3% NGS, 0.1% Triton-X 100 in PBS. Antibodies were directed to p19<sub>Arf</sub> [rat monoclonal immunoglobulin 5C3-1] [50], Sox9 (Millipore AB5335, 1:1000), BrdU (Santa Cruz sc32323, 1:100), cyclin D1 (Santa Cruz 72-13G, 1:750), Dmc1 (Santa Cruz H-100, 1:750), γ-H2AX (Cell Signaling 2577, 1:200), and SUMO2/3 (Cell Signaling S139, 1:500), and detected using antibodies to Ig-Alexa Fluor 555 or Ig-Alexa Fluor 488 (1:500 dilutions; Invitrogen). Slides were washed three times in PBS, and then incubated for 1 hour at room temperature in 3% NGS, 0.1% Triton-X 100 in PBS containing the relevant secondary antibodies conjugated to Ig-Alexa Fluor 555 or Ig-Alexa Fluor 488 (1:500 dilutions; Invitrogen). Slides were washed three times in PBS and mounted with Vectashield (Vector Labs) containing DAPI. Surface spread spermatocytes were visualized by a Marinas spinning-disc confocal microscope, and images were assembled and analyzed using Slidebook 5.0 SDC software (Intelligent Imaging Innovations, Denver CO). Meiotic spreads from three adult mice (age three months) were analyzed. One hundred spermatocytes were scored each from mouse.

Distinct staining patterns allow for classification of each stage of meiotic prophase [51,52]. Leptotene cells were categorized by short stretches of axial elements accompanied by intense γ-H2AX staining throughout the nucleus and the absence of a distinct sex body. Zygote cells also display intense γ-H2AX staining throughout the nucleus and lack a sex body, but can be distinguished by longer stretches of SYCP3 staining, some of which are synapsed. Pachytene cells have fully formed and synapsed axes that appear as thick, continuous SYCP3-stained threads, while displaying intense γ-H2AX staining only in the sex body. Dmc1 and Rad51 foci are normally present at leptotene and zygote, and largely disappear by pachytene. Diplotene cells have γ-H2AX localized only to the sex body, but fully formed axes are desynapsing and chiasmata are visible.

Immunoblotting

As previously described [53], detergent lysates were prepared, and protein concentration was quantified by bicinchoninic acid assay (Pierce). Samples (25–75 μg protein per lane) were electrophoretically separated on 4% to 12% Bis-Tris NuPAGE gels (Invitrogen), transferred to polyvinylidene fluoride membranes (Millipore), and detected using antibodies to γ-H2AX (Cell Signaling S139, 1:500), p19<sub>Arf</sub>MC (5C3-1; Bertwistle et al. 2004b), p53 (Cell Signaling 1C12, 1:500), and actin (Santa Cruz C-11, 1:500) to control for protein loading.

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Author Contributions

Conceived and designed the experiments: MLC IR MJ SK CJJS. Performed the experiments: MLC. Analyzed the data: MLC CJJS. Wrote the paper: MLC CJJS SK MJ.

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