Age-dependent alterations in spermatogenesis in *itchy* mice

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Abbreviations: PND, postnatal day; cFLIP, cellular FLICE-like inhibitory protein; TRAIL, TNF-related apoptosis-inducing ligand

Spermatogenesis is an intricate process in which spermatogonial stem cells divide and differentiate to produce mature sperm. This process strongly depends on protein turnover both in the developing germ cells and the supportive Sertoli cells, and recent evidence has demonstrated the role of the ubiquitin-proteasome system in this protein turnover in the testis. Itch, an E3 ligase important in the immune system, has been implicated in regulating the blood testis barrier. Although the specific role of Itch during spermatogenesis is not yet well understood, its ubiquitous expression and wide array of functional targets suggest multiple and tissue-specific roles. Here the testes of mice that lack Itch protein are evaluated at two developmental time points: peri-pubertal postnatal day (PND) 28 and adult PND 56. *itchy* mice demonstrate an increased germ cell apoptotic index compared with wild type C57BL/6J mice at both PND 28 and PND 56. A corresponding 27% reduction in the total number of spermatid heads produced in PND 56 *itchy* mice was also evident. A histological evaluation of *itchy* mice revealed a delay in spermatogenesis at PND 28 and disorganization of late stage spermatids at PND 56. An analysis of several apoptotic markers revealed an age-dependent change in cleaved caspase 9, an intrinsic apoptosis mediator. The breeding success of the *itchy* mice was also significantly decreased, possibly due to a developmental defect. Taken together, these findings indicate that Itch is required for functional spermatogenesis, and that it may play differing cellular roles during development.

Introduction

Spermatogenesis is the complex process in which diploid spermatogonial stem cells divide and differentiate to ultimately lead to the formation of haploid spermatids in the testis. Sertoli cells, the somatic cell type located within the seminiferous epithelium, tightly regulate this process through paracrine signaling, secretion of nutritional and hormonal factors, and close physical interactions. Tight junctions located between neighboring Sertoli cells isolate meiotic and post-meiotic germ cells from the surrounding testicular environment and create a specialized microenvironment that fosters their development.¹ This process relies heavily on the rapid turnover of short-lived proteins, both in the haploid germ cells where cellular remodeling and chromatin condensing is occurring, and in the supporting Sertoli cells, which must remove the residual waste that is left behind.²,³ Recent evidence has highlighted the importance of the ubiquitin-proteasome pathway in the turnover of proteins during spermatogenesis.⁴ Ubiquitin is a highly conserved 76 amino acid protein that is used to target specific protein substrates to a variety of cellular processes, including degradation by the 26S proteasome. In spermatids, histones are ubiquitinated and subsequently degraded to allow for the tight packaging of DNA during spermatid formation.⁵ In Sertoli cells, the ubiquitin-proteasome pathway allows for the dynamic changes in the proteins required for movement of preleptotene spermatocytes across the blood testis barrier.⁶ The covalent addition of ubiquitin to target proteins requires a three-step enzymatic process (reviewed in ref. 8). In short, first an E1 ubiquitin activating enzyme utilizes ATP to activate and covalently attach the ubiquitin molecule to itself. Second, the active ubiquitin is transferred from the E1 to an E2 ubiquitin conjugating enzyme. Lastly, an E3 ubiquitin ligase transfers the ubiquitin molecule from the E2 to the target protein, by either acting as a scaffold for direct transfer or as an intermediate, where ubiquitin becomes covalently attached to the E3 during transfer. There are two major classes of E3 ligases: RING finger ligases that do not form a ligase-ubiquitin intermediate and HECT ligases which facilitate transfer of the ubiquitin to themselves before attaching it to its substrate. Because the E3 confers the substrate specificity, there are hundreds of different ubiquitin E3 ligases but only a limited number of E2s. One such E3 ligase, termed Itch, belongs to the Nedd4-like family of HECT E3s. It is made up of a C2 lipid binding domain, four WW protein binding domains, and a catalytic HECT domain (reviewed in ref. 9).

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Major progress in deciphering the role of the E3 ligase Itch during development has stemmed from the generation and identification of Itch loss-of-function mutant mice. The initial study examined a set of mutants with a disruption in the coat color gene agouti that were randomly generated by ionizing radiation. Interestingly, one of these mutants also displayed an autoimmune-like phenotype, characterized by severe inflammation, infiltration of immune cells into various organs, and most apparently chronic dermatitis, which led to their classification as *itchy* mice.11 These mice displayed shortened lifespans compared with wild type, surviving to only 6 to 9 mo of age, which is thought to result from pulmonary inflammation. Although initially unknown, it was later discovered that this gene, which was disrupted by a chromosomal inversion that also decreased the expression of the neighboring *agouti* gene, encoded a novel E3 ubiquitin ligase, fittingly named Itch.11

Since its identification, Itch has been shown to play an important role in a variety of tissues and cellular pathways, most importantly in the immune system during adulthood. A number of Itch targets that span a wide array of cellular functions and tissue distributions have already been identified and characterized. In the immune system, Itch is responsible for the activation and differentiation of T cells, and a number of proteins are deregulated in the *itchy* mice.6 In the absence of Itch, increased levels of the transcription factors JunB and c-Jun results in a preference for T helper type 2 (TH 2) cells, resulting in severe autoimmune response observed in the *itchy* mice.12

Itch has been suggested to influence apoptosis, where it targets the anti-apoptotic protein cellular FLICE-like inhibitory protein cFLIP for degradation, preventing its inhibition of caspase 8.13 Death receptor signaling plays an important role in maintaining germ cell populations in the testis, where ligands presented on the surface of Sertoli cells bind corresponding receptors on neighboring germ cells, leading to binding and activation of caspase 8.14,15 Itch has also been linked to transcriptional regulation by targeting p63 and p73, two proteins in the p53 family of transcription factors.16,17 In the testis, Itch has been shown to induce occludin degradation, a tight junction protein important for regulating the blood-testis barrier. This wide array of targets leads to a broad spectrum of cellular pathways that Itch may influence during development, but little is known about the function of this protein in the control of germ cell apoptosis and regulation of spermatogenesis during various developmental time periods.

The aim of this study was to elucidate the role of Itch in the process of spermatogenesis by examining the reproductive and physiological characteristics of *itchy* loss-of-function mutants. Although these mice are fertile, *itchy* mice show reduced litter sizes and lower mature spermatid counts. Compared with wild type C57BL/6J mice, the *itchy* mice are smaller both in overall size and in testis weight. Although unexpected, germ cell apoptosis is increased in the *itchy* mice, which may be partially explained by activation of the intrinsic apoptotic-signaling pathway. Histological analysis reveals a variety of cellular dysfunctions in the testis, including possible changes in cell division and spermatid formation. Interestingly, these dysfunctions appear to change depending on the age of the animal, possibly pointing to a developmental stage-specific role of Itch in the testis. Examination of several known Itch targets failed to identify significant changes in the testis of *itchy* mice, suggesting that there may be as of yet unidentified substrates.

### Results

*Itchy* mice have smaller body weights and testis weights compared with wild type mice. For the initial analysis of *itchy* development, body weights and testis weights were recorded at PND 28 and PND 56 (Table 1). *Itchy* mice have significantly smaller body weights than C57BL/6J mice at both PND 28 (Table 1A, 13.64 ± 0.39 g and 15.60 ± 0.55 g) and at PND 56 (Table 1B, 20.84 ± 0.19 g and 25.16 ± 0.27 g). The testis weights were also significantly decreased in the adult (PND 56) *itchy* mice (0.088 ± 0.002 g and 0.098 ± 0.001 g), but were not different at PND 28 (0.045 ± 0.002 g and 0.046 ± 0.002 g), indicating that this difference in testis weight occurs after the first wave of spermatogenesis. A comparison of the testis to body weight ratios revealed that *itchy* mice have higher ratios than their wild type counterparts, both at PND 28 (3.34 ± 0.14 g/kg and 2.97 ± 0.05 g/kg) and at PND 56 (4.22 ± 0.12 g/kg and 3.92 ± 0.07 g/kg), which results from the body weights being more significantly impacted than the testis weights.

### Apoptosis rates are higher in *itchy* mice at both peri-pubertal and adult ages.

Itch has been linked to death receptor-mediated apoptosis through its targeted ubiquitination and subsequent proteasomal degradation of the anti-apoptotic protein cFLIP.13 In order to determine if Itch plays a role in the normal physiological apoptosis of germ cells, testicular cross sections from *itchy* mice were examined at PND 28 and PND 56 (Fig. 1A). TUNEL analysis of wild type C57BL/6J mice reveals an apoptotic index typical of normal, background apoptosis (6.91 ± 0.58% at PND 28 and 4.12 ± 0.34% at PND 56). Although it was predicted that a decrease in apoptosis would be observed with the loss of this pro-apoptotic protein, *itchy* mice actually show an increase
in apoptosis at both the peri-pubertal PND 28 (8.85 ± 0.43%) and at the adult PND 56 (7.20 ± 0.50%) age. This represents an increase of approximately 28% at PND 28 and 75% at PND 56 compared with wild type C57BL/6J mice.

The total number of mature spermatid heads produced is lower in itchy mice. Increased apoptosis of germ cells can ultimately lead to lower sperm production, therefore the total number of mature spermatid heads were counted at PND 56 for both C57BL/6J and itchy mice (Fig. 1B). The wild type C57BL/6J mice had an average of 1.2 x 10^7 ± 3.2 x 10^6 spermatid heads per testis counted, while the itchy mice averaged a significantly lower number of 0.9 x 10^7 ± 4.7 x 10^5 heads. Comparatively, this data indicate that itchy mice have a 25% reduction in total spermatid heads. Although a direct cause is unknown, this decrease corresponds closely to the increased rates of germ cell apoptosis observed in the PND 56 itchy mice.

Testicular histology of peri-pubertal (PND 28) itchy mice reveals alterations in meiosis and a delay in spermatogenesis. In order to further elucidate the role of the E3 ligase Itch during normal testis development, testicular cross sections were stained and histologically evaluated. At PND 28, itchy mice have more tubules with meiotic figures than wild type C57BL/6J mice (Fig. 2A–D). Quantification of essentially round tubules revealed that wild type mice have an average of 7.4 ± 0.4% tubules containing meiotic germ cells, while itchy mice have a significantly higher average of 10.7 ± 0.3% (Table 2A). Also notable in wild type mice at this age is the formation of mature spermatids, which can act as an indicator of normal physiology. Although C57BL/6J mice at PND 28 have completed the first cycle of spermatogenesis and contain elongated spermatids (Fig. 2E), the itchy mice appear to be developmentally delayed. Comparing similar tubule stages, mature spermatid subtypes that are present in wild type mice are lacking in the itchy mice (Fig. 2G). This could result from the altered meiosis that is also observed, where a change in cell cycle dynamics may alter the normally identifiable stages. Also possible is a delay in the initiation of the cell cycle, and therefore spermatogenesis, possibly by a late onset organization of the blood testis barrier. Examination of younger animals will be essential in determining the cause of this delay.

Testicular histology of adult (PND 56) itchy mice shows an increase in abnormal cells and a disorganization of spermatid heads. To determine if the alterations observed at PND 28 were age specific effects due to the loss of Itch, testicular cross sections from mice at PND 56 were also examined (Figs. 3–5). The presence of mature spermatids in the itchy knockout mice suggests that the developmental delay observed at PND 28 did not have a long lasting effect on spermatogenesis, but the meiotic phenotype remained (Table 2B), possibly pointing to an age-independent target of Itch. Cross sections from the itchy mice at PND 56 still contained a number of abnormalities, albeit different from those seen at PND 28, mainly because they were focused around development of the elongating spermatids. There appears to be an increase in the number of abnormal clusters present in the testis (Fig. 3B), although the cause remains unclear. These figures do not appear to be multinucleated germ cells, but rather clumps or large masses of cellular material, possibly clusters of leftover residual cytoplasm from released spermatids. They lack stage specificity (Fig. 3C and D), but may indicate a disruption in Sertoli cell phagocytosis that leads to their long-term retention. Observations of these clumps in TUNEL-stained and cleaved caspase 9 IHC slides show negative results, further supporting this hypothesis (data not shown). Along with this increase in leftover cellular material, some tubules show disrupted germ cell layers, where gaps in the germ cell layers appear in both early (Fig. 4B) and late (Fig. 4D) seminiferous tubule stages. This phenotype is indicated by a discontinuity in the observed “ring” of a distinct subset of germ cells in essentially round cross sections of seminiferous tubules, and gaps in early cell types may lead to missing late stage spermatids in the same regions.
Figure 2. For figure legend, see page 108.
irregularities were not frequently observed in the wild type C57BL/6J mice (Figs. 3A, 4A and C). There also appears to be a disruption of the later stage mature elongate spermatids (Fig. 5). In wild type C57BL/6J mice, elongate spermatids are positioned perpendicular to and uniformly around the lumen of each tubule (Fig. 5A and C). The itchy mice, however, show subtle alterations in this typical pattern, including elongate spermatids that are oriented parallel to the lumen rather than perpendicular (Fig. 5B) and appear to be clustered together and not uniformly arranged in the tubule (Fig. 5D). Although the role of Itch during testis development has not yet been identified, these observations suggest that Itch is important in normal spermatogenesis, possibly during spermatid organization and maturation, and that its role may be age dependent.

Western blot and immunohistochemical analysis point to an age-dependent but not a genotype specific change in apoptotic markers. Histological analysis of the incidence of germ cell apoptosis revealed that the itchy mice have higher basal levels of apoptotic germ cells than their wild type counterparts, but the mechanism behind this observed increase was not apparent. Therefore, the two major pathways of apoptosis were examined using western blot analysis and immunohistochemistry (Fig. 6). Extrinsic apoptosis, or death receptor-mediated apoptosis was evaluated by assessing the levels of two death ligands previously identified in our lab as contributing to the regulation of germ cell apoptosis, FasL and TRAIL (unpublished data) (Fig. 6A). These two proteins were expressed at higher levels at PND 56 in both the wild type and itchy mice, although they were expressed at similar levels between the two genotypes (Fig. 6B and C). Intrinsic apoptosis or mitochondrial-mediated apoptosis, was analyzed by the immunohistochemical detection of the cleaved form of caspase 9 (Fig. 7A–D). Interestingly, the loss of Itch appears to have an age-dependent effect on the cleavage of caspase 9 in germ cells, a linkage that has not been previously described (Fig. 7E). At PND 28 (Fig. 7A and B), wild type C57BL/6J mice have a basal level of 8.12 ± 0.64% of tubules with more than three cleaved caspase 9 positive germ cells, while the age-matched itchy mice have a reduced level of 4.95 ± 1.09%. This indicates that not only may Itch act to promote intrinsic apoptosis signaling, but that the increased apoptosis observed in the itchy mice most likely results from an extrinsic signaling mechanism. On the contrary, at PND 56 (Fig. 7C and D), where the number of cleaved caspase 9 positive cells is reduced in the wild type C57BL/6J mice (3.81 ± 0.76%) they are significantly increased in the itchy mice (8.53 ± 1.23%), which corresponds to the overall increased apoptosis seen in these animals. Therefore, the loss of Itch leads to age-dependent alterations in germ cell apoptosis, although the mechanisms that account for these differential changes remain unclear.

![Figure 2 (See previous page).](image)

Histological evaluation of the testis of wild type C57BL/6J and itchy mice at PND 28. Testicular cross sections (5 μm) from paraffin embedded tissue were examined using PAS-H (periodic acid-Schiff-hematoxylin) staining. (A and E) are from wild type C57BL/6J mice while (C and G) are from itchy mice. The outlined box in each section indicates the area that is magnified to the right (B, D, F and H). Itchy mice appear to have more tubules with meiotic figures (C, denoted with an “m”) and fewer tubules with mature spermatid heads (G, denoted with an “s”) than their wild type counterparts (A and E, respectively). The bar represents 100 μm.

| Table 2. Quantification of tubules with meiotic figures in wild type C57BL/6J and itchy mice at (A) PND 28 and (B) PND 56 |
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| A. Quantification of tubules containing meiotic figures in wild type C57BL/6J and itchy mice at PND 28 |
| **Genotype** | **n** | **Total tubules counted** | **Tubules w/ meiotic figures** | **Percent meiotic** |
|---------------|-------|--------------------------|-------------------------------|-------------------|
| C57BL/6J      | 6     | 257.5 ± 6.6              | 18.9 ± 0.9                    | 7.4 ± 0.4         |
| Itchy         | 7     | 271.9 ± 5.5              | 29.2 ± 1.0                    | 10.7 ± 0.3*       |
| **B. Quantification of tubules containing meiotic figures in wild type C57BL/6J and itchy mice at PND 56** |
| **Genotype** | **n** | **Total tubules counted** | **Tubules w/ meiotic figures** | **Percent meiotic** |
|---------------|-------|--------------------------|-------------------------------|-------------------|
| C57BL/6J      | 4     | 226.8 ± 7.3              | 10.6 ± 0.7                    | 4.7 ± 0.4         |
| Itchy         | 5     | 250.7 ± 8.2              | 21.2 ± 1.4                    | 8.4 ± 0.4*        |

Itchy mice have a higher percentage of tubules containing meiotic figures than wild type C57BL/6J mice. Values represent the mean ± SEM with an asterisk identifying a significant difference from control (*p < 0.05, Student’s t-test).

Western blot analysis of previously identified substrates reveals that Itch may have a testis-specific target involved in apoptosis. A very limited number of studies have been done to decipher the role of Itch in the testis, but several targets of the E3 ligase have been previously identified in other pathways and organ systems. These protein targets have been identified from a wide range of functional pathways, such as cFLIP in apoptosis, c-Jun in cell signaling, and occludin in cell junctions, and here they were examined for their presence and possible alterations in the testes of wild type C57BL/6J and itchy mice (Fig. 8A). Although the previous observation of disrupted apoptosis suggested a dysregulation in cFLIP levels in the itchy mice, cFLIP is only detectable at extremely low levels by western blot analysis in total testicular lysates. Although there are age-dependent changes in other two common targets of Itch, c-Jun and occludin, they do not appear to be significantly increased as expected between the wild type and itchy mice (Fig. 8B and C).

Itchy mice have fewer pups per litter, but it most likely results from a defect in embryo development rather than parental gamete formation. The reproductive ability of the itchy mice was examined by comparing the average number of pups born per litter. For C57BL/6J mice, 41 litters from 12 breeding females were counted, giving an average of 3.4 litters per female. For itchy mice, 46 litters from 19 breeding females were counted, giving a lower average of 2.4 litters per female. The number of itchy pups born per litter was significantly decreased compared with C57BL/6J (5.15 ± 0.23 and 7.93 ± 0.36 respectively), suggesting that there may be a reproductive role for Itch during conception and/or pregnancy (Table 3A). Partially reabsorbed and/or not fully developed pups were also found within the...
c-Jun, which has been well characterized and shown to be upregulated in the T cells of itchy mice, was not found to be altered in the testis of the itchy mice, although it was detectable (Fig. 8). Occludin, a previously identified testicular Itch substrate, did not appear to be significantly increased in the itchy mice at either PND 28 or PND 56, suggesting a low rate of protein turnover or possibly a compensating E3 ligase which replaces the role of Itch. The anti-apoptotic protein cFLIP, capable of blocking caspase 8 activation and therefore apoptosis, is expressed at very low levels under normal conditions in the testis of wild type mice. Even with the loss of Itch, cFLIP levels remain low at both of the ages examined, which was unexpected given the importance of the FasL death receptor signaling pathway during spermatogenesis.

Although it was originally hypothesized that the itchy mice would have lower basal levels of apoptosis due to the predicted stabilization of cFLIP protein, the exact opposite was observed, with the itchy mice having significantly higher levels at both the of the ages observed (Fig. 1A). Interestingly, a similar increase in the incidence of apoptosis was seen in a mouse model that lacks the uterine horns of several of these itchy females that were sacrificed the day after birth (data not shown). Cross-mating experiments were then conducted to determine if the smaller litter sizes could be attributed to problems in the males or females (Table 3B). Itchy females were bred to C57BL/6J males and itchy males were bred to C57BL/6J females for 4 consecutive litters, and the total number of pups born were counted. Although the itchy females did produce slightly smaller litters than the C57BL/6J females (6.93 ± 0.35 and 8.09 ± 0.48), these numbers did not account for the significantly smaller litter sizes observed in the homozygous itchy colonies. This suggests a possible failure in Itch−/− pup development that is not observed in the Itch+/− offspring.

**Discussion**

Much progress into deciphering the role of Itch has come from work in the immune system and the late onset autoimmune disease that occurs in the itchy mice, while its role in other tissues and systems remains less understood. Important immune targets like

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**Figure 3.** Histological evaluation of the testis of wild type C57BL/6J and itchy mice at PND 56. Testicular cross sections (5 μm) from paraffin embedded tissue were examined using PAS-H (periodic acid-Schiff-hematoxylin) staining. (A) is from wild type C57BL/6J mice while (B–D) are from itchy mice. At 56 d, itchy mice show an increase in abnormal cells (B) compared with wild type (A), and these cells appear to not be stage-specific (C). The outlined box indicates the area that is magnified to the right (D). Arrows indicate sites of described abnormal histology. The bar represents 100 μm.
germ cell apoptosis, and its loss therefore mimics the loss of these more common proapoptotic factors. Interestingly, although we did not measure an increase in death receptor signaling as indicated by western blot analysis of two common death ligands (Fig. 6), we did observe a difference in the cleavage of caspase 9 and the interpreted activation of the intrinsic signaling pathway (Fig. 7). C57BL/6J mice show a similar pattern of cleaved caspase 9 positive staining as they do TUNEL (Fig. 1A), with levels around 8% at PND 28 and around 4% at PND 56. This, however, is reversed in the itchy mice, which have lower levels of caspase 9 cleavage at PND 28.

![Figure 4](image)

**Figure 4.** Histological evaluation of the testis of wild type C57BL/6J and itchy mice at PND 56. Testicular cross sections (5 μm) from paraffin embedded tissue were examined using PAS-H (periodic acid-Schiff-hematoxylin) staining. (A and C) are from wild type C57BL/6J mice while (B and D) are from itchy mice. At PND 56, itchy mice have an increased number of tubules that lack distinct germ cell subtypes in both early (B) and late (D) stages, compared with wild type. This is characterized by gaps in the normally continuous “ring” of germ cell stages within the tubule. Arrows indicate sites of described abnormal histology.

proapoptotic death ligand FasL. The FasL gene deficient mice, which also display an autoimmune phenotype, have significantly higher levels of germ cell apoptosis than the itchy mice, but they show a similar pattern of delayed spermatogenesis and decreased spermatid head counts compared with wild type C57BL/6J mice. Unpublished observations from our lab of TRAIL gene deficient mice, another death ligand family member, show similar results, which may point to a general phenotype for disrupted germ cell apoptosis. Even though no changes in cFLIP protein levels were observed, Itch may be acting in a separate manner to promote germ cell apoptosis, and its loss therefore mimics the loss of these more common proapoptotic factors.

Interestingly, although we did not measure an increase in death receptor signaling as indicated by western blot analysis of two common death ligands (Fig. 6), we did observe a difference in the cleavage of caspase 9 and the interpreted activation of the intrinsic signaling pathway (Fig. 7). C57BL/6J mice show a similar pattern of cleaved caspase 9 positive staining as they do TUNEL (Fig. 1A), with levels around 8% at PND 28 and around 4% at PND 56. This, however, is reversed in the itchy mice, which have lower levels of caspase 9 cleavage at PND 28.
mice. This, however, does not hold true at PND 28. Although apoptosis levels are higher than the wild type C57BL/6J mice, caspase 9 cleavage is significantly lower, and there lacks an obvious increase in extrinsic death ligand signaling.

Histological analysis of the *itchy* mice revealed several subtle although consistent changes throughout the seminiferous epithelium. At PND 28, the loss of Itch seems to influence cell division, with *itchy* mice having more tubules with meiotic figures and less tubules with late stage spermatids. It is unknown whether meiosis is permanently arrested or whether the length of the cycle is extended, but the lack of spermatids points to a clear delay in normal spermatogenesis. At PND 56, the *itchy* mice are able to (~5%) and more than double at PND 56 (~8.5%) compared with wild type. Several inferences can be drawn from this apparent difference in apoptotic signaling. First, Itch appears to effect caspase 9 cleavage in an age dependent manner. At PND 28 in wild type mice, Itch may promote caspase 9 activation, and therefore in the absence of Itch there is less cleavage. On the other hand, at PND 56, Itch may switch roles, where its presence now inhibits caspase 9, causing significantly higher levels of cleavage in the *itchy* knockout mice. This falters, though, when these data are compared with the overall apoptotic indexes determined by TUNEL.

At PND 56, the high levels of caspase 9 cleavage are able to account for the corresponding high apoptosis levels in the *itchy* mice. This, however, does not hold true at PND 28. Although apoptosis levels are higher than the wild type C57BL/6J mice, caspase 9 cleavage is significantly lower, and there lacks an obvious increase in extrinsic death ligand signaling.

**Figure 5.** Spermatid head organization in wild type C57BL/6J and *itchy* mice at PND 56. Testicular cross sections (5 μm) from paraffin embedded tissue were examined using PAS-H (periodic acid-Schiff-hematoxylin) staining. (A and C) are from wild type C57BL/6J mice while (B and D) are from *itchy* mice. *Itchy* mice show alterations in spermatid head formation, including disorientated and disorganized spermatids. Arrows indicate sites of abnormal spermatids.
produce mature and functional sperm, suggesting that they can at least recover from the delay, although they still show alterations in meiosis. Itch appears to take on a different role during adulthood; with PND 56 *itchy* mice showing a series of spermatid head defects and a disorganization of the cell layers (Figs. 3–5). The large masses of cellular material left over in various stages of the seminiferous epithelium possibly indicate a failure of Sertoli cells to properly dispose of spermatid cytoplasm. The observed alterations of the late stage elongate spermatids may explain the decreased spermatid head counts as clumped material would less likely be counted. Although these phenotypes appear subtle, the combination of several smaller issues may lead to the more significant issues such as increased apoptosis, decreased testis weights, and decreased spermatid head counts.

Although male *itchy* mice display a higher incidence of germ cell apoptosis and lower numbers of mature spermatids (Fig. 1), mating experiments with wild type C57BL/6J females revealed no observable differences in their reproductive ability or number of viable offspring produced (Table 3). This is a very common finding in male reproductive disorders that lower sperm numbers, as many more sperm are produced than are actually required for fertilization. This led us to assume that there was a reproductive issue with the female *itchy* mice, but similar mating experiments to wild type C57BL/6J males did not fully replicate the significantly decreased litter sizes seen in the *itchy* colonies. This indicates there may be a problem during development of the *itchy* pups that does not occur in the *itchy* x C57BL/6J heterozygous offspring. In support of this, several *itchy* females were found to have partially reabsorbed fetuses following the birth of a small litter size (data not shown), but further work is needed to determine the role of Itch during this sensitive time period.

The alterations in spermatogenesis without an identifiable disrupted target has led to a few possible conclusions about the role of Itch in spermatogenesis and germ cell apoptosis. Although several of Itch’s common targets do not appear to be upregulated as expected, it may be that Itch has a yet unidentified, possibly testis-specific protein target. The ubiquitin-proteasome pathway is an important regulator during testicular maturation and spermatogenesis, and several testis specific enzymes have been discovered. The ubiquitin-conjugating enzyme UBC4-testis is responsible for protein turnover during spermatogenesis, and mice lacking this testis-specific E2 show a delay in testicular development.2 Another possible explanation for the data presented here is that it is a secondary effect, rather than the direct loss of Itch, that results in the observed phenotype. The autoimmune disease of the *itchy* mice has been well described, and one such defining characteristic is the buildup of IgG complexes.22 Although this is typically thought of as a late onset disease, our lab has observed increased IgG concentrations in the testes of the *itchy* mice as early as PND 28 (data not shown). The testis is thought to be an immunoprivileged organ due to the protection afforded the germ cells from the circulating immune cells, but increases in these
cells can lead to inflammation through the secretion of cytokines and disruptions in normal spermatogenesis. Further work is needed to determine whether the measureable effects observed during testis development in the itchy mice are due to the direct loss of Itch in the testis or as a secondary effect to the loss of Itch in another organ or system.

Figure 7. Immunohistochemical analysis of intrinsic apoptosis in wild type C57BL/6J and itchy mice at PND 28 and PND 56. (A–D) Immunohistochemical analysis of intrinsic apoptosis was performed using an antibody specific for cleaved caspase 9. Sections were analyzed from C57BL/6J and itchy mice at both PND 28 (A and B, respectively) and PND 56 (C and D, respectively). The bar represents 100 μm. (E) Quantification was performed by counting the number of positive cells in a minimum of three cross sections from each age group and from each strain, and a positive index was calculated as the percentage of tubules containing more than three positive cells. Itchy mice have lower intrinsic apoptosis signaling at PND 28 but higher at PND 56 than wild type mice. Values represent the mean ± SEM with an asterisk identifying a significant difference from control (*p < 0.05, Student’s t-test).
Materials and Methods

Mice. All mice used in the experiments described were housed in the Animal Resource Center at The University of Texas at Austin. The mouse room was kept at a constant temperature (22 ± 0.5°C) at 55–70% humidity with a 12L:12D photoperiod. To enhance breeding, mice were fed a high-energy diet containing 9% fat (5P06 Prolab RMH 2000) and water ad libitum. All experiments using mice were preformed in accordance with the guidelines of The University of Texas at Austin’s Institutional Animal Care and Use Committee in compliance with guidelines established by the National Institute of Health. Mating pairs of C57BL/6J were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in house to obtain pups. Itchy mice (Itch+/+) on a C57BL/6J background were a generous gift from Dr. Lydia Matesic at the University of South Carolina, Columbia, South Carolina.18 These non-agouti-lethal 18H (a18H) mice were originally generated through a radiation-induced chromosomal inversion18 and further characterized as having a disrupted gene that was later termed Itch.19 Heterozygous breeding pairs were initially set up, and from the F1 generation offspring, Itch+/+ (wild type C57BL/6J) and Itch−/− breeding colonies were established. Cross breeding experiments were performed using itchy mice from the breeding colonies and wild type C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME).

Genotyping PCR and primers. Wild type C57BL/6J and Itchy mice were confirmed using genotyping PCR with primers specific for the Itch gene (protocol from Dr. Lydia Matesic). Tail clippings were collected and digested overnight with Proteinase K. Total genomic DNA was ethanol precipitated and PCR was performed using Taq polymerase. The primers used included individual wild type (5'-ATC GTC TAC TCA CCC CAC ATA AGG-3') and mutant (5'-AAG AAG CAG CAG AGA CAA CGA GTG-3') forward primers that share a common reverse primer (5'-TCT ATG CTC TGT TGT CTC CCA TGC-3'). The wild type primer and common primer results in a 194 bp band, while the mutant primer mixed with the common primer results in a 294 bp band (data not shown).

Physiological and reproductive characterization. Litter sizes were determined by counting the total number of pups at postnatal day (PND) 0. A minimum number of 12 females/mating cages were used for each wild type and itchy group. Each female was left to breed until they ceased having pups for 2 mo or died. Body and testis weights were recorded at PND 28 and PND 56. These
Table 3. Litter sizes of (A) wild type C57BL/6J and itchy breeding colonies and (B) cross-breeding experiments

|                  | Male | Female | n litters | pups/litter |
|------------------|------|--------|-----------|-------------|
| **A. Litter sizes of wild type C57BL/6J and itchy mice** |      |        |           |             |
| C57BL/6J        | 41   | C57BL/6J | 46        | 7.93 ± 0.36 |
| itchy           |      |         |           | 5.15 ± 0.23* |

|                  | Male | Female | n litters | Pups/litter |
|------------------|------|--------|-----------|-------------|
| **B. Litter sizes of cross-matings between C57BL/6J and itchy mice** |      |        |           |             |
| C57BL/6J        | 11   | C57BL/6J | 14        | 8.09 ± 0.48 |
| itchy           |      |         |           | 6.93 ± 0.35 |

Itchy mating pairs have significantly smaller litter sizes than C57BL/6J pairs, but it may be due to a developmental defect. Values represent the mean ± SEM with an asterisk identifying a significant difference from control (*p < 0.05, Student’s t-test).

ages were selected in order to evaluate two important testicular developmental ages, peri-pubertal (PND 28) and adult (PND 56). The testes weights were expressed as an average of the right and left testis weights, and the testis/body weight ratio as an average testis weight in grams divided by the body weight in kilograms. A minimum number of five mice were used for each genotype in each age group.

Terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine triphosphate nick end labeling. The germ cell apoptotic index was determined as previously described in reference 19 and 20, through terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine triphosphate nick end labeling (TUNEL) analysis using an ApopTag™ kit (Chemicon, S7100). Paraffin-embedded testicular cross sections (5 μm) were enzymatically labeled and the slides were imaged using a Nikon E800 microscope. The apoptotic index was determined by calculating the percentage of essentially round tubules that contained more than three caspase 9-positive germ cells. At least 2 sections were counted from each animal, and at least 8 animals were counted in each age group.

**Spermatid head counts.** As previously performed,39 testes were collected from PND 56 mice and flash frozen in liquid N₂. Frozen testes were gently homogenized in a solution containing 0.9% w/v NaCl and 10% v/v dimethyl sulfoxide (DMSO). Homogenization-resistant spermatid heads were counted on a standard hemocytometer using a Nikon E800 microscope. The average number of spermatid heads for each genotype was determined using a single testis from 8 individual mice, and each testis sample was counted 3 times.

**Testicular histology and meiotic quantification.** Paraffin-embedded testicular cross sections (5 μm) from PND 28 and PND 56 C57BL/6J and itchy mice were examined using periodic acid-Schiff-hematoxylin staining. Sections were viewed on a Nikon E800 microscope and images were captured using a Nikon Digital DS camera and NIS Elements software. The percentage of essentially round seminiferous tubules containing meiotic figures were quantified as a percentage of the total number of tubules. At least 2 sections were counted from each animal, and at least 6 animals were counted in each age group.

**Total protein extraction and western blot analysis.** As detailed previously in reference 20, total protein was collected from two sets of whole testes homogenized in RIPA buffer and the concentration was determined using the Biorad DC Protein Assay Lowery Method. For each sample, 30 μg was separated using a 4–12% NuPAGE gradient gel, transferred to a PVDF membrane, and blocked using a 5% milk solution. Quantification was determined using antibodies specific for Itch (BD Trans, 611199, 1:1,000), FasL (Santa Cruz, sc834, 1:1,000), TRAIL (Zymed, 40–3900, 1:1,000), cFLIP (Dave-2, Alexis, ALX-804-127, 1:1,000), c-Jun (Cell Signaling, 9165, 1:1,000), and occludin (Abcam, ab31721, 1:1,000). Actin (Santa Cruz, sc1016, 1:1,000) was used as a loading control. Quantification was preformed using the ImageJ software (NIH).

**Testicular immunohistochemistry.** Caspase 9 cleavage as an indicator of activation was determined using paraffin-embedded testicular cross sections (5 μm) and an antibody specific for the cleaved form of mouse caspase 9 (Cell Signaling, 9509, 1:100). Briefly, sections were deparaffinized and rehydrated, and antigens were unmasked by boiling the sections in sodium citrate. Hydrogen peroxide was used to inhibit endogenous peroxidases, while horse serum (Vector laboratories, S-2000) was used to block nonspecific antibody binding. The cleaved caspase 9 antibody was diluted in blocking serum and slides were incubated overnight at 4°C. The primary antibody was detected using a biotinylated anti-rabbit secondary, Vectastain ABC reagent (Vector Laboratories, PK-6101), and DAB peroxidase substrate (Vector laboratories, SK-4100). The index was determined by calculating the percentage of essentially round tubules that contained more than three caspase 9-positive germ cells. At least 3 sections were counted from each animal, and at least 2 animals were counted in each age group.

**Statistical analysis.** Statistical results are expressed as mean ± SEM. The data were analyzed using JMP software (version 9, SAS Institute Inc.), first with the Shapiro-Wilk test to determine normality, then subjected to a Student’s t-test. Because of the nature of the data, litter sizes were also subjected to the Wilcoxon rank sum test as if the data were not normally distributed, and the data was only considered significant if both the parametric and nonparametric tests came to the same conclusion. All of the data was considered statistically significant when p < 0.05.

**Disclosure of Potential Conflicts of Interest**

The authors have disclosed all financial sources and confirm that there are no conflicts of interest.

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